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The Characterisation of Peripheral Blood and Intestinal Antigen Presenting Cells in Coeliac Disease

A dissertation submitted to Trinity College Dublin as requirement for the degree of Doctor of Philosophy (PhD)

BY

Louise Elliott

B.Sc (Hons)

Supervisor: Professor Conleth Feighery,
Department of Immunology
School of Medicine
Trinity College Dublin
Declaration

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Signed: Louise Elliott

Louise Elliott
Acknowledgments

I would like to express my special appreciation and thanks to my supervisor Professor Feighery "Con", you have been a tremendous mentor for me. I would like to thank you for the gentle encouragement and for allowing me to grow as a research scientist. I especially like to say thanks for all the hours you invested into proof reading my thesis making it possible for me to see the light at the end of the tunnel.

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Naturally, I would like to thank all the patients recruited during this project

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BDCA</td>
<td>Blood dendritic cell antigen</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD</td>
<td>Coeliac disease</td>
</tr>
<tr>
<td>CDP</td>
<td>Common DC progenitor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>cDMEM</td>
<td>Complete Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>CFSE</td>
<td>5(6)-Carboxyfluorescin diacetate N-succinimidy ester</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>cRPMI</td>
<td>Complete Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II associated invariant chain peptide</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony stimulating factor receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>Cy7</td>
<td>CyChrome 7</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBAO</td>
<td>Ethidium bromide and acridine orange</td>
</tr>
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra – acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EMA</td>
<td>anti-endomysium antibodies</td>
</tr>
<tr>
<td>ERAAP</td>
<td>Endoplasmic reticulum aminopeptidases</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMO</td>
<td>“Fluorescence minus one” control</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FLT-3</td>
<td>Fms-like tyrosine kinase 3</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>Hb/HP</td>
<td>Haemoglobin/Haptoglobin</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMB-PP</td>
<td>(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T-cell co-stimulator</td>
</tr>
<tr>
<td>iDC</td>
<td>Immature dendritic cell</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocytes</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant natural killer T cell</td>
</tr>
<tr>
<td>ITAMs</td>
<td>Immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer-cell immunoglobulin-like receptors</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhan</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeats</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>mDC</td>
<td>Mature dendritic cell</td>
</tr>
<tr>
<td>M-CSFR</td>
<td>Macrophage-colony stimulating factor receptor</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MDP</td>
<td>Monocyte/dendritic cell progenitor</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid DC</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation</td>
</tr>
<tr>
<td>Mtb</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide- binding oligomerisation domain</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBA</td>
<td>PBS containing BSA and azide</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein complex</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemaglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>Rh</td>
<td>Recombinant human</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3,5,5-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>tTG</td>
<td>Tissue transglutaminase</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumour necrosis factor receptor associated factor</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>αGalCer</td>
<td>Alpha galactosylceramide</td>
</tr>
<tr>
<td>B₂m</td>
<td>β₂-microglobulin</td>
</tr>
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5.4 Discussion

5.5 Conclusion

Chapter 6 The Investigation of Tissue Transglutaminase Expression in the Gut and in Monocyte derived Dendritic Cells
Chapter 1 Abstract

Antigen presenting cells, part of the innate immune system, play a central role in initiating and modulating the adaptive immune response. Coeliac disease is caused by aberrant activity of T cells specific for the dietary antigen gliadin that results in a complex inflammatory cascade, causing mucosal damage and villous atrophy. The overall objective of this thesis was to gain a better understanding of the role of antigen presenting cells in the immunopathogenesis of coeliac disease and the significance of tTG and CD163 expression in the coeliac lesion.

In order to address this, a triple immunofluorescence staining technique was carried out on duodenal sections from healthy controls and patients with treated or untreated coeliac disease using an array of monoclonal antibodies. Images were acquired by confocal microscopy and underwent quantitative analysis. In addition the main antigen presenting cells in the peripheral blood from healthy controls and patients newly diagnosed with coeliac from both adults and children were characterised using flow cytometry.

In the first part of the study, the main antigen presenting cells in the coeliac lesion and peripheral blood were characterised and quantified. Results demonstrated that myeloid dendritic cells accumulated in the untreated coeliac lesion. Complementary to this result we found that peripheral blood myeloid dendritic cells were decreased in untreated coeliac adults when compared to healthy subjects.

In the second part of the study, we investigated CD163 expression in different cell types in the duodenal mucosa and peripheral blood of healthy controls and coeliac patients. Comparison of the different macrophage subsets in the coeliac lesion demonstrated a skew in phenotype towards the M1 subset in the coeliac lesion compared to the healthy mucosa which was dominated by the M2 subset. Consistent with this finding is the elevated levels of soluble CD163, a molecule used to distinguish the M2 macrophage subset, found in the serum of untreated coeliac patients.

Finally, in the third part of the study, we examined the expression of tTG immunoreactivity in different cell types in coeliac disease small intestinal biopsies. Both Louise Elliott
mucosal macrophages and smooth muscle alpha actin positive myofibroblasts identified in the coeliac lesion were shown to over-express the autoantigen tissue transglutaminase. Thus, we conclude that the characterisation of antigen presenting cells in the future could help further our understanding of the pathogenesis of coeliac disease.
Chapter 2 General Introduction

2.1 The Immune System

The immune system is a highly intricate and sophisticated system that is fundamental to survival. It has evolved to allow an effective immune response to invading pathogenic organisms while remaining tolerant to self. The immune system can be divided into two branches, each with distinct characteristics. The innate immune system is considered to be the first line of defense against pathogens and is capable of a rapid and powerful response to harmful pathogens. It is often characterised as non-specific immunity, as it does not require prior sensitisation or memory. Nonetheless it discriminates very effectively between self and non-self via the detection of motifs on invading pathogens known as pathogen associated molecular patterns (PAMPs). Most importantly, innate immune cells such as granulocytes and antigen-presenting cells (APCs) are capable of activating the adaptive immune system.

The adaptive Immune system has the power to mount a specific immune response while generating a memory for that specific antigen allowing a quicker and stronger response the next time the pathogen is encountered. Innate cells such as antigen presenting cells can activate T cells and B cells, the adaptive effector cells, by directly presenting processed antigen to these cells. These two classes of lymphocytes carry out very different protective functions. B cells are responsible for the humoral arm, while T cells are responsible for the cell-mediated arm. Although the two have different functional characteristics, they are also intimately connected and interactions between the two are key in implementing a successful immune response against invading pathogens (Janeway & Medzhitov, 1998; Janeway, 2001).

2.1.1 Pathogen recognition receptors

In recent years there has been an explosion in innate immunity research resulting in the rapid understanding that the innate immune system possesses a comprehensive set of receptors that recognise conserved foreign molecular motifs, common to most pathogens. Collectively these receptors are called pathogen recognition receptors (PRRs) that allow non-specific recognition of PAMPs. There are four classes of PRRs: toll-like receptors (TLRs), C-type lectins (CLRs), nucleotide-binding oligomerization domain (NOD)
leucine rich repeat containing (NLRs) receptors and retinoic acid-inducible gene I protein (RIG-I) helicase receptors (Netea & van der Meer, 2011). Figure 2.1 summarises these receptors and their corresponding pathogen-associated molecular patterns. PRRs are expressed on many effector cells of the innate immune system such as antigen presenting cells and are located both intracellularly in the cytoplasm and endosome and extracellularly on the cell surface (Barton & Kagan, 2009; Blasius & Beutler, 2010). Once the PRR identifies a PAMP, the effector cells are triggered to perform their function such as phagocytosis of microbes, the activation of antimicrobial killing mechanisms and the production of inflammatory cytokines and chemokines that recruit and activate other immune cells that orchestrate the development of the adaptive immune response.

TLRs were first identified in 1996 in the Drosophila fruit fly (Rock, Hardiman, Timans, Kastelein, & Bazan, 1998) and since then 12 TLRs have been discovered, ten human and 12 mouse (Lee, Avalos, & Ploegh, 2012). TLRs are characterised by the presence of an extracellular domain containing leucine-rich repeats (LRR) and a cytoplasmic domain, similar to the cytoplasmic domain of the interleukin-1 receptor (Akira, 2003). The molecular mechanisms by which TLRs induce gene expression are now being rapidly elucidated. Microbial recognition promotes dimerization of TLRs triggering activation of signalling pathways. Central to this downstream signalling is the adaptor protein myeloid differentiation (MyD)88 (O'Neil, 2006; Takeda & Akira, 2005). MyD88 is essential for TLR signalling, as demonstrated by the observation in MyD88 deficient mice where TLR signalling was abrogated (O Takeuchi et al., 2000). MyD88 mediates downstream activation of IL-1 receptor associated kinases that interact with tumour necrosis factor receptor associated factor (TRAF)6 activating nuclear factor kappa-light chain-enhancer of activated B cell (NF-κB) and mitogen activated protein (MAP) kinases and transcription of pro-inflammatory cytokines (Akira & Takeda, 2004).

CLRs specifically recognise carbohydrate structures of microorganisms and endogenous ligands. They include dectin-1, macrophage mannose receptor, dendritic cell specific intracellular adhesion molecule 3-grabbing non-integrin, dectin 2, and the circulating mannose binding lectin. They have a role in the recognition of fungal pathogens. Some CLRs can induce signalling pathways that directly activate nuclear factor-κB, whereas others can induce signalling by TLRs. (Geijtenbeek & Gringhuis,
In addition to TLRs and CLRs, there are the cytoplasmic pattern recognition receptors RIG-I and NLRs. RIG-I helicase receptors mainly recognise nucleic acid of viruses (Osamu Takeuchi & Akira, 2008). The NLRs recognise the peptidoglycans of bacterial cell walls and can participate in the formation of inflammasomes and the processing of IL-1β (Fukata, Vamadevan, & Abreu, 2009). The NOD receptors, members of the NLRs and can be divided into NOD 1 and NOD 2 and are both structurally similar. Both have a NOD domain and a C-terminal LRR domain, NOD-1 possesses one caspase recruitment domain (CARD), whereas NOD-2 has two CARD domains. NOD 1 has been implicated in the detection of γ-D-Glu-mDAP (iE-DAP), a dipeptide present in the peptidoglycan (PGN) of all Gram-negative bacteria (Chamaillard et al., 2003; Strober, Murray, Kitani, & Watanabe, 2006). NOD-2 recognises muramyl dipeptide (MDP), minimal repeating structures in bacterial peptidoglycans found in almost all bacteria (Girardin et al., 2003). Both these components must be delivered intracellularly either by bacteria that invade the cell or via other cellular uptake mechanisms.
Figure 2.1: Illustrates the different pathogen recognition receptors and their ligands. The four classes are Toll Like Receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD) leucine rich repeats (LRR)-containing receptors (NLRs), and retinoic acid-inducible gene I protein (RIG-I) helicase receptors. (Image adapted Netea & Van der Meer, 2011)

2.1.2 Innate Lymphocytes

Unlike T cell and B cells innate lymphocytes act without antigen specific receptors and are important effector cells of innate immunity. Multiple types of innate lymphocytes have been identified that include natural killer (NK) cells, natural killer T (NKT) cells, invariant NKT (iNKT) cells and γδ T cells. They play essential roles in epithelial homeostasis, intestinal and mucosal defense, and tissue remodelling and repair to having the ability to induce tissue pathology. They are the forefront of innate immunological defense, as they are found in high numbers in the mucosal surfaces such as the intestinal lamina propria.

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Natural killer cells are a subset of cytotoxic innate lymphocytes. They express an array of inhibitory and activating surface receptors that enable them to distinguish stressed cells (tumor cells or infected cells) from healthy cells. The most important and well described is their inhibitory receptor, killer cell immunoglobulin-like receptors (KIRs), for the major histocompatibility complex (MHC)-I molecules. This interaction allows NK cells to recognise ‘missing self’ in target cells that have down regulated MHC-class I molecules as a result of viral infection or transformation (Vivier, Ugolini, Blaise, Chabannon, & Brossay, 2012). NK cells are also equipped with cell-surface activating receptors, such as NKG2D. NKG2D interacts with specific ligands, MHC class I polypeptide-related sequence (MIC) A and MICB, that are selectively upregulated in stressed cells. Importantly, NK cells express Fcγ receptor IIIA, CD16, which recognises antibody coated target cells via their Fc region and mediate antibody dependent mediated cytotoxicity (ADCC) (Vivier et al., 2012). Primary mechanisms of killing by NK cells are mediated via the production of perforin and granzymes, which create pores in targets cells, inducing apoptosis.

NKT cells primarily recognise lipid antigens presented by CD1d molecules (Speak, Cerundolo, & Platt, 2008). NKT cells express the αβ T cell receptor and CD3 molecule but also express NK cell markers such as CD56. They have been classified into 4 groups but only type I and type II are CD1d restricted. The most studied group is the invariant NKT cell (iNKT) or the type I cells. iNKT cells possess an invariant αβ TCR composed of a Vα24-Jα18 paired with a Vβ11 chain in humans resulting in limited clonal diversity (Berzins, Smyth, & Baxter, 2011). iNKT cells are responsive to glycolipid antigens presented by CD1d antigen presenting molecules. An example of such glycolipid antigens include α-galactosylceramide (αGalCer) (Speak et al., 2008). iNKT cells are highly cytotoxic cells and have been shown to lyse CD1d transfected Chinese hamster ovary (CHO) cells in vitro (Exley et al., 1998). It was later shown that human iNKT cells killed CD1d^+ tumour cell lines and that this killing was significantly enhanced by the presence of the glycolipid αGalCer (Metelista et al., 2001). Stimulated iNKT cells are capable of rapidly producing copious amounts of both Th1 and Th2 cytokines, generally IL-4, IL-13, IL-10 and IFN-γ (Burdin, Brossay, & Kronenberg, 1999). NKT cell defects have been implicated in a broad array of disease conditions ranging from cancer, autoimmunity, and infections. The most
important evidence to support this notion comes from mice that are completely deficient in NKT cells which are left predisposed to the development of autoimmune disorders and tumours (Bendelac, Savage, & Teyton, 2007; Berzins et al., 2011). To date iNKT cells are the focus of much research with the prospect of manipulating NKT cells for therapeutic advantage in treating cancers (Cerundolo, Silk, Masri, & Salio, 2009).

The majority of T cells express a T cell receptor (TCR) composed of an αβ heterodimer, while a minority expresses a γδ TCR. The γδ T cells show less TCR diversity. In humans there are only six expressed Vγ genes, five of which, Vγ2, 3,4,5,8 belong to the Vγl family while a single member VγII family contains Vγ9. γδ T cells account for 1-10% of circulating T cells (Stephen C De Rosa et al., 2004; Groh et al., 1989) and are particularly enriched at the epithelial surfaces (Komori, Meehan, & Havran, 2006). In the peripheral blood of healthy individuals, there is a marked predominance of Vγ9 together with the Vδ2, accounting for 50-95% of all γδ T cells (Stephen C De Rosa et al., 2004). Among the γδ in the skin and small intestine, T cells expressing Vδ1 are the most frequent (S C De Rosa, Mitra, Watanabe, Herzenberg, & Roederer, 2001). Largely, the antigen specificity of these cells is not known, except for the Vγ9Vδ2. Vγ9Vδ2 recognise the phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP). This is produced by many different bacteria and its corresponding levels determines the magnitude of γδ cell expansion in vitro (M. Hintz et al., 2001). In combination with their TCR, γδ cells possess the NK activating receptor, NKG2D that can detect stress-induced signals in target cells, which subsequently activate their effector functions (Vantourout & Hayday, 2013). γδ cells can kill infected, activated or transformed cells via the engagement of death inducing receptors, such as CD95, TNF-related apoptosis-inducing ligand receptors (TRAILR), and the release of cytotoxic effector molecules such as perforin and granzymes (Beetz et al., 2008). Activated γδ T cells also release immunomodulatory cytokines IFN-Y and tumour necrosis factor (TNF)-α that are involved in protective immunity against viruses, intracellular bacteria, and parasites (Born, Reardon, & Brien, 2006; Hayday, 2000). More recently, it has been shown that IL-23 producing γδ cells can exacerbate inflammatory responses by inhibiting the suppressive functions of T regulatory cells in the mouse model for experimental autoimmune encephalomyelitis (EAE) (Petermann et al., 2010).
2.1.3 Phagocytes

Phagocytes are a critical component of the innate immune system. Their key feature is rapid response in order to eliminate pathogens before they can establish infection. Phagocytes are composed of three major subsets; granulocytes, macrophage/monocyte, and dendritic cells. Granulocytes are made up of neutrophils, basophils, eosinophils, and mast cells. Collectively, the monocyte, macrophage and dendritic cell are referred to as antigen presenting cells.

Granulocytes

Neutrophils are the most prominent leucocyte in the peripheral blood. They are rapidly recruited from the blood to the site of infection and constitute the first line of defense against invading pathogens. They are involved in acute inflammation, as well as chronic inflammatory disorders and adaptive immune responses (Mantovani, Cassatella, Costantini, & Jaillon, 2011). They are essential for innate immunity against pathogens as demonstrated by the detrimental effects associated with defects in neutrophil function associated with leukocyte adhesion deficiency (LAD) and chronic granulomatous disease. Their main function is to recognise, engulf and destroy extracellular pathogens. Upon activation, neutrophils migrate to the tissue at an increased rate in response to chemotaxis via transendothelial migration. Tissue neutrophils display more phagocytic activity compared to their blood counterparts. They are highly effective at generating reactive oxygen species by a process known as respiratory burst essential for the degradation of internalized antigen and bacteria (DeChatelet, 1978; Kolaczkowska & Kubes, 2013; Shepherd, 1986). In addition to their production of reactive oxygen intermediates, neutrophils can form neutrophil extracellular traps (NETs). NETs are networks of extracellular fibers made up of DNA and proteins from neutrophil granules. They act as a mesh, which trap and kill microorganisms independent of phagocytic uptake. These traps are also believed to prevent the spread of microbes and neutrophil derived effector molecules, thus limiting tissue damage.

Basophils play an important role in allergic reactions and host defense against parasite infections. They are important IL-4 producers for Th2 differentiation. It has been suggested by some researchers, although controversial, that basophils can act as antigen presenting cells as they express MHC II and co-stimulatory molecules (CD80 & Louise Elliott

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More recently they have been shown to have a role in the generation of B cell memory response (Denzel et al., 2008).

Eosinophils are largely known for their role in helminthes immunity, allergic responses, and in the pathogenesis of asthma where they are recruited to the lung tissue and cause airway dysfunction and tissue remodelling (Fulkerson & Rothenberg, 2013). They have ability to modulate the function of other leucocytes. They can secrete IL-4, IL-13, and IFN-γ that can promote either Th2 or Th1 responses (Blanchard & Rothenberg, 2009). Eosinophils are also capable of priming B cell responses for the production of antigen specific IgM via the production of APRIL, a proliferating – inducing ligand, and IL-6 which are crucial for the support of long-lived plasma cells (Chu et al., 2011; Rosenberg, Dyer, & Foster, 2013).

Mast cells possess an array of effector and immunomodulatory functions and are widely distributed throughout the tissue in particularly the mucosal surfaces exposed to the external environment. They are mostly known for their ability to promote inflammation and other tissue changes in IgE mediated disorders (S. N. Abraham & John, 2010; Dumitrașcu, 1996). Mast cells have ability to induce immunosuppressive and pro-inflammatory immune responses via the production of histamine. Histamine can exert both negative and positive immune effects dependent on the receptor it acts through; H1 receptor promotes Th1 whereas the H2 receptor suppresses both Th1 and Th2 activation (Galli, Grimaldeston, & Tsai, 2008).

2.2 Antigen Presenting Cells - Monocytes, Macrophages and Dendritic Cells

There are three main antigen-presenting cells capable of antigen presentation to T cells, including dendritic cells, macrophages/monocytes and B cells. Antigen presenting cells express co-stimulatory molecules necessary and essential for efficient T cell activation (section 2.4). The most powerful APC is the dendritic cell as they provide vital links between the innate and adaptive immune systems (Lewis & Reizis, 2012). They have the ability to prime naive T cells and are abundantly found in the T cell areas of lymph nodes and spleen. They express high levels of co-stimulatory molecules, as well as MHC class I and II molecules, which interact with CD8+ and CD4+ T cells, respectively. Unlike DCs,
macrophages and B cells only express sufficient amount of co-stimulatory molecules for T cell activation upon infection.

2.2.1 Origin of Monocytes, Macrophages and Dendritic Cells

The most difficult challenge in human (DC) biology is understanding whether human DCs arise from distinct haematopoietic precursor cells as mouse DCs. Due to the lack of information on the existence of a committed DC precursor in humans, we will discuss the origin and development of the mouse DC.

Most dendritic cells are believed to derive from a haematopoietic bone marrow progenitor cell referred to as a common myeloid progenitor (CMP). Thus, all DCs share a common myeloid origin with monocytes, and macrophages indicating a close relationship between these cells. The CMP further differentiates into a monocyte/dendritic cell progenitor (MDPs) that is then succeeded by a common DC progenitor (CDP) (Collin, Bigley, Haniffa, & Hambleton, 2011; Frederic Geissmann, 2007; Liu & Nussenzweig, 2010). Both of these cell types are proliferating cells that express high levels of Fms-like tyrosine kinase 3 (FLT-3), a cytokine receptor expressed on haematopoietic progenitor cells essential for cell survival, proliferation and differentiation. The importance of FLT-3 in DC survival was confirmed by the observation made in FLT-3 deficient mice that exhibited a decrease in DC numbers (McKenna et al., 2000). They also express macrophage- colony stimulating factor receptor (M-CSFR), a cytokine receptor for colony stimulating factor 1 (CSF1), which controls the production, differentiation, and function of macrophages (Satpathy, Wu, Albring, & Murphy, 2012; Schmid, Kingston, Boddupalli, & Manz, 2010). Monocytes derive directly from the MDP while the CDP can directly differentiate into plasmacytoid DCs and pre- DCs. Pre-DCs are a progenitor cell to the myeloid subset and can be found in the blood, secondary lymphoid organs and tissue where they mature into myeloid DCs. Mature DCs are believed to have a life span of 5-7 days. Figure 2.2 shows the origin and differentiation of dendritic cells, monocytes and macrophages from precursor cells in the bone marrow.
Figure 2.2: The origins of human dendritic cell, monocytes, macrophages. HSC=Haematopoietic stem cell, CMP= common myeloid precursor, MDP= Myeloid/dendritic cell precursor, CDP= Common dendritic cell progenitor, moDC= monocyte derived DC.

2.2.2 Monocyte

Monocytes originate from the bone marrow and circulate in the peripheral blood for a few days before migrating to the tissue where they differentiate into a range of tissue macrophages and potentially DCs. Thus the primary role for monocytes is to replenish the pool of tissue resident macrophages in a steady state and in response to inflammation (Tacke & Randolph, 2006). Monocytes are a heterogeneous population of cells and can be subdivided into three subsets, each subset exhibiting specialised functions (Ziegler-Heitbrock et al., 2010).

According to their relative expression of CD14 and CD16 monocytes can be divided into three main subsets. The CD14\(^+\)CD16\(^-\) monocytes, which are referred to as classical monocytes, are the most prevalent subtype in the blood and express CCR2. The CD16 subset comprises of two subsets, the CD14\(^+\)CD16\(^+\) and CD14\(^-\)CD16\(^+\) also known as the intermediate and non-classical subsets, respectively (Shi & Pamer, 2011).

The relationship between the three subsets is not entirely understood but recent genome wide studies suggest that the non-classical and intermediate subsets are more closely related to each other than to the classical subset. The finding that there is a greater gene expression overlap between the former two supports the notion that the
intermediate and non-classical subsets have a closer developmental relationship (Frankenberger, Hofer, & Marei, 2012; Ingersoll et al., 2010; Ziegler-Heitbrock & Hofer, 2013)

<table>
<thead>
<tr>
<th>Human</th>
<th>Markers</th>
<th>Function</th>
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<tbody>
<tr>
<td>Classical</td>
<td>CD14^^CD16</td>
<td>Pro-inflammatory roles</td>
</tr>
<tr>
<td>Intermediate</td>
<td>CD14^&quot;CD16&quot;</td>
<td>Patrolling, antiviral roles, Pro-inflammatory?</td>
</tr>
<tr>
<td>Non-Classical</td>
<td>CD14^CD16^</td>
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Table 2.1: Human monocyte subsets

**Monocyte: Functional Properties**

To date there is a lot of conflict regarding cytokine production by the different monocyte subsets. Several reports have indicated that the intermediate subtype preferentially produces TNF-α along with IL-6 and IL-1 in response to LPS stimulus when compared to the classical and non-classical subset. In contrast, Wong et al, (2011) reported that the non-classical subset produced the greatest amounts of TNF-α in vitro in response to LPS stimulation. Likewise, Belge et al, (2002) employing a whole blood intracellular assay demonstrated that CD14 low monocytes produced the highest amounts of TNF-α, thus suggesting the non-classical subset are the dominant TNF-α producers. Reports regarding IL-10 production by the different subtypes are also inconsistent. Skrzeczynska-Monczik et al. (2008) found that intermediate monocytes exhibited the highest IL-10 production in response to LPS, while Belge and colleagues (2002) reported that the classical subset were the main IL-10 producers. In agreement with Belge, Szafalarska et al. (2004) found that the classical subset produced the highest amounts of IL-10 in response to tumour cells, in-vitro.

Concerning their antigen processing and presentation capabilities, the intermediate subset has been shown to express the highest amount of surface HLA-DR and CD40. Thus suggesting that this subset is the most efficient subtype at antigen presentation. This was confirmed by functional studies carried out by Grage-Griebenow and colleagues (2001), which showed that the intermediate subset demonstrated a higher
capacity for antigen mediated T cell proliferation.

It has been observed that the CD16+ monocytes are more motile than their CD16-counterparts (Randolph, Sanchez-Schmitz, & Liebman., et al 2002). This suggests that these monocytes may play a role in patrolling the endothelium primed to respond and transmigrate in response to inflammation. However, this study did not distinguish between the two different CD16+ monocyte subsets, thus further work is required to establish whether the intermediate or the non-classical subset is the main migratory subset.

Monocyte: Implication in Disease

In clinical studies the CD16+ monocyte subset or the intermediate subset has been shown to increase in numbers in several disease. Baeten et al. (2000) found an increase in the CD16+ subset in patients with rheumatoid arthritis that correlated with C-reactive protein and erythrocyte sedimentation rate while Kawanaka et al. (2002) found an increased frequency of the intermediate subset in patients with active disease. Cooper et al. (2012) noted an expansion in the intermediate subset in patients with long standing RA and also found that this increase was associated with reduced response therapy to methotrexate.

Koch et al. (2010) demonstrated in Crohn’s patients an increase in the CD16+ monocyte subset that correlated with a high disease activity index and colonic involvement thus suggesting that they maybe a crucial pro-inflammatory cell population in Crohn’s disease. Additional studies identified these cells as the intermediate subset CD14++^h^CD16+^lo^ . This subset was found to express CCR2, which responds to the chemokine CCL2 detected at high levels in inflamed tissue (Grip, Bredberg, Lindgren, & Henriksson, 2007). Another studied carried out in asthmatic patients demonstrated that the frequency of CD14++^h^CD16+^lo^ monocytes was significantly higher in patients with severe asthma compared mild asthmatics (Moniuszko, Bodzenta-Lukaszyk, Kowal, Lenczewska, & Dabrowska, 2009).

Thus, the finding that that CD14+^h^CD16+^lo^ monocyte subset are found to be expanded in an array of diseases, show a higher antigen presenting capacity and are

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potent producers of TNF-alpha, one may speculate that these cells may play a role in promoting inflammation in disease.

2.2.3 Macrophages

Macrophages are a highly heterogeneous population of cells with both beneficial as well as destructive roles in tissue homeostasis and host defense. In humans, CD68 antigen, an intracellular vacuolar marker, is the most used marker for the identification of macrophages. Macrophages are nonmigratory, tissue resident cells that are generally inefficient at antigen presentation. In steady state, macrophages are strategically located throughout the body where they play an important role in immune surveillance. They constantly survey their surroundings for signs of danger while ingesting apoptotic cells and toxic materials ensuring tissue homeostasis is maintained (Bain & Mowat, 2011; Weber, Saurer, & Mueller, 2009). Macrophage colony stimulating factor (M-CSF) is a major growth, differentiation, and survival factor for macrophages.

Classically macrophages have been categorised into organ specific subsets such as bone osteoclasts, liver kupffer cells, splenic red pulp macrophages, lung alveolar macrophages and brain resident macrophages known as microglia (Mosser & Edwards, 2008). The gut is populated with multiple types of macrophages that have distinct phenotypes and functions, but work together to maintain tolerance to the micro flora and dietary antigen. Due to the high degree in overlap of surface markers, macrophages have been characterised according to their specific gene expression profiles. The M1, classical activated macrophage and the M2 subset, alternatively activated macrophage each display pro-inflammatory and anti-inflammatory phenotypes, respectively (Sica & Mantovani, 2012). M1 macrophage phenotype is generated in response to microbial products or IFN-γ. They produce IL-1β, IL-6 and TNF-α and mediate cellular immunity in response to bacteria, protozoa, and viruses and have roles in antitumour immunity. In contrast, M2 macrophages produce low IL-12, IL-23 and high IL-10 and transforming growth factor beta (TGF-β) and express high levels of CD163, the haemoglobin haptoglobin (Hb/Hp) scavenger receptor. M2 macrophages are found during the healing phase of acute inflammation, as they are associated with angiogenesis, wound healing and fibrosis. The M2 derived TGF-β stimulates fibroblast differentiation into myofibroblasts contributing to tissue regeneration and wound repair. However, it is Louise Elliott
assumed that macrophages when stimulated represent a spectrum of activated phenotypes rather than distinct steady subsets. In fact recent studies have demonstrated that M1 macrophages can convert into the anti-inflammatory M2 macrophage in response to certain stimuli.

It is vital for health that tissue macrophages maintain a state of hyporesponsiveness to external stimulation. Thus, in the absence of inflammation, tissue macrophages possess intrinsic anti-inflammatory functions that ensure the macrophage does not respond inappropriately to their external environment. For example, colonic macrophages are conditioned with IL-10 rendering them unresponsive to normal gut flora and their products. Disruption to this IL-10 source leads to overt inflammation in the gut (Ueda et al., 2010). In addition to this, macrophage IL-10 is important in maintaining regulatory T cell differentiation in the intestine which is essential for maintaining tolerance to micro flora and food antigen (Mowat & Bain, 2011). Activated macrophages produce high amounts of TNF-α, IL-1 and nitric oxide, which enhance their microbicidal activity that contribute to kill invading pathogens but can also be highly destructive to surrounding tissue. The M1 macrophage is believed to play a role in an array of chronic inflammatory and autoimmune disorders. For example, in rheumatoid arthritis TNF produced by M1 macrophage trigger cytokine production by synovial cells, leading to the development of chronic polyarthritis, thus reinforcing the importance in controlling macrophage activation in preventing excessive tissue damage to the host (Murray & Wynn, 2011).

2.2.4 Dendritic Cells

Ralph Steinman first described dendritic cells 40 years ago. He described a novel cell having distinct morphological features due to their contorted shape and extending processes of varying length, hence the term dendritic cell (Steinman & Cohn, 1973). Although they are found at relatively low percentages in the peripheral blood (<1%) and in tissue (1-2%), DC are the most potent APC (Nagaraj, Ziske, & Schmidt-Wolf, 2004). They are the only APC capable of priming naïve T cells, bridging the gap between innate and adaptive immune systems (Lewis & Reizis, 2012; Ralph M Steinman, 2007). Why DCs are so efficient in T cell activation seems to be related to their high MHC expression, which is 10-100 times higher on DCs compared to other APCs and one DC has the ability to...
to activate 100-3000 T cells (Inaba & Inaba, 2005). DCs represent a heterogeneous cell population, residing in most peripheral tissues, particularly at the interface with the environment such as the skin, the mucosa lining the lungs and intestine, and peripheral blood. Dendritic cells are continuously traveling through the blood and tissue, sampling large amounts fluid containing proteins and particulates from the environment via macropinocytosis. Subsequently DCs then home to the lymph nodes where they display antigen to naïve T cells to induce an immune response that is matched to the challenge at hand (Reis e Sousa, 2004; Sallusto & Lanzavecchia, 2002; Ralph M Steinman, 2012).

DCs differ in their anatomic distribution, cell surface marker expression (pathogen recognition receptors, chemokine receptors & integrins), and function. This is reflected in the difference in requirements necessary for establishing viral immunity compared to that necessary for the elimination of bacteria, fungi, and parasites. DCs can be sub-dived into four categories: Langerhan DCs, myeloid DCs or conventional DCs, plasmacytoid DCs, and monocyte derived DCs (Table: 2.2).

Peripheral blood dendritic cells are detected using a combination of cell surface markers dependent on their presence and absence. These include the presence of class II MHC molecules and the absence of various lineage markers such as CD3 (T cells), CD19 and CD20 (B cells) CD14 (monocytes), CD56 (natural killer cells) and CD16 (neutrophils, NK cells and monocytes) (Rovati, Mariucci, Manzoni, Bencardino, & Danova, 2008).

The majority of dendritic cell knowledge has been gained about the origin, phenotype, and function of mouse DC subsets. The challenge is now to translate this knowledge to the human immune system and their relevance to human health and disease. Unfortunately, much less is known about human DCs due their rarity, lack of specific markers and restricted access to tissues. The majority of the information discussed in this section is based on human work, unless otherwise stated.

**Dendritic Cell Subsets**

**Langerhan Dendritic Cells**

Langerhan cells (LCs) were first characterised in the skin in 1868 by a medical student in Germany, Paul Langerhan. Humans have about $10^9$ epidermal LCs, located above the basal layer of proliferating keratinocytes (Merad, Ginhoux, & Collin, 2008).
Langerin/CD207 is a C-type lectin with mannose binding specificity and serves as a valuable marker for LCs. Langerin is localised in the Birbeck granules, a unique pentalamellar cytoplasmic organelle (Valladeau et al., 2000).

Through their extended dendrites, LCs forms a continuous cellular network that surveys the epidermis for foreign antigen. Interestingly, unlike other DCs, LCs can self renew in-situ independent of bone marrow or blood precursors. This was demonstrated in mice that were lethally irradiated and reconstituted with bone marrow cells. LCs from these mice were only partially eliminated and repopulated the skin independently of donor circulating precursor cells (Merad et al., 2008).

Myeloid DCs

Myeloid DCs (mDC) or conventional DCs, are one of the two major subsets found in the peripheral blood but are also found in secondary lymphoid organs, and peripheral tissues. They are characterised based on their lack of expression of lineage-associated markers and the presence of CD11c. CD11c, a αx integrin is a member of the leukointegrin family involved in cell-cell interaction. CD11c is found associated with CD18, which forms the ligand for CD54 and fibronectin and possesses the ability to mediate phagocytosis of iC3b-opsonised particles (Sadhu et al., 2007).

Blood mDCs can be further divided dependent on their expression of blood dendritic cell antigen (BDCA)-1 (CD1c) or BDCA-3 (CD141). CD1c is a glycoprotein that mediates presentation of lipid antigens whereas, CD141 has been identified as thrombomodulin and can mediate coagulation by interaction with thrombin and protein C (Sadler, 1997). However, nothing is known about its function on the CD141+ DC subset. CD1c is expressed on the majority of peripheral blood dendritic cells, found on about 80% of myeloid DCs.

The minor subset, CD141+, is a potent producer of interferon-λ and β (Lauterbach et al., 2010) (Yoshio et al., 2012) and expresses TLR3 and TLR8 (Jongbloed et al., 2010). They can be found in various tissues such as dermis, liver, and lung and mediate cross presentation of exogenous antigens important for the activation of cytotoxic lymphocytes (Joffre, Segura, Savina, & Amigorena, 2012; Jongbloed et al., 2010). More recently, it has come to light that these cells may a play role in viral immunity, especially in hepatitis C, as they are found in a higher frequency in the liver compared to blood and
produce high amounts of IFN-α when stimulated with HCV protein (Yoshio et al., 2012).

Myeloid DCs are highly phagocytic and are very effective at antigen presentation and T cell activation. Myeloid DCs express TLR 2 and TLR 4 and produce high amounts of IL-12 and TNF-α upon activation. These cells encounter and capture antigens in peripheral tissue and subsequently migrate to the secondary lymphoid organs where they initiate an adaptive immune response. Therefore they are seen as mobile sentinels capable of bringing antigen to the corresponding antigen specific T cell (R M Steinman & Inaba, 1999). In addition to their ability to recognise and eliminate foreign antigen, mDCs are fundamental in the induction of tolerance to self, discussed in section 2.4.

Plasmacytoid DC
In addition to the mDC subsets, a second subset can be found in the blood known as plasmacytoid DCs (PDC). They reside primarily in the lymphoid organs in a steady state, and enter the lymph nodes via the blood where they are primarily located in the T cell zones.

PDCs express CD123⁺ (the IL-3 receptor) and are low or negative for the integrin CD11c. More recently several specific plasmacytoid markers have been established for the identification of plasmacytoid DC including BDCA-2 and BDCA-4 (Dzionek et al., 2000). BDCA-2 also known as CD303/neurophilin-1, is a novel type II transmembrane C-type lectin. PDCs can take up ligands via CD303, allowing them to process and present the ligands to T cells. They depend on IL-3 and CD40L for survival and differentiation into mature DCs (Grouard et al., 1997). They display poor antigen-presenting capacity compared to their myeloid counterparts due to their high MHC II turnover on their cell surface (Villadangos & Young, 2008).

Plasmacytoid DCs express endosomal nucleic acid sensing toll like receptors 7 and 9 and produce large amounts of type I interferons, up to 1000 fold more than other cells, in response to viral infections (Colonna, Trinchieri, & Liu, 2004; Swiecki & Colonna, 2010). Human PDCs produce all the subtypes of type 1 interferons, IFNα, IFNβ, IFNλ, IFNω and IFNτ. These powerful immunomodulatory functions of PDCs contribute to the recruitment and activation of other immune cells such as NK cells, mDCs, T cells and B cells thus are responsible for orchestrating and initiating both the innate and adaptive
immune response (Gilliet, Cao, & Liu, 2008; McKenna, Beignon, & Bhardwaj, 2005). PDC derived IFNs have been shown to trigger IL-12, IL-15, IL-18, and IL-23 production by mDCs and to induce monocytes differentiation into moDCs. They can also enhance mDC ability to cross present exogenous antigens to CD8+ T cells. In the presence of IL-6, IFNs can drive B cells to differentiate into antibody secreting plasma cells (Reizis, Bunin, Ghosh, Lewis, & Sisirak, 2011).

Plasmacytoid DCs are also implicated in the induction of central and peripheral tolerance. It is believed that circulating PDCs may present self-antigen in the thymus, contributing to T cell deletion of auto reactive T cells. Martin-Gayo and Colleagues (2010) showed that PDCs in the human thymus, when activated with CD40L and IL-3 efficiently promoted the generation of natural T regulatory cells from autologous thymocytes. Moreover, these Treg cells exhibited similar IL-10 and TGF-beta cytokine expression to that of Treg cells primed by myeloid DCs. In mice, PDCs were shown to prevent T cell priming while promoting systemic tolerance of CD4(+) and CD8(+) T to dietary antigen. In fact, systemic depletion of PDCs prevented induction of tolerance by antigen feeding, suggesting that oral tolerance depends on antigen presentation by PDCs (Goubier et al., 2008). In vitro, PDCs have been shown to promote the induction of IL-10 producing T regulatory cells via the interaction of inducible costimulator ligand (ICOSL) with ICOS expressed on naïve T cells (Tomoki Ito et al., 2007).

Plasmacytoid DCs have been most extensively studied in hepatitis C viral infection (Liang et al., 2009; Libri, Barker, Rosenberg, & Semper, 2009; Mengshol et al., 2009). It has been demonstrated that HCV-infected cells can trigger robust IFN responses in plasmacytoid DCs via viral replication, direct cell-cell contact, and toll-like receptor 7 signalling (Takahashi et al., 2010). The same group showed that activated PDC supernatants inhibited HCV infection in an IFN dependent manner. In contrast, another group demonstrated that HCV could specifically impair plasmacytoid DC function thereby compromising T cell responses allowing HCV to escape the immune surveillance (Yonkers et al., 2007).
Monocyte derived Dendritic Cells

Due to their rarity, DCs were difficult to study until the late 1990s when researchers learned how to generate DCs from CD14+ monocytes cultured with IL-4 and GM-CSF, referred to as monocyte derived DCs (moDC) (Chapuis et al., 1997). They express high levels CD11c and HLA-DR, however, these DCs are morphologically and functionally distinct from myeloid DCs isolated from the peripheral blood (Andersson, Cirkic, Hellman, & Eriksson, 2012; Osugi, Vuckovic, & Hart, 2002). moDCs are larger and more granular cells than myeloid DCs and also produce higher amounts of tumour necrosis factor-alpha (TNF-α) and IL-12 when stimulated with TLR antagonists. In-vitro monocyte derived DCs demonstrate potent antigen-presenting capacity.

In mice under inflammatory conditions, peripheral monocytes are believed to migrate to sites of inflammation and differentiate into cells that exhibit many phenotypical features of DCs. However, due to the lack of reliable markers to distinguish classical myeloid DCs from moDCs, it has remained difficult to prove that this phenomenon occurs in vivo in humans. However, Choeng et al. (2010) identified DC-SIGN positive DCs in the lymph node of mice stimulated with LPS. DC-SIGN is a lectin receptor expressed on human moDCs generated in-vitro but not on myeloid DCs (Geijtenbeek et al., 2000). Choeng et al believed they were moDCs as they were unidentifiable in mice depleted of monocytes and present in mice that were FLT3 deficient, which lack the classical myeloid subset thus, suggesting that this marker may be useful in the identification of moDC in vivo.

Not previously mentioned but noteworthy, monocytes have been described as a precursor to a subset of DCs, named TipDCs identified in mouse only. TNF and iNOS (inducible nitric oxide) producing DC (TipDC) are specialised in innate immunity against pathogens and display remarkable microbicidal activity. They were first identified in spleens of Listeria monocytogenes infected mice recruited to the site of infection in a CCR2 dependent manner (Serbina, Salazar-Mather, & Biron, et al., 2003). TipDCs are not required for T cell priming but rather exert innate effector functions through the production of soluble mediators and microbicidal activity (Schmid, Wege, & Ritter, 2012).
<table>
<thead>
<tr>
<th>Name</th>
<th>Markers</th>
<th>Cytokines</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Langerhan Cell</td>
<td>Langerin, (CD207)</td>
<td></td>
<td>Maintain tolerance and Immunity in the skin</td>
</tr>
<tr>
<td>Myeloid CD141+ subset</td>
<td>CD11c+, CD11c+(BDCA-3),</td>
<td>IFN-λ, TLR3</td>
<td>Cross presentation CD8 T cell responses</td>
</tr>
<tr>
<td>Myeloid CD1c+ subset</td>
<td>CD11c+, CD1c</td>
<td></td>
<td>MHC-class II restricted antigens, CD4+ T cell responses</td>
</tr>
<tr>
<td>Plasmacytoid DC</td>
<td>CD123+, CD11c-, TLR 7,</td>
<td>Type I</td>
<td>Immunity against virus Interferons Oral tolerance</td>
</tr>
<tr>
<td></td>
<td>TLR 9</td>
<td></td>
<td></td>
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<tr>
<td>Monocyte derived DCs</td>
<td>CD11c+</td>
<td>IL-12 TNF-α</td>
<td>Function in vivo unknown May represent a proinflammatory subset</td>
</tr>
<tr>
<td>TipDCs</td>
<td></td>
<td>TNF-α, iNOS</td>
<td>antimicrobial defense (mouse)</td>
</tr>
</tbody>
</table>

Table 2.2: Dendritic cell subsets

2.3 Antigen Recognition by T cells

2.3.1 T cell Receptor

T cells recognise antigen via specialised cell surface antigen receptors - T cell receptors (TCRs) and are highly restrictive in their antigen recognition capabilities. TCRs recognise short peptides, which are presented by major histocompatible complex (MHC) molecules on the surface of host cells. The T cell receptor is a heterodimer composed of two chains, 95% of T cells express the αβ TCR and the remaining 5% express the γδ TCR. Both TCRs resemble immunoglobin structures and consist of a large extracellular domain, transmembrane segment and a short cytoplasmic tail. Each α and β extracellular domain is composed of a constant region which links the two chains together, and a variable region for binding antigen (Bentley & Mariuzza, 1996). Each T lymphocyte carries ~100,000 T cell receptors (TCR) on its surface (Sykulev et al., 1994).

To form a complete functional and stable T cell receptor the αβ TCR must...
associate with the CD3 complex, a series of polypeptide chains. The CD3 complex is made up of a $\gamma$, $\delta$, 2 $\varepsilon$ and $\zeta$ chains. The CD3 chains are assembled as heterodimers of $\gamma\varepsilon$ and $\delta\varepsilon$ subunits with a homodimer of $\zeta$ chains (Figure 2.3). All chains except for the $\zeta$ chains are composed of an external Ig-like C domain, a transmembrane segment, and a long cytoplasmic tail. The function of these accessory molecules is to communicate the signal from the T cell receptor into the cell (San José, Sahuquillo, Bragado, & Alarcón, 1998). The $\zeta$ chains are structurally unrelated to the other CD3 components. The $\zeta$ is composed of a small extracellular domain, transmembrane domain, and a long cytoplasmic tail which contain subunits called immunoreceptor tyrosine-based activation motifs (ITAMs). These ITAMs become phosphorylated upon TCR interaction with a MHC peptide complex and are essential for T cell activation (Baniyash et al., 1988; Smith-Garvin, Koretzky, & Jordan, 2009).

![Figure 2.3: Structure of T cell receptor complex. TCR: T cell receptor](Taken from Mayer & Nyland (2010))

### 2.3.2 Major Histocompatibility Complex (MHC) Restriction

The major histocompatibility complex contains 128 functional genes making it one of the most gene dense regions of the human genome (Beck et al., 1999). It is also the region with the most disease associations with the majority of autoimmune diseases having a connection with genes in the MHC (Wandstrat & Wakeland, 2001) MHC genes Louise Elliott
MHC Class I Molecules

The MHC class I gene complex contains three loci, human leucocyte antigen (HLA) A, B, and C, each of which codes for a \( \alpha \) chain (Neefjes et al., 2011). The MHC class I molecule consists of two polypeptide chains; the alpha chain encoded by chromosome 6 and the non-covalently associated \( \beta 2 \) microglobulin. It is composed of 4 domains, three made from the alpha chain, and one from the beta microglobulin. The \( \alpha 1 \) and \( \alpha 2 \) domains fold to make the peptide binding cleft accommodating peptides up to 10 amino acids in length. All nucleated cells express MHC class I molecules and present endogenous antigen synthesised within the cell to CD8\(^+\) cytotoxic T cells (Shastri et al., 2005). This mechanism is particularly important in the recognition of intracellular bacteria and viruses that live within the cell hidden from the immune system.

The MHC- class I requires the merger of two distinct pathways. In the cytosol, peptides are generated from proteasomal degradation from normal cellular proteins as well as intracellular bacteria and viruses. These peptides are subsequently transported from the cytoplasm to the endoplasmic reticulum (ER) by transporter-associated antigen (TAP), a heterodimer ATP-dependent transporter (Neefjes et al., 2011; Pamer & Cresswell, 1998; van den Elsen & Rudensky, 2004). TAP preferentially transports peptides 8-12 amino acids in length. In the ER specialised proteases, ER aminopeptidases (ERAP) 1, trim peptides to an appropriate length for class I binding (Mellman &
In the ER, the early stage of MHC class I maturation involves the binding of the MHC heavy chain (HC) with chaperone calnexin. When the HC binds the \( \beta_2 \) microglobulin, calnexin is replaced with calreticulin. Both chaperones are involved in the correct folding of the HC, facilitating its interaction with \( \beta_2 \) microglobulin (Jensen, 2007). An additional chaperone involved in the folding and stabilisation of the MHC-molecule is ERp57 (Neefjes et al., 2011). When the MHC class I dimer is formed, it binds peptides transported into the ER to stabilize protein conformation. If the peptide fits the groove well dependent on amino acid composition and size, the class I molecule will stabilise and will be transported to the cell surface (Cresswell, Ackerman, & Giodini, et al., 2005). Tapasin associated with TAP supports peptide editing for the selection and presentation of stable-peptide MHC-class I complexes (Jensen, 2007; Shastri et al., 2005) Figure 2.4 illustrates the antigen-processing pathway for endogenous antigen.

![Antigen-processing pathway](image-url)

**Figure 2.4:** The MHC class I antigen presentation pathway. ER: endoplasmic reticulum, TAP: transporter associated with antigen presentation, ERAD: ER-associated protein degradation, TCR: T cell receptor. Image depicted from Neefjes et al., (2011)

**MHC- Class II Molecules**

There are three MHC class II molecules in humans, these are known as HLA-DQ, DR, and
Each of these codes for one α and a variable number of β chains (Neefjes et al., 2011). MHC-class II molecules are heterodimers composed of a non-covalent complex of two α/β chains. The peptide-binding cleft is formed from the α1 and β1 chains. The groove is open ended so it can accommodate peptides up to 30 amino acids long. The MHC-class II molecules present peptides derived from proteins phagocytosed from the extracellular environment. The exogenous antigen is proteolytically processed via the endocytic route and derived peptides are subsequently presented on the surface for CD4 T cells to recognise (Al-Daccak, Mooney, & Charron, 2004).

The short antigenic peptides are generated within the endosomal and lysosomal compartments. The acidic environment characteristic of these compartments provides an optimal environment for antigen processing. The low pH contributes to protein unfolding and denaturation, while the acidic proteases participate in antigen proteolysis (Neefjes et al., 2011).

To ensure MHC-class II molecules intersect with the appropriate peptides generated, a chaperone invariant chain associates with newly synthesised MHC-II molecules in the ER where they are subsequently transported to the endocytic pathway. Once in the endocytic pathway the invariant chain is cleaved as a result of lysosomal proteases (cathepsin) and low pH, leaving a small fragment called CLIP (class II-associated invariant chain peptide) bound to the peptide-binding cleft (Li et al., 2005; Mellman & Cresswell, 2010). CLIP is dislodged from the αβ dimer and exchanged for resident antigenic peptides, a reaction catalysed by Human leukocyte antigen (HLA)-DM. HLA-DM performs the vital function of editing the repertoire of MHC II class associated peptides to ensure selection of those with maximum binding affinity (Fig 2.5) (Jensen, 2007; Elsen & Rudensky, 2004).

MHC-class II molecules are normally expressed on a subset of specialised cells referred to as antigen-presenting cells (APC) composed of mainly B cells, monocytes, macrophages and dendritic cells (Al-Daccak et al., 2004).
Cross presentation

Classically it is stated that MHC-I molecules bind peptides from cytosolic proteins, while MHC-II molecules bind peptides from external sources. However, this dichotomy is not always the case, as a specialised subset of dendritic cells can phagocytose exogenous antigens and display peptides from them in the context of a MHC-I molecule. This process is known as cross presentation and is fundamental in priming CD8+ T cell responses to viruses and other pathogenic organisms that do not directly infect antigen-presenting cells (Ackerman & Cresswell, 2004; Cresswell et al., 2005).
2.4 Dendritic cells: Versatile controllers of the immune system

2.4.1 T cell activation

It is now abundantly clear that innate immune responses are essential for the generation of T cell responses and central to this process are the dendritic cells. They can both create and curtail immunity and are the only APC capable of priming naïve T cells (Steinman, 2007). They can direct T cell effector differentiation thus ensuring specificity of the innate immune response is translated into an equally specific adaptive immune response (Bakdash et al., 2013).

For APCs, specifically DCs, to sufficiently elicit an adaptive immune response, DCs must transform from an immature antigen capturing APC into a fully functioning APC capable of presenting high amounts of MHC/peptide complexes on its cell surface (Banchereau & Steinman, 1998; Cella et al., 1997). Mature dendritic cells deliver three crucial signals essential for the expansion and differentiation of naïve T cells (Bakdash et al., 2013; Kapsenberg, 2003; Mosmann & Livingstone, 2004; Ralph M Steinman, 2007). DCs can be activated in a PAMP dependent manner or independent manner mediated by inflammatory cytokines such as TNF-α and IL-1 (Reis e Sousa, 2004). DCs can also respond to DC-T cell interactions. Upon activation DCs change in morphology, downregulate their endocytic capacity, increase MHC/peptide complexes and co stimulatory molecules, such as CD80 and CD86 on the cell surface (Cella et al., 1997; Reis e Sousa, 2006). Mature DC are also characterised by cytokine production (IL-2, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α, IL-1β and IL-6). Maturation also results in loss of adhesive structures and upregulation of CCR7, a chemokine receptor, which is responsible for migration of the DC to T cell rich areas in lymph nodes (LN) (Banchereau et al., 2000; Bousso, 2008). It is important to note that different subsets of DCs express different cell surface markers and cytokines that selectively determine the type of immune response induced.

Signal one is composed of the antigen-specific signal derived from the interaction of a specific peptide:MHC complex on the APC with the T cell receptor (Fig 2.6). The initial encounter of T cells with APC is by non-specific binding mediated through adhesion molecules (Bakdash et al., 2013). This allows the T cell to encounter a large
number of different MHC-peptide complexes and in the absence of a specific interaction
the T cell dissociates. The specific MHC molecule-peptide-TCR interaction alone is not
sufficient to trigger T cell activation (Reis e Sousa, 2006).

A second signal is required, referred to as co-stimulation. The most potent co-
stimulatory molecules are B7.1 and B7.2 also referred to as CD80 and CD86, respectively
and are crucial for T cell activation (Fig 2.6) (Bakdash et al., 2013; Coquerelle & Moser,
2010; Sharpe & Freeman, 2002). B7 molecules are found exclusively on APCs and their
ligand CD28 is found on the cell surface of the T cell (Fig 2.6) (Greenwald, Freeman, &
Sharpe, 2005). The CD28 receptor is constitutively expressed on T cells. Ligation of CD28
on naïve T cells by either CD80 or CD86 ligands provide a potent costimulatory signal to
T cells (Carreno & Collins, 2002; Caux et al., 1994). Binding of CD28 increases
transcription of IL-2mRNA, a cytokine that drives the proliferation and differentiation of
T cells, expression of CD25, the receptor for IL-2, and entry into the cell cycle (Sallusto &
Lanzavecchia, 2002). CD28 co-stimulation is essential for the activation of naïve T cells
and blockade of this interaction results in ineffective T cell activation (Caux et al., 1994).

An additional co-stimulatory molecule found on activated T cells is ICOS, which
interacts with ICOS ligand on APCs mainly DCs and B cells (not shown in Fig). ICOS+ T
cells are predominantly located in the germinal centres and surrounding T cell zones of
lymphoid tissue. The ICOS/ICOSL pathway appears to play an important role in isotype
switching of B cells and germinal center formation (Berkel & Oosterwegel, 2006).
Moreover activated plasmacytoid DCs express higher levels of ICOSL and low levels of
CD86/CD80. These DCs have been shown to promote T regulatory differentiation in an
ICOS dependent fashion via the production of IL-10 (Tomoki Ito et al., 2007; Tuettenberg
et al., 2009)

For optimal expression of T helper-cell polarizing molecules, the primed DC
requires intimate cross talk with the T cell mediated through the ligation of CD40 with
CD40L (Fig 2.6). CD40L is rapidly upregulated on the T cell following signal one and two,
and interacts with CD40 on activated DCs or other APCs (Kapsenberg, 2003). Ligation of
CD40 results in increased expression of co-stimulatory and MHC molecules and
promotes the production of T cell stimulatory cytokine IL-12 (Haenssle et al., 2008).
The B7 molecules can alternatively bind to CTLA-4; this molecule is not detected on naïve T cells and is upregulated upon T cell activation (not shown in Fig). CTLA-4 functions as an inhibitory receptor essential for downmodulating immune responses (Greenwald et al., 2005). The critical role for CTLA-4 has been illustrated in CTLA-4 deficient mice that die 3 weeks after birth due to a massive T cell infiltration and tissue destruction. CTLA-4 binds to B7 molecules with a much higher affinity compared to that of CD28 limiting the degree of T cell activation (Bakdash et al., 2013; Carreno & Collins, 2002; Chen, 2004; Sharpe & Freeman, 2002).

An additional co-inhibitor is programmed cell death-1 (PD-1), which has two ligands, PD-L1 and PD-L2. PD-L1 and PD-L2 are upregulated on the cell surface of DCs upon activation (not shown in Fig). The interaction of T cells with DCs via PD-1 results in the delivery of an inhibitory signal to the T cell, which inactivates PI3-K. IFN-gamma production by the T cell is dampened and apoptosis is induced (Bakdash et al., 2013; Parry et al., 2005). Evidence for PD-1 involvement in negative regulation of T cell responses was illustrated in PD-1 deficient mice, which spontaneously developed
autoimmune disease (Chen, 2004).

The third signal delivered by the chemical environment at the time of the T cell/APC interaction influences the differentiation and polarisation of the naïve CD4+ T cell into the different effector T cells. There are several subsets of effector T cells with a variety of functions defined by their cytokine production. The main functional classes are Th1, Th2, Th17 and regulatory T cells (Treg) (Zhou, Chong, & Littman, 2009; Zygmunt & Veldhoen, 2011). Th1 development is induced when signal 3 is composed of cytokine IL-12. These effector T cells will produce copious amounts of IFN-γ upon interaction with their specific antigen, which in return activates macrophages prompting them to destroy phagocytosed microorganisms (H. Yamane & Paul, 2012). Th2 cells are driven by IL-4 and are controlled by the GATA-3 transcription factor. They produce IL-4, -5, -6, -9, -10 and -13, which provide B cell help and trigger antibody production and isotype switching (Allan, 2008; H. Yamane & Paul, 2012). Th17 cells arise in the presence of cytokines IL-23, IL-6, and transforming growth factor but in the absence of IL-4 and IL-12. These cells promote inflammation and have been implicated in the pathogenesis of several autoimmune diseases (Bettelli et al., 2006; Kimura & Kishimoto, 2010). Regulatory T (Treg) cells have also been described, which produce cytokines that dampen or halt inflammatory processes (IL-10, TGF-β), thus these cells prevent unwanted immune responses and development of autoimmunity. Treg cells are identified by the expression of the transcription factor forkhead box P3 (Foxp3), which plays a critical role in specifying and maintaining the functional program of Treg cells (Sakaguchi et al., 2010). TGF-β drives the development of T regulatory cells, while the presence of IL-6 inhibits TGF-beta induced Treg differentiation (Kimura & Kishimoto, 2010). Overproduction of IL-6 can lead to the development of autoimmune disorders, such as rheumatoid arthritis where Th17 cells are believed to play a pathogenic role (Kim et al., 2013; Pesce et al., 2013). Figure 2.7 describes the different T cell effector cells and their function.
2.4.2 Tolerance - Dendritic Cell

It has been proposed that dendritic cells may play a pivotal role in the maintenance of both central and peripheral tolerance. The thymus steadily produces thymocytes expressing newly assembled T cell receptors, some of which are reactive with components of self (Klein et al., 2009). High affinity auto-reactive thymocytes are eliminated upon encountering self-MHC peptide (Starr, Jameson, & Hogquist, 2003). Along with the deletion of auto reactive T cells, the thymus is responsible for the development of natural occurring T regulatory cells central in maintaining tolerance (Sakaguchi, 2004). It has been postulated that peripheral DCs that recirculate into the thymus can directly present self-antigen to developing thymocytes inducing thymocyte deletion or the generation of induced Foxp3+ Treg cells. However it is unknown whether this mechanism is relevant in vivo as depletion of myeloid DCs in mice did not disrupt central tolerance (Proietto et al., 2009). There are several limitations to the theory of central deletion, such as the requirement for the autoantigen to be present in the thymus, as well as the more stringent negative selection becomes, the greater the risk of
dangerously narrowing the repertoire available to respond to foreign pathogens. Therefore, additional mechanisms responsible for maintaining tolerance in the periphery are necessary to prevent loss of tolerance and development of autoimmunity via the activation of self-reactive T cells that escape elimination in the thymus. This phenomenon is known as peripheral tolerance (Walker & Abbas, 2002).

Several mechanisms mediated by dendritic cells are central to the developmental of peripheral tolerance. DCs have been shown to interact with T cells even in the absence of infection. In fact self-proteins are continuously sampled by immature DCs and presented to self-reactive T cells enforcing T-cell tolerance by several mechanisms. Such mechanism include apoptosis of effector T cells, the generation of Treg cells and T cell anergy, and the alteration of T cell phenotype (Amodio & Gregori, 2012; Gregori, 2011; Hawiger et al., 2001). T cell activation in the absence of co-stimulatory molecules can lead to T-cell tolerance by inducing T cell anergy, clonal deletion of auto reactive T cells or the induction of Treg cells. T cell anergy induces a state of "hyporesponsiveness" characterised by low affinity TCR signaling and decreased cytokine production. These T cells are unable to respond upon re-encounter with their antigen, even in the presence of co-stimulation molecules (Cools et al., 2007; Lutz & Schuler, 2002).

In addition, different subsets of tolerogenic myeloid DCs have been described in different tissue compartments where they promote tissue homeostasis via the differentiation and proliferation of Treg cells. The most well described phenotype is the CD103+ subset (discussed in section) whereas a minor subset expressing CD163 has been identified in the blood (Maniecki et al., 2006). Their exact mechanism of suppressive function is still much debated, however, four different modes of actions have been proposed: 1) suppression by inhibitory cytokines IL-10 and TGF-beta (Mills & McGuirk, 2004) 2) suppression by cytolysis, 3) suppression by metabolic disruption, and 4) suppression by modulation of DC maturation or function (Bordon, 2012; Vignali, Collison, & Workman, 2008). Thus, defects in the activation of tolerogenic or immunogenic DCs may contribute to the breakdown in self-tolerance leading to autoimmunity.
2.4.3 Dendritic cells and autoimmunity

Considering that antigen presenting cells, especially dendritic cells, are key regulators that determine the outcome of the adaptive immune response be it immunity or tolerance, it comes as no surprise that a system as complex as the dendritic cell system may suffer dysregulation that results in the loss of tolerance to self-resulting in autoimmunity. Autoimmunity can be defined as the breakdown in tolerance, which allows the immune system to attack self-tissue resulting in tissue damage. This is characteristic of many diseases, such as type 1 diabetes, systemic erythematosus lupus, multiple sclerosis, rheumatoid arthritis and coeliac disease. The perplexing issue of what allows the immune system to attack self-tissue is not well understood and is the continuing focus of research today.

It is hypothesised that the aberrant presentation of self or harmless antigen by APCs, in particularly the DC, to naive T cells can skew the adaptive immune response driving autoimmunity. Several studies carried out in an array of autoimmune diseases indicate that abnormal DC activation or functions is associated with self reactive responses and inflammation (Amodio & Gregori, 2012; Bayry et al., 2004; Drakesmith, Chain, & Beverley, 2000).

In rheumatoid arthritis (RA) patients, it has been reported that both myeloid and plasmacytoid DC are increased in the peripheral blood and synovial fluid compared to healthy controls. Whereas myeloid DCs are found closely related to T lymphocytes in the inflamed joints (Lutzky, Hannawi, & Thomas, 2007; Sarkar & Fox, 2005). Moreover, these DCs exhibited a more mature phenotype and functional profile compared to healthy controls expressing higher levels of MHC II class molecules and co-stimulatory molecules CD80, CD54, and CD40 (Santiago-Schwarz, 2004; Takakubo et al., 2008). These myeloid DCs were involved in the promotion of synovial inflammation via the production of pro-inflammatory cytokines (Jongbloed et al., 2006). The infiltration of DCs into the synovial allows DCs to take up, process and present antigen locally, contributing to disease exacerbation.

In patients with systemic lupus erythematosus (SLE), reduced numbers in both peripheral blood plasmacytoid and myeloid DCs were observed when compared to healthy controls (Robak et al., 2004). Myeloid DCs displayed an activated phenotype
with higher levels of co-stimulatory molecules CD80 and CD86 (Chan et al., 2012; Gerl et al., 2010). As for plasmacytoid DCs, their reduction in blood correlated with their infiltration into nephritic kidneys and inflamed skin lesions suggesting their migration to sites of inflammation (Mori et al., 1994; Tucci et al., 2008). Interestingly, peripheral leucocytes of SLE patients exhibited an upregulation in IFN-inducible genes that correlated positively with disease activity. Plasmacytoid DCs are potent producers of IFN-α further supporting the notion that DCs have a central role in driving SLE development (Rönnblom, Eloranta, & Alm, 2003). One proposed mechanism by which plasmacytoid DCs drive autoimmune responses in SLE is by unabated activation of myeloid DCs via the production of IFN-α (Palucka, Banchereau, Blanco, & Pascual, 2002). Furthermore in a mouse model of lupus dendritic cells were shown to be necessary for T cell expansion and differentiation but not for their initial activation (Teichmann et al., 2010).

In clinically defined multiple sclerosis (MS) patients, both myeloid and plasmacytoid DCs are found elevated in the cerebrospinal fluid (CSF) (Pashenkov et al., 2001). Furthermore, myeloid DCs were shown to respond chemotactically in response to RANTES (chemokine ligand 5) and MIP-1β (Macrophage inflammatory protein), which are expressed in the MS lesion (Pashenkov et al., 2002). An increased frequency of myeloid DCs are found within the demyelinating lesions suggesting that DCs play a role in the activation of myelin specific T cells upon entry to the CNS (G. F. Wu & Laufer, 2007).

DCs expressing high levels of CD83 have been shown to accumulate at sites of inflammation in both ulcerative colitis (UC) and Crohn’s disease, whereas both myeloid and plasmacytoid DCs subsets are depleted in the peripheral blood. One study demonstrated that peripheral dendritic cells from these patients showed increased T cell stimulatory capacity compared to healthy controls (Middel et al., 2006). Furthermore the infiltration of DCs into the inflammatory lesion was shown to positively correlate with the degree of crypt inflammation (Watanabe et al., 2007). DC recruitment into the inflammatory lesion may be the result of increased expression of intracellular adhesion molecule-1 (ICAM-1), and chemokine receptors CCR6 and CCR7. CCR6 is the receptor for CCL20 that promotes the CCR6-dependent recruitment of DCs into the mucosal surfaces.
Taken together, all of these studies clearly show that accumulation and hyperactivation of DC may be a key factor in promoting the generation of auto-reactive T cell responses. Thus, DCs are recruited to sites of inflammation where they are primed by the pro-inflammatory milieu to produce pro-inflammatory cytokines or to express high levels of co-stimulatory molecules that induce activation of antigen specific auto-reactive T cells (Amodio & Gregori, 2012).

For the majority of autoimmune disorders the causative agents have not yet been identified. However, coeliac disease (CD), to date, is the only autoimmune disorder where the genetic component, auto-antigen, and environmental trigger have been all identified contributing to its uniqueness. CD also shares many features with other autoimmune disorders making it an interesting human model for studies of autoimmunity and immunopathology.

2.5 Coeliac disease

2.5.1 Introduction

Coeliac disease is defined as a chronic small intestinal immune, mediated enteropathy induced by dietary gluten in genetically susceptible individuals (Gujral, Freeman, & Thomson, 2012; Sollid & Jabri, 2013). It is one of the most common autoimmune disorders found in both children and adults with a prevalence of 1% of the population in Europe and the USA (Gujral, Freeman, & Thompson, 2012). The only course of treatment for CD is a life long gluten free diet, which leads to histological and clinical improvement in the majority of patients (Rodrigues & Jenkins, 2008). However, a small number of patients do not respond to a gluten free diet, and are classified as having refractory coeliac disease. Refractory coeliac disease can be subdivided into categories, type I and II (Kagnoff, 2012; Malamut, Murray, & Cellier, 2012), the latter is now considered as an intraepithelial lymphoma with poor response to treatment and is frequently the precursor to overt T cell lymphoma (Malamut et al., 2012).

Coeliac disease is associated with a wide spectrum of clinical presentations often influenced by the age of onset and duration of disease. Children diagnosed with coeliac disease at a very young age present more often with "classical" symptoms marked by Louise Elliott
diarrhoea, abdominal distension, and failure to thrive. Older children and adolescents present with atypical gastrointestinal symptoms such as pain, vomiting, and constipation (Fasano, 2005). In adults the diagnosis of coeliac disease tends to be more complicated and often goes undiagnosed for many years. Some patients are asymptomatic and are diagnosed incidental due to the recognition of villous atrophy during endoscopy performed for any reason or serological screening of high-risk groups. Other patients present with atypical symptoms such as abdominal pain, diarrhoea, constipation, fatigue and these individuals are often mistaken as having irritable bowel syndrome (Reilly & Green, 2012; Hopper, Hadjivassiliou, Butt, & Sanders, 2007). Other presentations include anemia due to iron deficiency, osteoporosis, dermatitis herpetiformis, infertility and neurological problems (Lionetti & Catassi, 2011).

There is a strong hereditary component associated with coeliac disease and several studies have shown that 4-10% of first-degree relatives have the disease with the greatest risk among siblings (Lionetti & Catassi, 2011). Similar to other autoimmune disorders women are two or three times more likely to be diagnosed with CD compared to men. It is well established that there is strong association between coeliac disease and other autoimmune diseases as 15-20% of patients with CD have or will develop other autoimmune diseases the most common being type I diabetes and autoimmune thyroid disease (Reilly & Green, 2012; Feighery, 1999; Marchese, Lovati, Biagi, & Corazza, 2013; Pham-Short, Donaghue, Ambler, Chan, & Craig, 2012). Another important finding is the increased prevalence of CD in Down syndrome children with studies reporting a six fold increased risk of developing CD in this susceptible population (Cogulu et al., 2003; Márild et al., 2013).

Coeliac disease is one of the best-understood autoimmune diseases that can be described as a multifactorial disease with genetic, environmental, and immunological factors contributing to disease pathogenesis. The key components are the specific genetic background (HLA-DQ2.5 and HLA-DQ-8), environmental trigger (prolamins), and the autoantigen (tissue transglutaminase).
2.5.2 Diagnosis of Coeliac Disease

Serology

At present, diagnosis and screening of CD begins with the use of serological markers: IgA anti-tissue transglutaminase (tTG) and anti-endomysial antibodies (EMAs) while the patient is on gluten containing diet. Testing of patients on gluten free diet can lead to false negative serology tests. Anti-gliadin antibodies are less sensitive and are no longer recommended in the diagnostic workup for CD (Nelsen, 2002; Presutti, Cangemi, Cassidy, & Hill, 2007). Recently, there has been the commercial development of tests that are targeted against deaminated gliadin peptides (DGP) that yield a sensitivity of 94% and specificity of 99% (Harris et al., 2012). The role of DGP in diagnosis has yet to be established but could be proven to a useful test when used in combination with tTG. IgA EMA and tTG antibodies have been shown to have a sensitivity and specificity of greater than 95% for coeliac disease, illustrated in table 2.3 (Gujral et al., 2012; Nelsen, 2002).

Approximately 2% of individuals with coeliac disease have selective IgA deficiency compared with 0.5% of the general population. Therefore, if the serum IgA tTG result is negative but clinical suspicion for the disease is high, a total serum IgA measurement should be obtained (McGowan, Lyon, & Butzner, 2008).

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG, IgA anti-gliadin antibody</td>
<td>70%</td>
<td>97%</td>
</tr>
<tr>
<td>IgA anti-endomysial antibody</td>
<td>85-98%</td>
<td>97-100%</td>
</tr>
<tr>
<td>IgA anti-tissue transglutaminase antibody</td>
<td>95-98%</td>
<td>94-95%</td>
</tr>
</tbody>
</table>

Table 2.3: Diagnostic specificity of serological markers.

Novel Serological Markers

In recent years, additional biomarkers for coeliac disease have been identified, both previously studied in our lab. IgA autoantibodies directed against smooth muscle (SM) actin were found in serum of a subset of patients with coeliac disease. They were first identified in patients' serum that presented with an obscure classic endomysial antibody pattern, used for the diagnosis of coeliac disease. This skewed pattern was later found to be due to the presence of SMα-actin autoantibodies. Levels of SMα-actin IgA autoantibodies were found to correlate with Marsh classification and were more

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pronounced in individuals with refractory coeliac disease (Granito et al., 2004; Pedreira et al., 2005; Porcelli, Ferretti, Vindigni, Scapellato, & Terzuoli, 2013).

In addition, the soluble form of CD163, the haemoglobin/haptoglobin scavenger receptor found on the surface of monocytes and macrophages (Kristiansen et al., 2001) was found elevated in the serum of coeliac patients. Similar to SMα-actin antibodies, soluble CD163 correlated with lesion severity with higher levels detected in patients with Marsh II and III lesions (Daly et al., 2006).

### 2.5.3 Pathology of the Coeliac Lesion

Patients with positive serology and clinical features of CD are recommended to undergo an upper endoscopy to confirm the diagnosis. It is recommended that six biopsies be taken from the distal duodenum to allow for sufficient sampling, as mucosal changes in CD can be patchy (Harris et al., 2012; Rodrigues & Jenkins, 2008).

In general the histological features of CD are increased intraepithelial lymphocytes (IELs), villous atrophy, crypt hyperplasia and infiltration of inflammatory lamina propria cells. The utility of these features in combination allows the pathologist to grade the lesion according to Marsh staging system, which was first introduced in 1992 by M.N. Marsh and was then subsequently modified in 1999 by Oberhuber (Oberhuber, Granditsch, & Vogelsang, 1999; Oberhuber, 2000).

IELs are probably the most sensitive indicator of the effects of gluten on the mucosa. In normal duodenal mucosa the number of IELs has been reported as 11 to 23 IELs to 100 epithelial cells and the threshold for abnormal IELs according to the modified Marsh system is 40 IELs. The modified Marsh system is composed of 5 categories that cover the spectrum of mucosal changes. Marsh type 0 displays histological features that resemble those of the normal bowel mucosa with tall villi, no crypt or IEL abnormalities. These patients may be asymptomatic and only have serological changes. Marsh type 1 lesion is identified by an infiltrate of more than 40 IELs per 100 enterocytes and this is the only histological change. This finding alone is very non-specific and may seen in other conditions such as duodenditis, and drug-induced injury, thus correlation with clinical and serological information is necessary. Changes associated with modified Marsh 2 include crypt hyperplasia and increased IELs with preserved villous architecture. This lesion is rarely seen in clinical practice. Changes observed in the modified Marsh Louise Elliott
Type 3 lesions show villous atrophy of varying degrees in combination with crypt hyperplasia, a decreased villous height to crypt depth ratio (normal, 3-5:1) and increased IELs. This category is further subdivided to communicate the severity of the lesion into 3a (mild villous atrophy), 3b (moderate villous atrophy), and 3c (subtotal villous atrophy). Finally, modified Marsh type 4 represents a rare hypoplastic lesion characterised by villous atrophy with normal crypt height and IELs. This lesion represents the irreversible extreme end of the coeliac disease spectrum, however, this category has been made obsolete by the finding that this lesion is associated with aberrant clonal proliferation of IELs (Gujral et al., 2012; Lukás, 2004; Oberhuber, 2000; Harris et al., 2012). Figure 2.8 illustrate the key mucosal changes associated with each March classification.

Figure 2.8: The Marsh staging system of intestinal damage in coeliac disease (Image adapted from Am J clin Pathol, 2002)
2.5.4 Immunopathogenesis of Coeliac Disease

Genetic factors: Human Leukocyte Antigen

It is well known that a specific MHC class II molecule, HLA-DQ, determines susceptibility to CD. These HLA class II molecules, encoded by the MHC genes DQA1 and DQB on chromosome 6, are found on the surface of antigen presenting cells and their function is to bind peptides and present them to CD4+ T cells. The strongest association is observed with HLA-DQ2.5, with more than 90% of patients possessing one or two copies of HLA-DQ2.5, encoded by HLA-DQA1*0501 (α-chain) and HLA-DQB1*02 alleles (β chain). The remaining patients carry DR4DQ8 haplotypes and express a DQ8 molecule, encoded by DQA1*03/DQB1*0302 (Lundin, Qvigstad, Sollid, & Thorsby, 1989; Megiorni & Pizzuti, 2012). HLA-DQ2.5 molecules may be inherited together on the same chromosome (cis configuration) or separately on two homologous chromosomes (trans configuration). Usually HLA-DQA1*0501 and DQB1*02 are present in cis on DR3 haplotype (DRB1*03:01-DQA1*05:01-DQB1*02:01) or in trans on DR5/DR7 haplotypes (DRB1*11/12-DQA1*05:05-DQB1*03:01;DRB1*07-DQA1*02:01-DQB1*02:02). DQ8 heterodimers are generally in combination with DQA1*03 variant in cis position on DR4 haplotype (DRB1*04-DQA1*03:01-DQB1*03:02) (Koning, 2012; Meresse, Malamut, & Cerf-Bensussan, 2012) (Figure 2.9). HLA-DQ2.5 and HLA-DQ8 have positively charged pockets, which preferentially bind negatively charged residues at anchor positions P4, P6, and P7 for HLA-DQ2.5 and P1, P4, and P9 for HLA-Q8. HLA-DQ2 molecules prefer to bind peptides with a left-handed polyproline II helical configuration, which is characteristic of the environmental trigger, gluten (Kagnoff, 2007). Of the population 30% to 40% possess the HLA-DQ2/DQ8 serotype, but only a small proportion of these individuals develop CD suggesting that additional environmental, genetic factors must influence CD penetrance.
Figure 2.9: Human leukocyte Antigen class II association with coeliac disease. Image depicted from Meresse et al., 2012)

Non-HLA genes

Considering that 30% of the Caucasian population have the HLA-DQ2 phenotype (a van Heel & West, 2006) with an estimated risk effect of 36-53% for CD, suggests that additional genetic and environmental factors contribute to disease development (Megiorni & Pizzuti, 2012). The search and identification of additional genetic factors have been facilitated greatly by the recent application of genome-wide association studies (GWAS), an approach that can test thousands of single nucleotide polymorphisms (SNPs) across the whole genome (Pandey, 2010). With the introduction of GWAS, 40 new CD-associated genetic regions marked by SNPs have been identified (Figure 2.10) and 54% of its heritability can now be explained (Kumar, Wijmenga, & Withoff, 2012; Östensson et al., 2013). The regions identified contain genes largely involved in innate and adaptive immunity, many which are associated with other autoimmune disorders and chronic diseases (Kumar et al., 2012; Östensson et al., 2013; Zhernakova et al., 2011). However, this genetic predisposition depends on a multitude...
of genes, each of them adding only a small contribution to disease development.

The first GWAS for coeliac disease was performed in 2007 on a UK cohort testing 778 coeliac cases and 1,422 population controls. Excluding HLA, 13 regions in the genome were identified containing genes and genetic variants associated to CD. This study identified risk variants in the region of chromosome 4q27 containing the genes for IL-2 and IL-21 (David a van Heel et al., 2007). Both cytokines play an important role in T cell function. IL-2 plays an important role for the homeostasis and function of T cells while IL-21 regulates many immune and non-immune cells. Interestingly, Fina et al demonstrated an increased expression of IL-21 in the intestinal mucosa of patients with active coeliac disease but not of treated patients, supporting a biological mechanism for these genetic variants (Fina et al., 2008; Meresse, Verdier, & Cerf-Bensussan, 2008).

A follow up study carried out by the same group with a larger cohort went on to identify seven new risk regions, two of which encoded for the IL-18 protein receptor (Hunt et al., 2008). Interestingly, IL-18 possesses the ability to induce T cells to synthesize IFN-γ, a key cytokine involved in driving the pro-inflammatory response observed in the untreated coeliac lesion. Furthermore, IL-18 is expressed in the intestinal mucosa of untreated patients with coeliac disease but not in healthy controls (León et al., 2006; Salvati et al., 2002).

In 2010, van Heel et al performed a second-generation GWAS using more than six times as many samples as the previous GWAS. They identified 13 new risk regions with genome-wide significant evidence of association, including regions containing the CCR4, CD80, CIITA-SOC51-CLEC16A, ICOSLG, CD247, IRF4, TLR7-TLR8, and TNFRSF9, which have obvious immunological functions in T cell development, T cell and B cell stimulation and chemotaxis (Dubois et al., 2010).

Most recently, a GWAS employing an immunochip-based technique identified 13 new coeliac disease risk loci. These newly identified regions located on chromosome 2q33 contained genes involved in T cell regulation such as the co-stimulatory molecules CD28 and ICOS as well as the negative co-stimulator molecule CTLA4 (Trynka et al., 2011).
Now that an overabundance of CD susceptibility factors have been identified, the challenge is to locate the causal variants from each locus, and to prove that these variants affect the function of tissues and cell types involved in CD.

The Role of Dietary Proteins in Disease Pathogenesis

Proteins found in dietary cereal grains wheat (gluten), rye (secalins), and barley (hordeins) are the known environmental triggers for the activation of CD (Koning, 2005). These prolamins, particularly gluten, are rich in the amino acids proline and glutamine, which are pivotal for the initiation in the cascade of events that results in coeliac disease (Molberg et al., 2003). Their high proline content renders these proteins resistant to complete degradation by gastric, pancreatic and intestinal brush-border membrane

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proteases that lack propyl endopeptidase activity, allowing large immunogenic peptides to reach the mucosal surface and potentially to come in contact with immune cells (Shan et al., 2002). A large number of T-cell stimulatory peptides in gluten have been identified and studies have demonstrated that T cell responses to the α gliadin are mainly directed towards the epitopes clustered within an immunoreactive 33-mer peptide, LQLQPFPQPQLPYPQPQLPYPQPQPF. This stable 33-mer peptide is generated by digestion with gastric and pancreatic enzymes *in vivo* and *in vitro* and is resistant to *in vitro* digestion with preparations of brush border enzymes (van de Wal et al., 1999). Finally, proline residues introduce structural resistances that are compatible with the peptide-binding groove of HLA-DQ2.5/8 molecules and no other MHC-class II molecules.

**Tissue transglutaminase (tTG): Role in Driving the Adaptive Immune Response.**

As mentioned previously, the HLA-DQ2.5 or HLA-DQ8 molecules have a preference for peptides with negatively charged amino acids. However, such negatively charged amino acids are absent from native gluten peptides. This raised the question - how can antigen-presenting cells efficiently present gliadin peptides to T cells? This puzzle was solved after the discovery of tTG, the main autoantigen associated with CD (Dieterich et al., 1997; Mäki, 1997; O. Molberg, McAdam, & Sollid, 2000).

This multifunctional enzyme is strongly expressed in many tissues most importantly in the small intestine where is can be found both intra and extracellularly. tTG plays a role in tissue repair and cross-links proteins by forming isopeptide bonds between glutamine and lysine residues (Lentile, Caccamo, & Griffin, 2007). tTG has high affinity for gluten peptides and under certain conditions (low pH) and in the absence of lysine residues, tTG deamidates glutamine residues in gluten peptides within a Q-X-P sequence (Q and P denotes glutamine and proline, respectively) to negatively charged glutamate residues (Fleckenstein et al., 2002), thereby strongly increasing peptide avidity for HLA-DQ2.5 and HLA-DQ8 (Quarsten et al., 1999; Sjöström et al., 1998; van de Wal et al., 1998).

The 33mer peptide product has the highest specificity for deamination by tTG and the resulting products are extremely stimulatory for all of the HLA-DQ2 restricted α gliadin specific T cells (Quarsten et al., 1999; Sjöström et al., 1998). The 33-mer peptide

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carries multiple copies of 3 epitopes, rich in proline and glutamine (PFQPQLPY, PQPQLYPYQ and PYPQPQLPY), and has been demonstrated by several groups to be potent T cell stimulators after deamidation by tTG (Arentz-Hansen et al., 2000; Di Sabatino et al., 2012; Qiao, Iversen, Ráki, & Sollid, 2012; Shan et al., 2002; L. W. Vader et al., 2002)

2.5.5 Adaptive Immune Response in CD

Once bound to HLA-DQ2 or HLA-DQ8, the gluten peptide:MHC complexes can activate gluten specific T cells in the mucosa of the small intestine (Schuppan, Esslinger, & Dieterich, 2003). It is well established that the adaptive immune response plays a central role in the pathogenesis of coeliac disease. The coeliac lesion is characterised by an infiltration of T cells in both the epithelium and the lamina propria. CD4+ memory T cells, bearing the α/β T cell receptor, are the dominant T cell found in the lamina propria. In the epithelium, IELs consist of CD8+ αβ T cells and γδ T cells that are either CD8+ or CD4-CD8- (Gianfrani, Auricchio, & Troncone, 2005; Gujral et al., 2012; Qiao et al., 2012; Wal et al., 2000). The CD8+ αβ T cells are the major cell subset of the three present in the epithelium and upon gluten free diet this subset returns to normal while the γδ T cells remain increased for years (Meresse et al., 2012).

After in vitro challenge with gliadin, gliadin specific CD4+ αβ T cells can be readily isolated from biopsies from coeliac patients but not controls (Kagnoff, 2007; Qiao et al., 2012; Sollid, 2002). IFN-γ production by gliadin specific T cells is a signature of coeliac disease and has a key role in mucosal damage (Lionetti & Catassi, 2011; Nilsen et al., 1998; Wapenaar et al., 2004). Neutralisation of IFN-γ has been shown to prevent gluten induced mucosal damage in-vitro (Przemioslo et al., 1995). Furthermore, the transcription factor T bet, which drives Th1 cell lineage commitment, is upregulated in the mucosa of untreated CD patients (I. Monteleone et al., 2004). The release of IFN-γ and other cytokines maintain the ongoing proinflammatory response to gliadin in the mucosa. They alter key mucosal functions such as intestinal permeability resulting in the increased influx of gliadin peptides into lamina propria (Bethune et al., 2009). They can also cause the release of enzymes such as matrix metalloproteinases (MMPs) that further contribute to mucosal damage resulting in the loss of villous structure and crypt hypertrophy (Rachele Ciccocioppo et al., 2005). Interestingly IL-12, a cytokine Louise Elliott
responsible for driving a Th1 response, is undetectable in the coeliac lesion (Green, Jabri, & Kasarda, 2005; A. J. León, Garrote, & Arranz, 2005; Sollid, 2002), provoking the question, what cytokine is responsible for initiating and maintaining the pro-inflammatory response and what cells are involved in antigen presentation and the subsequent priming and activation of these T cells?

2.5.6 Innate immunity: Antigen Presenting Cells in Coeliac Disease

Recently, observations also point towards a central role for the innate immune response in the pathogenesis of coeliac disease (A-C R Beitnes et al., 2011; Ann-Christin Røberg Beitnes et al., 2012; Di Sabatino et al., 2007; Melinda Råki et al., 2006; Rescigno & Sabatino, 2009). The induction of an adaptive immune response is tightly controlled by innate immunity. Central to this phenomenon is the APC, especially the DC. T cell priming usually takes place in the lymph node where antigen loaded dendritic cells travel from the peripheral tissue to generate effector or tolerogenic T cells, which then operate in the peripheral tissue (Qiao et al., 2012).

The APC importance in coeliac disease development is emphasised by the observation that HLA-DQ2 homozygous individuals have a fivefold higher risk of developing coeliac disease compared to heterozygous individuals (Koning, 2012; W. Vader et al., 2003). This finding correlated with the ability of APCs from homozygous individuals to elicit stronger gliadin specific T cells than APCs from heterozygous subjects. It is clear that without signals provided by intestinal APCs, no gluten specific T cell responses could develop.

Intestinal Macrophages

Macrophages are the most prominent APC found in the lamina propria of the duodenum and are preferentially located in the sub epithelial compartments, the main site of antigen entry. They are non-proliferating cells that stem from circulating monocytes. In a steady state, blood monocytes migrate to the lamina propria where they replenish the macrophage pool in response to IL-8 and TGF- beta (Smythies et al., 2006). In contrast to their blood counterparts, under homeostatic conditions, intestinal macrophages lack expression for CD14, TLR-4, and Fc receptors IgA and IgG, rendering Louise Elliott

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them refractory to microbe associated patterns (Bain & Mowat, 2011; Smith et al., 2001). They display potent phagocytic activity making them instrumental in the effective clearance of lumen bacteria and apoptotic cells with minimal signs of inflammation. *In-vitro*, when stimulated with LPS, intestinal macrophages, fail to secrete pro-inflammatory cytokines TNF-alpha, IL-6, and IL-1 suggesting that these cells do not facilitate mucosal inflammation in a steady state (Qiao et al., 2012; Smythies et al., 2005). Intestinal macrophages are highly adaptable and the local tissue environment crucially determines their applied functions. It is believed that an array of intestinal cells such a myofibroblasts, epithelial cells, and LP cells produce soluble factors such as TGF-beta, and IL-10, which condition the phenotype and functional properties of intestinal macrophages helping to main tissue homeostasis (Weber et al., 2009). This was illustrated *in-vitro*, where monocytes exposed to stromal cell conditioned media displayed reduced expression of several innate immune receptors as well as inducing inflammatory anergy. Interestingly, TGF-beta antibodies efficiently blocked these effects advocating the importance of this cytokine in tissue health (Smythies et al., 2010). However, it is more than likely that TGF-beta alone is not sufficient to induce inflammatory anergy in intestinal macrophages and is more likely to depend on a combination of intestinal derived factors and cell-cell interactions.

Intestinal macrophages are not thought to serve as professional antigen presenting cells due to their low CD80, CD86 and CD40 surface expression (Rugtveit, Bakka, & Brandtzaeg, 1997). This was confirmed by the observations made by Raki and colleagues (2006) who found intestinal macrophages to be poor presenters of gluten peptides to gluten reactive T cells *ex-vivo* when compared to intestinal DCs. Thus, the exact role for macrophages in the pathogenesis of coeliac disease remains elusive but is likely that their cytokine production help sustain the inflammatory cascade within the coeliac lesion.

Classically human macrophages are identified as CD68 positive but recently a novel marker, CD163 a haemoglobin/haptoglobin scavenger receptor, has been used in the identification of intestinal macrophages (Beitnes et al., 2011). Interestingly, our lab has detected elevated levels of the soluble form of CD163 in the serum of patients with untreated coeliac disease (Daly et al., 2006). However, it has not yet been elucidated...
what cell or what tissue compartment contributes to this source of soluble CD163. It is most likely derived from the coeliac lesion, characterised by high levels of IFN-γ and TNF-α, proinflammatory cytokines known to induce ectodomain shedding of CD163 from the cell surface (Buechler et al., 2000). In agreement with this hypothesis, is the observations made by Beates et al, 2011, who reported a decrease in CD163+ macrophages in the coeliac lesion compared to healthy controls. The shedding of CD163 from the surface of macrophages within the coeliac lesion may reflect their activation status along with a change in the overall macrophage phenotype switching from the alternatively activated subset to the classically proinflammatory activated macrophage.

Intestinal Dendritic Cells

Due to extensive research in the last decade our understanding of intestinal dendritic cells is slowly improving. However, due to the complexity of isolating dendritic cells from the gut in humans, most information about these cells has been gained from mice studies. In mice, several subsets of gut lamina propria myeloid DCs have been characterised, according to their ability to differentially activate lymphocytes and on the basis of their expression of CD103+ (Persson, Jaensson, & Agace, 2010; Rescigno & Sabatino, 2009; Rescigno, 2011; Schulz et al., 2009; Varol et al., 2009). CD103 also known as integrin alpha E binds to E-cadherin, an adhesion molecule found on epithelial cells. The CD11c+ CD103+ DC subset stem from pre DCs, express high levels of CCR7 and are the main migratory DC subset. After uptake of antigen they home to lymph nodes to prime T cells (Johansson-Lindbom et al., 2005). They can induce the development of Foxp3+ T regulatory cells via retinoic acid (RA), a metabolite of vitamin A, and TGF-β (Coombes et al., 2007). Ex-vivo experiments comparing isolated CD103+ and CD103- DCs confirmed that only the CD103+ DCs could induce Foxp3 Tregs in the presence of TGF-β, as this was the only subset capable of producing sufficient amounts of RA. In contrast, the CD103- subset could only induce Tregs cells when both TGF-β and RA were present ex-vivo (Schulz et al., 2009).

There is conflict in the literature regarding the nature of the second subset of DCs, defined as CD11c+ CD103- CX3CR1+. Some believe they are DCs while others consider them to be macrophage, due to their inability to migrate to lymph nodes, their
development from monocytes, and their inferior capacity to prime naïve T cells (Bogunovic et al., 2009; Niess, 2010). These cells have been well identified in mice and have been shown to be capable of extending protrusions across the epithelial barrier allowing them to sample antigen directly from the lumen, a rare finding in humans (J. H. Niess et al., 2005). Due to their inability to migrate, they may directly exert their biological functions in the lamina propria. For example, the capture of antigen may result in cytokine secretion that modulates the surrounding microenvironment promoting CD103+ subset migration to the mesenteric lymph nodes or they may directly pass on sample antigen to other cells (Bogunovic et al., 2009). In the human duodenum, there are no reports on the expression of CX3CR1. Figure 2.11 describes the main mucosal DCs found in the mouse gut lamina propria.
**Dendritic cells and Coeliac Disease**

In the lamina propria of patients with coeliac disease the characterisation of DCs has proven to be complex. Several studies have been conducted, all reporting conflicting results. The first study conducted by Raki et al., 2006, reported an increase in the number of CD11c+ myeloid DCs in the lamina propria of untreated coeliac patients compared to treated coeliac patients and normal controls. The same study did not identify the presence of the CD123+ plasmacytoid DC subset in either coeliac or healthy mucosa. Employing T cell proliferation assays the study found that isolated mucosal CD11c+ DCs were far superior in presenting the 33-mer peptide to gliadin specific T cells when compared to intestinal macrophages. In contrast, Di Sabatino et al., 2007 identified CD123+ plasmacytoid DCs as the major DC population in the lamina propria from healthy controls. Furthermore this group noted that this subset was largely responsible for the increase in DCs found in the mucosa from untreated coeliac patients. A more recent study conducted by Raki and colleagues found that plasmacytoid DCs only represents less than 1% of APCs in duodenal mucosa from both normal and untreated coeliac individuals (M Ráki et al., 2013). Thus due to the small number of studies and varying results, further studies are warranted to elucidate the importance of the different DC subsets in the pathogenesis of coeliac disease. Despite these inconsistencies, both studies show that the number of DCs is increased in the coeliac lesion, which reverts to normal numbers upon elimination of gluten from the diet, thus indicating the potential importance of dendritic cells in the pathogenesis of coeliac disease.

In addition to the well-identified macrophage and classical myeloid DC, additional subsets of DCs have been identified in the coeliac lesion expressing both markers CD163 and CD11c, referred to as an “intermediate DC” subset. It has been
postulated that this intermediate subset is the equivalent counterpart to the mouse myeloid DC subset characterised as CD11c+ CX3CR1+ (Beitnes et al., 2011). In mice this subset appears to originate from monocytes rather than DC precursors (Liu & Nussenzweig, 2010). Interestingly, Beitnes and colleagues (2011) found the monocyte marker CD14 expressed on about 65% of CD163+CD11c+ cells detected in lamina propria of the duodenum, indicating that these cells stem from monocytes similar to their mouse counterpart. In addition to this, it has also been previously shown that a minority of DCs in the peripheral blood expresses CD163 (Maniecki et al., 2006), thus suggesting that a proportion of this “intermediate subset” may simply represent a population of newly recruited DCs that have yet to down regulate or shed CD163 from their cell surface. Furthermore, it was shown that the density of these intermediate subsets were increased within the coeliac lesion, which preceded the architectural changes of the intestine as well as the surge of intraepithelial lymphocytes, suggesting that these cells may take part in disease initiation (Ann-Christin Røberg Beitnes et al., 2012).

Beitnes and colleagues (2011) also identified a minor DC subset expressing CD103 in the normal duodenal mucosa. This subset was found decreased in the untreated coeliac lesion compared to healthy controls. Therefore, based on the assumption that the CD103+ subset identified in this study is comparable to its mouse counterpart it is plausible to suggest that the decrease may due to an increased rate in migration to the lymph node exacerbated by the ongoing inflammation in the gut. Upon arrival in the lymph node these DCs can prime gliadin specific T cells and induce the expression of intestinal homing receptors resulting in the migration of gliadin specific T cells to the duodenum lamina propria.

Although, innate cells are clearly implicated in coeliac, it is still not known what stimulates the innate arm of the immune system to induce an immunogenic response towards gluten as opposed to a tolerogenic one. It is plausible that gliadin it self may directly effect the innate immune system under certain conditions that result in the loss of tolerance promoting inflammation.
Innate Triggers

It has been proposed that a viral or bacterial infection in genetically susceptible individuals may be responsible for triggering the innate immune response, which disrupts the balance between tolerance and immunity resulting in the development of coeliac disease (Pavone, Nicolini, Taibi, & Ruggieri, 2007; Troncone & Auricchio, 2007). However, more recently another hypothesis has come to light, which suggests that gliadin peptides possess the ability to directly activate innate immune cells such as DCs. This has been illustrated by several studies where gliadin peptides have been shown to induce activation of innate immune cells. One study found that gliadin peptides were capable of inducing interleukin (IL)-6, IL-8, and IL-12 (p40) secretion in monocyte derived DCs and the upregulation of HLA-DR, CD83, and CD86 in all subjects irrespective of their genotype or the presence of disease. However, gliadin-stimulated dendritic cells from active coeliac patients showed enhanced stimulation of autologous T cells compared to the other groups (Rakhimova et al., 2009). The mechanism by which gliadin can directly stimulate the DC remains unknown but it is postulated to interact with a PRR. Additional studies using gliadin and macrophages have produced similar results to that observed above. Proteolytic fragments of gliadin were shown to induce IL-8 and TNF-alpha production in human monocytic line THP-1 cells (Jelinkova et al., 2004). Likewise a further study showed that peritoneal macrophages stimulated with gliadin fragments exhibited a significant increase in NO production as well as TNF-alpha and IL-10 production (Tuckova et al., 2002). In addition to these gliadin-mediated effects on APCs, gliadin peptides have been reported to stimulate the migration of human DCs employing transwell studies, which was accompanied by rearrangement of the cytoskeletal structure (Chladkova et al., 2011).

Both macrophage and DC function is dependent on the local environment and in the absence of inflammation these cells are conditioned by TGF-beta, thymic stromal lymphopoietin, and retinoic acid to a tolerogenic state (Iliev et al., 2009; Rimoldi et al., 2005). Recently, it was illustrated in a mouse model for coeliac disease, by Jabri and colleagues that retinoic acid in combination with high levels of IL-15, a feature of the coeliac lesion, was capable of disrupting oral tolerance. This breakdown in tolerance resulted in the generation of immunogenic DCs capable of promoting a IFN-gamma Th1
response (DePaolo et al., 2011). An additional cytokine implicated in the loss of tolerance to gluten is IFN-alpha. IFN-alpha is detected at higher levels in the coeliac lesion and is believed to provide an alternative mechanism in promoting the loss of tolerance to gluten via the activation of gliadin specific T cells (Di Sabatino et al., 2007; G. Monteleone et al., 2001). Thus, it is very likely that the strong Th1 response associated with coeliac disease is coupled with an aberrant innate component that drives the adaptive immune response. However, much work is required to identify the innate trigger capable of altering the microenvironment in such a way that allows gliadin to induce the activation of the mucosal immune system (Sollid & Jabri, 2013).

**Antigen Presenting Cells and Tissue Transglutaminase**

As discussed previously, the posttranslational modification of glutamine to glutamic acid mediated by tTG at specific sites is crucial for T cell recognition of gliadin peptides (Di Sabatino et al., 2012). However, where the necessary gluten deamidation takes place is still unknown. It has been hypothesised that tTG on the surface of antigen presenting cells may be directly involved in the deamidation of gluten peptides, as well as facilitating antigen uptake and processing for presentation to gliadin specific T cells. Supporting this hypothesis is the detection of surface tTG on antigen presenting cells such as macrophages and dendritic generated in vitro when cultured in the presence of M-CSF or IL-4 and GM-CSF, respectively (Hodrea et al., 2010; Ráki et al., 2007). However, the study carried out by Raki et al (2007) found that the monoclonal antibody 6B9 employed in their study was not directed against surface tTG but rather CD44 (Stamnaes et al., 2008), thus further studies are warranted to determine the relationship between tTG and APCs and the potential involvement of surface tTG in coeliac disease. An additional finding that substantiates a role for surface tTG in the deamination of gliadin peptides is the expression of surface tTG on individual lamina propria cells in duodenum sections taken from healthy controls and coeliac patients (Esposito et al., 2003; Gorgun et al., 2009; Mercan & Celiac, 2003). Although these cells have yet to be identified in-situ it is strongly suspected that they are macrophages and possibly dendritic cells. Both these cells are found in normal duodenum and increased in the coeliac mucosa (Ráki et al., 2006) and are central in controlling the outcome of the adaptive immune response, especially the dendritic cell. Moreover, the dendritic cell has been shown to efficiently
activate gluten-specific T cells (Ráki et al., 2006). These results suggest that mucosal DC are directly involved in the local T cell activation in CD.

As tissue transglutaminase is significantly increased in the lamina propria in the coeliac lesion (Gorgun et al., 2009; Skovbjerg et al., 2004; Villanacci et al., 2009) and possesses a vast array of functions (Belkin, 2011) it was not surprising to find that tTG was shown to have a direct effect on DC maturation (Dalleywater, Chau, & Ghaemmaghami, 2012). Higher concentrations of tTG were associated with a more mature DC phenotype along with increased ability to stimulate T cells. This phenomenon clearly shows additional functions in which tTG can drive and maintain the pro-inflammatory cascade observed in untreated coeliac patients. Thus a relationship between antigen presenting cells and tTG may co-exist and may be shown to be central in the pathogenesis of coeliac disease via the initiation of the adaptive immune response.
2.6 Objectives of Thesis

The overall objective of this thesis is to gain a better understanding of the role of antigen presenting cells in the immunopathogenesis of coeliac disease. Further elucidation of the phenotype of these cells will further aid our understanding of the role of the innate immune system in coeliac disease. To achieve this objective, this project had the following aims:

1) Characterisation of dendritic cell and macrophage subsets in duodenal biopsies from untreated and treated coeliac subjects and healthy controls.

2) Characterisation of dendritic cell and monocyte subsets in whole blood, with respect to their frequency and resting phenotype from healthy and coeliac subjects

3) Characterisation of CD163 positive antigen presenting cells in the duodenal mucosa and peripheral blood and to identify the main source of soluble CD163 in coeliac patients and healthy controls.

4) Evaluate tTG expression on peripheral blood monocyte derived dendritic cells using flow cytometry, RT-PCR, and Western blots.

5) Evaluate tTG expression in the duodenal mucosa from coeliac and healthy controls and determine whether antigen presenting cells in duodenal mucosa express tTG, supporting the hypothesis for surface tTG in the uptake of gliadin and its subsequent presentation of gliadin to T cells.
### Chapter 3  Materials and Methods

#### 3.1 Materials

#### 3.1.1 Equipment

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</thead>
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Louise Elliott
3.1.2 Plastics

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<td>Roskilde, Denmark</td>
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3.1.3 Reagents

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</tr>
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<td>-----------------------</td>
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<td>Ripa Buffer</td>
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</tr>
<tr>
<td>Protease inhibitor cocktail</td>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
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### 3.1.4 Cell stimulators

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<td>Lipopolysaccharide (LPS)</td>
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### 3.1.5 Recombinant human cytokines

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### 3.1.6 ELISA Reagents

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### 3.1.8 Cell Proliferation

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### Antigens List of antibodies used for flow cytometry

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<tr>
<td>CD8α</td>
<td>APC</td>
<td>Biolegend</td>
<td>San Diego, CA, USA</td>
</tr>
</tbody>
</table>
3.1.10 List of antibodies used for tissue Immunofluorescence studies

<table>
<thead>
<tr>
<th>Name (Clone)</th>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c (EP1347Y)</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CD123</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CD68 (KP1)</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CD163 (10D6)</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CD163</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CD103</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Tissue Transglutaminase (TG100)</td>
<td>Antibodies on line</td>
<td></td>
</tr>
<tr>
<td>Tissue Transglutaminase (Cub7402)</td>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Tissue Transglutaminase (pAB)</td>
<td>Immunotools</td>
<td>Friesoythe, Germany</td>
</tr>
<tr>
<td>Tissue Transglutaminase 2G3H8</td>
<td>Dr. Fernando Chirdo</td>
<td>Universidad Nacional de la plata, Argentina</td>
</tr>
<tr>
<td>Tissue Transglutaminase 5G7G6</td>
<td>Dr. Fernando Chirdo</td>
<td>Universidad Nacional de la plata, Argentina</td>
</tr>
<tr>
<td>Anti-rabbit 488</td>
<td>Invitrogen</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Anti-rabbit 568</td>
<td>Invitrogen</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Anti-mouse 488</td>
<td>Invitrogen</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Anti-mouse 568</td>
<td>Invitrogen</td>
<td>Paisley, UK</td>
</tr>
</tbody>
</table>

3.2 Samples

3.2.1 Tissue samples

Paraffin embedded tissue blocks were selected from the files of the routine histology labatory. All samples were fixed in 10 per cent formalin and processed according to the
standard operating procedures of the histology laboratory. The selected blocks were representative of untreated and treated coeliac patients and normal individuals. Details of the patients investigated in the study are given in Table 3.1.

<table>
<thead>
<tr>
<th>M:F ratio</th>
<th>Healthy Controls (n=20)</th>
<th>Treated (n=15)</th>
<th>Untreated (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean)</td>
<td>63.5</td>
<td>54</td>
<td>58</td>
</tr>
<tr>
<td>tTGF/EMA</td>
<td>Negative</td>
<td>&lt;40/Negative-Positive</td>
<td>&gt;90/Positive</td>
</tr>
<tr>
<td>Marsh</td>
<td>Healthy Duodenal Mucosa</td>
<td>Normal Duodenal Mucosa Marsh I-II</td>
<td>Marsh III</td>
</tr>
</tbody>
</table>

Table 3.1 Patient details for tissue samples used for fluorescence studies

3.2.2 Subjects

Adult cohort

All patients were enrolled consecutively at the coeliac Day clinic at St. James’s hospital, and each patient gave informed consent to participate in the study. The study was approved by the local Ethical Committee. The numbers of patients studied in each analysis (n) are shown in each result chapter. Therefore the frequencies of any cells of interest were not done for all patients. Details of the patients investigated in the study are given in Table 3.2.
Healthy controls | Coeliac patient on a GFD | Newly diagnosed coeliac patient
--- | --- | ---
Sex (F:M ratio) | 3:1 | 1.4 : 1 | 3.3 : 1
Age (years) range (mean) | 21-60 (30.8) | 28 – 80 (50.8) | 22 - 66 (36.45)
Anti-endomysial antibody | Negative | Negative | Positive
Anti-tTG | Negative | <7 | 23.5 - >128 (75.8)
Histology Marsh | | | Marsh II - III

Table 3.2: Patient details for Adult cohort

**Paediatric cohort**

All patients were enrolled at the coeliac day clinic at Our Lady’s Children’s Hospital, Crumlin. Consent was obtained from the consenting guardian via the research nurse. A total of 50 patients were enrolled in this study. The numbers of patients studied in each analysis (n) are shown in each result chapter. The paediatric cohort consisted of 30 healthy controls and 20 newly diagnosed patients with coeliac disease. All newly diagnosed patients with coeliac disease were confirmed by positive serology and histology. Details of the patients investigated in the study are given in Table 3.3
<table>
<thead>
<tr>
<th>Healthy controls</th>
<th>Newly diagnosed coeliac patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F:M ratio)</td>
<td>1 : 2.3</td>
</tr>
<tr>
<td></td>
<td>1.6 : 1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>&lt;1 : 16</td>
</tr>
<tr>
<td>(mean +/- SEM)</td>
<td>(8.4)</td>
</tr>
<tr>
<td></td>
<td>4 - 12</td>
</tr>
<tr>
<td></td>
<td>(7.18)</td>
</tr>
<tr>
<td>Anti-endomysial antibody</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Anti-tTG</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>&gt;128</td>
</tr>
<tr>
<td>Histology Marsh</td>
<td>Normal Duodenal mucosa</td>
</tr>
<tr>
<td></td>
<td>Coeliac Disease on Biopsy</td>
</tr>
</tbody>
</table>

Table 3.3: Patient details for paediatric cohort

**Buffy coats**

Buffy coats were obtained from The Irish Blood Transfusion Services, St. James’s Hospital, Dublin 8 for the generation of monocyte derived dendritic cells.

**Specimen Details**

Blood samples were collected into K3EDTA BD or serum vacutainer tubes. Samples for phenotyping were processed for flow cytometry within 4 hours. Serum samples were left at room temperature for 30 minutes before they were spun at 1000g for 15 minutes. After centrifugation the serum was removed and stored at -20°C.

**Preparation of intestinal tissue**

Small intestinal epithelial and lamina propria cells were released from biopsies as previously described (O’Keeffe et al., 2004). Briefly, duodenal biopsies were agitated for 1 hour in a shaker at 37°C in calcium- and magnesium-free Hanks Balanced Salt Solution (Gibco) supplemented with 5% foetal calf serum (FCS) (BioWest), 1 mM EDTA (Sigma-Aldrich) and 1 mM DTT (Fisher). This treatment removes the epithelial layer of cells, leaving the lamina propria attached to the basement membrane. The resulting single-cell suspension was filtered through a 40μM filter (BD Biosciences), washed in complete...
RPMI 1640 solution (Gibco; with Glutamax, supplemented with 10% v/v foetal calf serum, Penstrep, Fungizone and HEPES). Tissue remaining in the filter was collected into complete cRPMI solution containing 130 U/ml collagenase (Type IV-S, Sigma-Aldrich), and rotated for 3 hours at 37°C. Cells were then filtered, washed and enumerated by ethidium bromide and acridine orange staining.

3.3 Immunohistochemistry/Immunofluorescence

3.3.1 Sample preparation

All biopsy specimens were cut in 4μm thick sections, collected on superfrost slides and allowed to dry for 1 hour at 50 degrees Celcius (°C), to ensure optimal adhesion. Prior to immunofluorescence staining, sections were dewaxed by immersing slides in 2 changes of xylene for 10 minutes each. Next slides were rehydrated by immersing sections in different grades of alcohol ranging from 100% to 50% for 5 minutes each. Finally slides were rinsed in distilled H₂O and incubated for 5 minutes in PBS.

3.3.2 Antigen retrieval

Tissue fixation can alter protein biochemistry by masking the epitopes of interest and preventing the binding of primary antibodies. Masking of the epitopes is caused by crosslinking of amino acids within the epitope or to unrelated peptides and alteration of the epitope conformation. Antigen Retrieval can reverse this epitope masking restoring antigen antibody interaction. Heat induced epitope retrieval (HIER) was the choice of antigen retrieval used. HIER is believed to reverse crosslinks restoring the secondary and tertiary structure of the epitope.

Rehydrated slides were placed in a plastic slide holder with all slots occupied to ensure equal heat distribution. The slides were then immersed in preheated 10 mM Citrate buffer at pH 6 in a plastic container. The container was covered with cling film and pierced to allow steam to escape. The container was processed in a microwave at full power for 20 minutes. After the 20 minutes the container was removed and allowed to cool at room temp for a further 20 minutes. The slides were rinsed in running tap water for 5 minutes and then placed in PBS.

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3.3.3 Cytospin Preparations

A cell suspension of \(1 \times 10^6\) of freshly isolated monocytes, and monocyte derived DCs were prepared in PBS. Slides were mounted with the paper pad and the cuvettes in the metal holder provided. 200\(\mu\)l of the suspension was loaded into each cuvette and spun at 400rpm for 10 mins. The slide, paper and cuvette were removed from the centrifuge without disturbing the prep. Slides were air-dried and quick diff was performed to examine cell morphology or cells were stained with tissue transglutaminase monoclonal antibodies described in Table 3.4 using the protocol described in 3.3.4.

<table>
<thead>
<tr>
<th>Name</th>
<th>Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG100</td>
<td>447-538</td>
</tr>
<tr>
<td>Cub7402</td>
<td>447-478</td>
</tr>
<tr>
<td>5G7G6</td>
<td>548-558</td>
</tr>
<tr>
<td>2G3H8</td>
<td>314-329</td>
</tr>
<tr>
<td>FITC conjugated</td>
<td>Not known</td>
</tr>
</tbody>
</table>

Table 3.4: Tissue transglutaminase monoclonal antibodies

3.3.4 Indirect Fluorescent staining

Slides were blocked in blocking buffer (2% BSA and 5% GS) for a minimum of 1 hour and rinsed in PBS. Primary antibodies were diluted in 2% BSA to obtain optimal concentration and 50\(\mu\)l of each dilution were added to each slide and incubated overnight at 4°C. Slides were washed 3 times in PBS for 5 minutes each. Secondary antibodies diluted in 2% BSA were applied to each slide and incubated at room temperature for 1 hour. All slides were counterstained with dapi for 1 minute and then rinsed in PBS. Slides were mounted and cover slipped with anti-fading mounting medium and allowed to dry overnight at room temperature. For long-term storage slides were stored at 4°C protected from the dark.

3.3.5 Controls

Appropriate controls are critical for the accurate interpretation of immunofluorescence results. Certain tissues have inherent biological properties resulting in background staining that may lead to misinterpretation of results. Lipofusion has autofluorescent

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properties that overlap with the excitation and emission spectra of commonly used fluorochromes (Schnell, Staines & Wessendorf 1999). In order to correct for autofluorescence interference, prior to image acquisition a blank slide for each slide to be analysed was prepared. The blank slide was used to adjust the confocal settings discussed in section 3.5.2, so that minimal autofluorescence was detected. Therefore when the corresponding stained slide was acquired any fluorescence detected was considered specific staining.

Negative controls, were the primary antibody was replaced with staining buffer, were used to control for non-specific staining of secondary antibodies. Additional controls were employed to support the specificity of staining such as tissue type controls when antibodies were being optimised.

3.3.6 Troubleshooting

CD103, CX3CR1 and CD83 antibodies were not suitable for immunofluorescence staining on paraffin sections. As CD103 and CX3CR1 are the two main dendritic cell subsets found in the mouse lamina propria and are speculated to be found in the human intestine (Schulz et al., 2009). We investigated whether these two subsets could be identified in healthy and coeliac mucosa. However, although the CD103 antibody employed in this study was previously shown to work on paraffin sections in one other study (Divella et al., 2010), the results achieved with this antibody were unsatisfactory as a huge amount of background staining was observed making it difficult to interpret results. CX3CR1 strongly stained everywhere including the epithelium and lamina propria at several dilutions. From this it was concluded not to continue with the experiment as the findings were difficult to interpret. Initial experiments employing the monoclonal antibody CD83 showed very little staining in the duodenum and required the antibody to used at very high concentration therefore it was eliminated from our analysis.
3.4 Confocal Microscopy

Confocal microscopy was the choice of image acquisition for this study. The confocal microscope is an invaluable tool for high-resolution fluorescent microscopy. The key features of confocal microscopy are point illumination and its ability to reject out of focus light. The integration of two pinhole apertures positioned at the focal plane and at the detector is the heart of the design (Inoue, 2006).

Marvin Minsky first founded the idea of confocal microscopy in 1955. He created a concept based on the principle that allowed an image to be constructed by focusing a point of light sequentially across a specimen collecting the return rays within the confocal plane only.

3.4.1 Principles of Confocal Microscopy

Confocal microscopy is built on the ideas of point-by-point illumination of the specimen and rejection of out of light focus light rays. Light emitted by a laser passes through a pinhole that is strategically placed in confocal plane with a scanning point on the specimen. The purpose for this pinhole is to focus the laser light onto a very small area on the specimen (Inoue, 2006; Paddock, 2000; Robinson, 2001). This focused beam is then scanned across a defined area in a raster motion controlled by a dichromatic mirror. One mirror moves the beam along the x-axis while the other mirror moves beam along the Y-axis. The laser light excites the dye in the specimen and fluorescence is emitted. The same mirrors that were used to scan the excitation light descan the emitted fluorescent light (Inoue, 2006; Paddock, 2000; Robinson, 2001). The fluorescence light is then focused as a confocal point at the detector pinhole. A detector such as a photomultiplier tube then measures this light. Since the images are taken point-by-point, there is never a complete image of the specimen because only one point at time is acquired. Thus, for visualization the detector is attached to a computer that reconstructs the image (Inoue, 2006; Paddock, 2000; Robison, 2001). Figure 3.1 provides a schematic overview of the principle confocal microscopy.

In conventional microscopy a large proportion of the specimen is flooded with light at once. In contrast confocal microscopy focuses a beam of light onto a small point onto the specimen (focal point) (White, Amos, & Fordham, 1987). This focal point is
aligned with the pinhole aperture placed in front of the detector, allowing only the light from that point to reach the detector while eliminating light from below and above the focal point.

**Figure 3.1: The confocal principle** Light (blue line) from a point source is focused on a point in the specimen. Light from the illuminated point (green line) is focused on, and passed through, a detector aperture. (Image adapted from Weeks/Semwogerere, Emory University)

### 3.4.2 Confocal Set Up

Prior to image acquisition several settings had to be adjusted to obtain optimal results. First the appropriate dyes used in the experiment were chosen from the list of fluorescent dyes. Since more than two dyes were examined at any one time, images were recorded in a sequential mode to prevent spectral overlap between channels during acquisition. The following parameters were defined for each dye used: Laser percentage, high voltage (HV), Gain, and Offset.

The laser % is the amount of laser power that is used to excite the specimen. HV affects the sensitivity of the photomultiplier tube (PMT). The PMT collects the released...
photons of light from the laser-excited sample on the slide. Raising the HV increases the sensitivity of the PMT thus making the image brighter. However, increasing the HV above 750 will only introduce noise into the image, therefore the HV was never adjusted above 650. The gain increases the brightness of the image and is only adjusted after the laser and HV have been corrected. Finally the Offset decreases the level of background detected. All four parameters are adjusted to generate the optimum signal/noise ratio.

After all settings were adjusted a 2D image could be acquired. All images were acquired at 40x oil objective. Using the eyepiece the specimen was focused and an area of interest was located. The lasers were turned on and the sample was focused until the brightest fluorescent signal was detected. When all the necessary adjustments were made and the image was ready to acquire the laser was temporarily turned off to turn on the kalman filter. The kalman filter functions by removing background noise. The Aspect ratio was increased to 1024x1024 to ensure optimal resolution. Finally the image was acquired and saved for further analysis.

3.5 Tissue Evaluation

All specimens were analysed using imaging analysis software, BITPLANE IMARIS version 7.4.2. Imaris is a scientific software for the visualisation, analysis, and interpretation of fluorescence images. It provides a large variety of tools that allows the user to identify, separate, and visualise individual objects that can be further processed by the Imaris measurement Pro module to extract statistics.

Prior to analysis, the intensity for each colour channel in the image was interactively adjusted to eliminate background noise and to brighten the image. After the background noise was eliminated a “surface object” was applied to each fluorescence colour in the data set. A surface object is a computer-generated representation of a specified region which statistics can be accurately calculated from. Figure 3.5 shows the surface object display window.

For each image an area of interest was selected and the antigen expression within that area was analysed. This was achieved using the contour feature, which allowed the manual selection of specific areas such as villus/superficial epithelium, cryptal
epithelium, and lamina propria as shown in figure 3.3. Artifacts such as autofluorescence or the mucosa muscularis were excluded from analysis when appropriate (Figure 3.4).

Figure 3.3: Mark up image highlighting the areas in yellow to be evaluated, areas in blue were excluded from analysis. Figure A and B highlights the lamina propria and cryptal epithelium to be analysed, respectively.

Figure 3.4: Example of areas excluded. When carrying out tissue transglutaminase expression analysis, positive staining within the mucosa muscularis, area beneath the broken white line, was excluded to prevent skewness of results.
Figure 3.5: Surface object display window: A, Original image of smooth muscle alpha actin staining (left), image on the right is the identical image with a surface area applied shown in grey. All areas in grey are measured. B, shows the display window that allows the user to manually create the surface for each channel in the data set.

3.5.1 Co-localisation quantification

Imaris software provides an accurate “Coloc” tool that allowed obtainable reliable information about the relationship of tissue components. Prior to co-localisation analysis, intensity ranges below a certain level were excluded in the data set in order to eliminate background noise. This was achieved by applying a threshold to the source channels used for each image. This was manually selected using the histogram or dotplots shown in figure 3.6. After the thresholds were selected, a channel was
generated that contained only the voxels colocalised from the data set. After a channel is generated and updated, ImarisColoc produced an array of statistical parameters to document the degree of overlap. (Figure 3.6). For the purpose of this study the percentage of channel A and B volume colocalised above the threshold co-localised was determined for each image.

As the co-localisation channel is displayed as a separate colour channel it can be quantified like any of the other channels employing the surface object tool. Therefore the total surface area of co-localisation expression was determined for each image processed and was expressed as a ratio against the total lamina propria surface area.

Figure 3.6: ImarisColoc display window. 1 shows the image being analysed. 2 & 3 are the histograms and dot plots that set the background threshold. 4 shows the statistics display window. (5) The display adjustment window.
3.6 Cellular preparation

3.6.1 General tissue culture procedures

All tissue culture techniques were performed in sterile conditions in a class II laminar airflow unit, using culture grade plastic ware and reagents. Cells were kept at 37°C at 5% CO₂ (Carbon dioxide) in an incubator. Cells were grown in complete RPMI medium (RPMI 1640 with glutamax, supplemented with 10% foetal calf serum, heat inactivated, 50ug/ml streptomycin, 50U/ml penicillin, 2µg/ml fungizone and hepes buffer, stored at 4°C.

3.6.2 Peripheral blood mononuclear cell isolation

PBMCs were isolated using a density gradient centrifugation technique using lymphoprep solution. Lymphoprep contains sodium Diatrizoate and polysaccharide and has a density gradient of 1.077g/ml, less than mononuclear cells thus preventing their penetration through the lymphoprep layer. The solution aggregates the erythrocytes, increasing their sedimentation rate leaving lymphocytes and monocytes in solution, in the form of a distinct band at the sample/medium interface.

Buffy coats were diluted in sterile PBS in a 1:5 ratio. Fifteen millilitres of lymphoprep was added to a 50ml Falcon and 35mls of diluted blood was carefully layered on top. Tubes were centrifuged for 25mins and 400g with brake off. Theuffy coat was removed using a pasteur pipette into a new falcon, which was topped to the 50ml mark with sterile PBS. Cells were centrifuged for 5 mins at 800g. The supernantant was discarded and the pellet was vortexed. The cells were again washed with sterile PBS and centrifuged for 10 mins and 400g. The supernantant was discarded, and cells were resuspended in complete RPMI medium.

3.6.3 Cell enumeration and viability

Cell counts and visibility were determined using ethium bromide. Ethium bromide is an intercalating agent, which binds DNA and fluoresces under UV light. It stains the DNA of live cells green and the DNA of dead cells orange. Cell counts were performed using a haemocytometer shown in figure 3.8. 10 µl of the cell suspension was diluted in EBAO at Louise Elliott
a range of 1:2 to 1:20 and then mounted on an improved Neubauer Haemocytometer and visualised using a fluorescence microscope. The average number of live cells in one section (0.1 mm x 1 mm x 1 mm) of the haemocytometer is calculated and multiplied by $10^4$ and then by the dilution factor to give the number of cells in 1 ml of your cell suspension.

![Diagram of a Neubauer Haemocytometer](image)

Figure 3.8: Schematic diagram of a Neubauer Haemocytometer
Four 1 mm x 1 mm cell counting areas have an area of 0.1 mm$^3$. Cells are counted within this area and cell concentration was calculated. The volume of liquid held within each counting area is 1 mm x 1 mm x 0.1 mm.

3.6.4 Magnetic Bead Separation

Monocytes (CD$^{14^+}$ cells), T cells (CD$^{3^+}$, CD$^{4^+}$ or CD$^{8^+}$ cells), were enriched using Miltenyi microbeads. All reagents were pre-cooled and cells were kept cold to prevent non-specific cell labeling and capping of the antibodies. Total PBMCs were magnetically labeled with Microbeads (Miltenyi) and incubated for fifteen minutes at 4°C. After centrifugation 10 ml of cold miltenyi buffer was added and the suspension was centrifuged at 300g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 500μl of miltenyi buffer. Columns were primed with 3 ml of cold miltenyi buffer prior to cell addition. A pre-separation filter was fitted to the top of the column. The cell suspension was loaded onto a MACS column, which was placed in the magnetic}

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field of the MACS separator. The column was washed 5 times with 3 mls of cold miltenyi buffer each time to remove any unbound cells. After the last wash was completed 5 mls of miltenyi was added and the column was removed from the magnetic field and the positive cell fraction was eluted. Cell purities were assessed by flow cytometry and cell yield was determined.

3.7 Antibodies and flow cytometry

All of the antibodies used during the course of this work are listed in tables 3.2.7. Antibodies were titrated in order to determine the optimal concentration to use for staining. Fluorescence-minus-one (FMO) controls were used to set gates where necessary.

3.7.1 Principles of flow cytometry

Flow cytometry is a powerful technique that allows the multi-parametric analysis of individual cells in a heterogeneous population. It is based on several principles, hydrodynamic focusing, light scattering and emission of fluorochrome molecules. Cells in a suspension are injected and enclosed by sheath fluid that exerts a force that causes the sample stream to compress. This effect allows the cells to be delivered to the laser one at a time and is called hydrodynamic focusing. Once the cells pass through the laser they scatter light or emit fluorescence (if the cell is labeled with a fluorophore). This scattered light is collected and can provide information about cell properties. Light scattered in the forward direction, forward scatter channel) corresponds to cell size and the side scattered channel provides information on granular content within a cell.

Cells may also be labeled with antibodies conjugated to fluorescent probes and when passed through a laser of the right wavelength they become excited to a higher energy state and re-emit light at a longer wavelength. The fluorescence emitted by a fluorochrome conjugated to a mAb for a specific cell surface marker is interpreted by the flow cytometer software and given as mean fluorescence intensity (MFI), which correlates with the quantity of that specific marker on the cell surface.

Figure 3.9 shows a schematic diagram of the internal components of the flow cytometry employed in this study.

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3.7.2 Whole blood staining

100µl of whole blood was stained with directly conjugated antibodies for 20 minutes at room temperature in the dark. After incubation the red cells were lysed with 2ml of 1x FACS Lysing solution and all tubes were vortexed thoroughly. Tubes were spun for 7 mins @ 300g. The supernatants were discarded and washed twice with 2ml of PBA. The cell pellets were resuspended in 5 drops of 0.5% PFA and stored in the fridge until ready for acquisition.
3.7.3 Labeling of cells with fluorochrome-labeled antibodies

Cells were counted and suspended in PBA buffer (phosphate buffer containing 1% BS, and 0.02% sodium azide). The phosphate buffer is isotonic to the cell composition, BSA blocks non-specific antibody binding and the sodium azide prevents mAb endocytosis. Approximately 0.2x10^5 cells suspended in PBA were pipetted into each facs tube and the optimum amount of each fluorochrome-labeled antibodies were added. Cells were incubated for 20 mins at room temp in the dark, then washed with PBA buffer and centrifuged for 7 mins at 300g. The supernatants were discarded; the cell pellets were resuspended in 500µl of PBA and stored at 4 degrees until acquisition on the flow cytometer.

3.7.4 Labeling of cells with unconjugated antibodies

For monoclonal antibodies not directly conjugated to a fluorochrome, indirect staining with a secondary antibody, fluorochrome conjugated, was carried out. 2x10^6 cells suspended in 80µl of PBA were pipetted into each facs tube, purified unconjugated antibody was added at the optimised concentration. Cells were incubated for 15 mins at room temperature in darkness. Cells were washed 3 times in PBA buffer, supernatants were discarded and the cell pellet was vortexed. A fluorochrome conjugated secondary antibody was added to the cells at 10µl per tube, as per manufacturer's guidelines. Cells were incubated for 20 mins at room temperature in the dark, and were washed in PBA. Normal mouse serum (1/25 DF) was added to cells at 50µl per tube, and incubated for 15 mins in order to block non-specific binding sites on the secondary antibody. Cells were stained with mAbs, which were directly conjugated to fluorochromes, as described in section 3.8.3.

3.7.5 Intracellular staining

Cells were stimulated for 24 hours with medium alone, PMA (10ng/ml) and Ionomycin (1µg/ml). Brefeldin A (BFA) was added to each sample at a concentration of 10µg/ml. Cells were then washed using 2ml of cold PBA, and then centrifuged at 300g for 10 minutes. Cells were resuspended in 80ul of PBA per sample. The appropriate amount of each surface staining mAB was added and incubated at room temperature for 15 mins. Cells were washed with 2 ml of cold PBA to remove any unbound antibody and Louise Elliott
centrifuged at 400g 7 mins. Next 500μl of 4% paraformaldehyde (PFA) was added to each sample for 10 minutes at room temperature to fix cells (stop internalisation of surface mAb). Cells were washed with 2 ml of cold PBA, and then centrifuged at 400g for 7 mins. Cells were permeabilised by adding 1ml of 0.2% w/v saponin in PBA to each sample and incubated for 10 minutes at room temperature in the dark. Tubes were centrifuged to remove excess saponin. Cells were stained for the presence of intracellular cytokine by adding the appropriate amount of anti-human intracellular cytokine diluted in 50μl of 0.2% w/v saponin and incubated for 20 minutes at room temperature in the dark. Excess antibody was diluted and removed by centrifugation. Samples were resuspended in 500μl of 1% PFA and acquired by flow cytometry.

3.7.6 Instrument set up

All flow cytometry acquisition was carried out on a CyAn ADP flow cytometer and analysed using FlowJo software. The Cyan was set up for cell acquisition as recommended in the user manual. The gain and voltage were set on unstained control cells for FSC and SSC respectively to distinguish the different cell types based on size and granularity. The negative populations were then set on each of the fluorescent channels being used by gating on the cell population of interest based on FSC/SSC and adjusting the voltage until the peak of each fluorescent channel was between the 10^0 and 10^1 marks on the flow cytometry histogram. Compensation controls consisting of BD compensation beads stained with a single fluorochrome conjugated mAb were run. Single stain controls are used to compensate for spectral overlap of fluorochromes that have similar light emission wavelengths. After single stain controls were run for each fluorochrome, FMO controls were used when necessary to delineate negative and positive populations.

3.7.7 Phenotypic analysis of antigen presenting cells by flow cytometry

Cell surface staining was used to investigate antigen presenting cell phenotypes in coeliac patients and controls. Appropriate fluorescence minus one (FMOs) controls were used as a gating control, essential for the accurate discrimination of cells particularly when antigen expression is low. For the identification of dendritic cells, cells were visualised first on a forward and side light scatter plot and a gate was drawn around the
total live cell gate. This gate was then plotted against a lineage negative cocktail antibody against HLA-DR separating dendritic cells from all lineage positive cells.

For the identification of monocytes, cells were visualised first on a forward and side light scatter plot and a gate was drawn around the monocyte population. This gate was then plotted against HLA-DR to exclude natural killer cells. The resultant population was then visualised on a plot comparing CD14 against CD16 and a quadrant was used to identify the different subsets of monocytes.

3.7.8 Cell culture and measurement of proliferation by cell trace violet.

All cultures were done in 96 well flat-bottomed plates at 37°C in CO₂. Prior to cell culture, freshly isolated T cells were stained with T cell trace violet to evaluate cell proliferation. Cell Trace Violet diffuses into the cells where it is cleaved by intracellular esterase to yield a highly fluorescent compound. The compound then binds to intracellular amines.

T cells were prepared according to manufacturers guidelines. Briefly, 1μl of 5mM Cell Trace stock solution was added to each mL of cell suspension. Cells were incubated for 20 minutes at 37°C in the dark. After the incubation, 5 times the initial volume of complete medium was added to the cell suspension to quench any unbound reagent and left to incubate for 5 mins. After, the cell suspension was centrifuged at 400 g for 7 mins. The supernatant was discarded and the pellet was resuspended with pre-warmed medium.

Stained T cells (1x10⁵) resuspended in complete RPMI were plated onto anti-CD3/CD28 coated plates with and without varying concentrations of rhCD163 (2μg/ml & 10μg/ml). T cells alone were cultured alone in 200μl RPMI to serve as a negative control. Each culture was performed in triplicate and incubated for 6 days prior to evaluating T cell proliferation by flow cytometry (Figure 3.10).
Figure 3.10 Measurement of T cell proliferation using CellTrace violet dye. Naïve T cells are labelled with CellTrace violet before being stimulated for 6 days. Non-proliferating cells maintain a high CellTrace violet signal while proliferating cells lose this signal as they divide. Each peak on the histogram represents one round of cell division.

3.8 Human Dendritic Cells

3.8.1 Generation of monocyte derived dendritic cells (moDC)

PBMC were isolated from buffy coats and enumerated as described in previous sections. Monocytes were magnetically separated, as described in section 3.7.4, washed and resuspended at a concentration of 1 million per ml in cRPMI containing HyClone Foetal Calf Serum. HyClone defined FCS was used to prevent maturation of the DC due to endogenous endotoxins such as LPS. For the generation of monocyte derived DCs, isolated CD14+ monocytes were plated in 6 well culture plates to give a final concentration of 3X10^6 cells per 3 ml. GM-CSF and IL-4 were added to a final concentration of 50ng/ml and 70ng/ml, respectively. Cultured cells were incubated at 37°C, 5% CO2 for 6 days. On day 3, 1ml of medium from each well was removed and replaced with 1.5ml of cRPMI containing 50ng/ml of GM-CSF and 70ng/ml of IL-4. On day 6, cells were carefully harvested from wells by gentle flushing and agitation using cRPMI. Monocyte derived DCs were enumerated and resuspended to a final
concentration of $1 \times 10^6$ cells per ml in RPMI. Flow cytometry was used to verify that
differentiation into iDC had taken place and cells expressed HLA-DR and CD11c but not
CD14.

3.8.2 Stimulation and analysis of dendritic cells with LPS

Freshly isolated moDCs were plated in cRPMI and 1μg/ml of LPS was added to each well.
Cultures were incubated for 24 hours at 37 °C with 5% CO$_2$.

3.8.3 Analysis of dendritic cell maturation

After 24 hour stimulation, 180μl of supernatant was removed from each well for
cytokine analysis, IL-10 and IL-12p70 production. 80μl of PBA was added to each well,
resuspended and removed to a FACS tube. Cells were stained with a panel of antibodies
shown in table 3.4.

<table>
<thead>
<tr>
<th>Tube A</th>
<th>Tube B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c APC</td>
<td>CD80-APC</td>
</tr>
<tr>
<td>CD14 FITC</td>
<td>CD83-PE</td>
</tr>
<tr>
<td>HLA-DR PE</td>
<td>CD86-FITC</td>
</tr>
<tr>
<td></td>
<td>CD40-APC</td>
</tr>
</tbody>
</table>

Table 3.4: DC maturation staining panel of mAb

3.9 Enzyme linked Immunosorbent Assay (ELISA)

DuoSet ELISA kits from R&D systems were used for the identification of secreted
cytokine in assay supernatants and soluble CD163 in the serum from healthy controls
and coeliac patients. The working concentrations for the antibodies and standards used
were obtained from the manufacturer’s instructions and varied depending on the
cytokine being measured. Each kit consisted of a capture and detection (biotin
conjugated) antibody specific for different epitopes of a particular cytokine as well as a
known standard quantity of the cytokine being measured and a streptavidin-horseradish
peroxidase (HRP) solution.

In summary, 96 well flat-bottomed plates were coated with 100μl per well of
specific capture antibody at the recommended concentration outlined in the manufacturer's protocol. The plates were sealed and left to incubate overnight at room temperature. After the overnight incubation, excess antibody was removed by washing 3 times with PBS and TWEEN and the plates were thoroughly dried. After, the plates were blocked by adding 300μl of the 1% BSA and were incubated for a minimum of one at room temperature. The wash step outlined was repeated and the plates were dried by blotting on tissue paper. Next 100μl of each standard and samples were added per well in triplicate. A high standard using recombinant cytokine (at a given concentration) was serially diluted to give a 7-point standard curve. Samples and standards were incubated for 2 hours at room temperature. The plates were aspirated and washed three times as described before. A 100μl of specific detection antibody (at the manufacturers recommended concentration) was added to each well and incubated at room temperature for 2 hours. To remove excess antibody the plates were washed as described before. A 100μl of streptavidin-horseradish peroxidase (HRP) at the appropriate dilution was added to each well and left to incubate for 20 minutes. The plates were washed as before. Next a 100μl of substrate solution Tetramethylbenzidine (TMB) was pipetted to each well and left to stand for 20-30 minutes at room temp in the dark. 50μl of stop solution (2N H2SO4) was added to each well and the plates were read using the TECAN sunrise microplate reader set to 450nm and 620nm. The principle of ELISA is demonstrated in figure 3.11 show standard curves for IL-10 and IFN-gamma generated in accordance with protocols provided.
Figure 3.11 Graphs showing a set of standard curves used for ELISA. Serial dilutions of a standard cytokine solution are prepared and assayed alongside unknown samples in order to generate a standard curve for relative cytokine quantification. Graphs shown are chosen for a single representative experiment. Standard curves were generated for each ELISA performed.

3.10 Western Blot Analysis

Western blot analysis is based on a protein-protein hybridisation technique that is used for immunodetection of specific antigens of interest. The principle of Western blotting is as follows: (1) a protein mixture is first separated based on molecular weight on an SDS-PAGE gel. The separated proteins are immobilised on a nitrocellulose membrane. The antigen-antibody complex is visualised by incubation with a second antibody that is fluorescently labeled, bound to an enzyme with a colorimetric substrate, or is radioactively labeled.

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3.10.1 Whole Protein Isolation of monocytes and moDCs

Cells were pelleted and washed twice in cold PBS at 2,500 x g for 5 mins. After the last wash the supernatant was discarded and 1 ml of RIPA buffer for 5 million cells was added. Prior to the addition of RIPA buffer, protease inhibitor was added. The mixture was gently agitated for 15 mins at 4°C and then centrifuged at 14,000 x g for 15 minutes to pellet cell debris. The supernatant was transferred to a new tube and stored at -20°C until further analysis.

3.10.2 Membrane Protein extraction of monocytes and moDCs

Membrane protein was isolated from both monocytes and monocyte derived dendritic cells employing a membrane extraction kit (Pierce Thermo Scientific) according to manufacturers instructions. In summary, 5 million cells (monocytes and moDCs) were harvested and centrifuged at 850 x g (RCF) for 2 min. The supernatant was discarded and the cells were washed in PBS in 1.7 ml microcentrifuge tubes. The supernatant was carefully removed and 150 μl of reagent A containing protease inhibitors was added to cell pellet. The homogenous cell suspension was incubated for 10 minutes at room temp with occasional vortexing. After incubation the cells were placed on ice and 450 μl of diluted reagent C was added to lysed cells. The cells were incubated on ice for 30 mins and vortexed at 5 minute intervals. Tubes were centrifuged at 10,000 x g for 3 mins at 4 degrees and the supernatant was transferred to a new tube. The supernatant was incubated for 20 minutes at 37 degrees to separate the membrane protein fraction. Tubes were then centrifuged at room temperature for 2 mins at 10,000 g to isolate the hydrophobic fraction (the fraction containing membrane proteins of interest) from the hydrophilic fraction. The hydrophilic phase (top layer) was removed from the hydrophobic protein phase (bottom layer) and saved in a new tube. The layers had to be separated quickly as possible because the interface between the layers slowly disappear at room temp. Fractions were stored at -20 until further analysis.

3.10.3 Dialysis

Before the protein concentration could be determined in the membrane fraction, dialysis was performed to remove the interfering detergent. The slide-A-Lyzer Dialysis Cassettes (Thermo Scientific) were used for this purpose. Before the sample was added Louise Elliott

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to the cassette, the cassette required hydration, to increase membrane flexibility. To hydrate, the cassettes were immersed in dialysis buffer (PBS containing CHAPS) for 1-2 minutes. The cassette was removed from the buffer and excess water was wiped off. The syringe was filled with sample, due to the small working volumes the syringe was filled with a small volume of air before sample uptake to prevent sample loss in the syringe’s dead volume. With the needle the gasket was penetrated through one of the syringe ports and sample was injected. With the needle still in the cassette, the piston was drawn up to remove air, compressing the membrane windows so the sample contacts the greatest surface area. The syringe was removed and the cassette was placed in the dialysis solution. The sample was dialysed for 2 hrs at room temp, the dialysis buffer was changed and the sample was again dialysed overnight at 4 degrees. A dialysis volume of 200-500 times the volume was used. After the dialysis was completed the syringe was filled with air and inserted into the cassette cavity. The air was discharged into the cavity to separate the membranes and the unit was turned upside down so that the sample is indirect contact with the tip of the needle and was withdrawn into the syringe.

3.10.4 Protein quantification with Bicinchoninic acid (BCA) protein assay

The Bicinchoninic acid reagent was used to generate a standard curve using a BSA standard. This curve was used to measure the concentration of total protein isolated from cell lysates.

BCA working reagent was prepared by combining BCA reagent A with BCA Reagent B in the ratio of 50:1, to yield a clear, green colour. All standards and samples were prepared in triplicate. Cell lysates or standards were added to working BCA reagent at a ratio 1:8 and incubated for 2 hours at 37°C. Colorimetric absorbance readings at 620nm were obtained using the TECAN Sunrise plate reader.

GraphPad prism v5.0 was used to draw the standard curve and obtain protein concentration values for the cell lysates. A standard curve was prepared by plotting the average-blanked-corrected 562nm measurement for the BSA standards vs. its concentration in milligrams per millilitre.
3.10.5 Sodium Dodecyl sulphate polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate (SDS) is an anionic detergent that denatures and coats proteins, generating a negative charge. Thus all proteins in solution separate based upon their molecular weight in an electrical field.

Prior to use the electrophoresis plates were rinsed with ethanol. A spacer plate and a short plate were placed into the casting frame and the pressure cams were closed. The shorter plate was facing outwards and it was ensured that both plates were level with the surface. The casting frame was positioned onto the rubber gaskets in the casting stand and was locked in position. The comb was placed between the two plates and the glass was marked 1cm down from the end of the comb. The comb was removed and the 12% resolving gel was prepared in the following order (Table 3.5).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3ml dH20</td>
<td></td>
</tr>
<tr>
<td>4.0ml Bis-30%</td>
<td></td>
</tr>
<tr>
<td>2.5ml 1.5M Tris-HCLpH8.8</td>
<td></td>
</tr>
<tr>
<td>100ul 10% APS</td>
<td></td>
</tr>
<tr>
<td>100ul 10% SDS</td>
<td></td>
</tr>
<tr>
<td>5ul TEMED</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5: Recipe for resolving gel

The resolving gel was quickly poured between the glass plates up to the 1 cm mark. The resolving gel was overlayed with 1ml of distilled water and the casting stand was gently tapped. The gel was allowed to set for 40 minutes at room temperature. When gel was set the water was removed and the stacking gel was prepared in the following order as shown in Table 3.6.
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1ml dH2O</td>
<td></td>
</tr>
<tr>
<td>0.5ml Bis</td>
<td></td>
</tr>
<tr>
<td>370ul 0.5M Tris HCL pH</td>
<td></td>
</tr>
<tr>
<td>30ul APS 10%</td>
<td></td>
</tr>
<tr>
<td>30ul SDS 10%</td>
<td></td>
</tr>
<tr>
<td>3ul TEMED</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.6: Recipe for stacking gel**

The stacking gel was mixed and pipetted between the glass plates until solution reached the top. The comb was added carefully ensuring to air bubbles developed. The stacking gel was allowed to set for 40 min at room temp. After the gel sandwich was removed from the casting frame and the glass plates were fasted with the gel support with the shorter plate facing inwards. The clamping frame and fastened gel cassettes were placed into the electrophoresis rig. The clamping frame required two plates to create a functioning assembly. If an odd number of gels were being used the buffer dam was placed in the empty spot. 1x SDS-PAGE running buffer was poured up to the mark in the outer reservoir and in the inner reservoir between the 2 plates. The comb was carefully removed and the protein samples and the molecular weight markers were loaded in to their designated wells.

Before loading, 2x loading buffer was mixed with each protein sample. Samples were vortexed, and heated at 95°C for 5 minutes. After heating samples were centrifuged and loaded. Empty wells were loaded with 10μl of loading buffer to ensure that that sample lanes did not spread out. The leads were attached and the gel was run at 140V at 30mAMPs until the bromophenol blue dye reached the bottom of the gel.

**3.10.6 Gel Stain**

For the visualisation of the protein bands the gel was stained with brilliant blue stain. Prior to staining, the gel was incubated in fixing solution (7% glacial acetic acid, 40% (v/v) methanol) for 30 minutes. The gel was incubated overnight in staining solution (4 parts Brilliant Blue G-Colloidal [Sigma] and 1 part methanol) with continuous shaking. The gel was destained (10% acetic acid and 25% methanol) for 60 seconds with gentle
agitation and then rinsed with 25% methanol. Finally, the gel was incubated in 25% methanol for 3 hours.

3.10.7 Immunoblotting

The stacking gel was removed from the gel and its measurements were recorded. Ten pieces of filter paper and one PVDF membrane were cut to the size of the gel. It is important that the blotter paper and the membrane are the same size of the gel to prevent inefficient transfer. Five pieces of filter paper were soaked in transfer buffer and placed on the bottom plate of the blotting apparatus. The PVDF membrane was activated by soaking it in methanol for 60 seconds. The membrane was then saturated in transfer buffer and placed on top of the filter paper. The gel was briefly soaked in transfer buffer and placed on top of the membrane. Another 5 pieces of filter paper were saturated in transfer buffer and stacked on top of the gel. The top plate of the blotting apparatus was placed over the sandwich and the electrodes were connected to a power supply. A current of 100mAmp/per gel was applied. The PVDF membrane was carefully removed from the apparatus and incubated in blocking solution for 1 hour at room temp with continuous agitation. The primary antibody was diluted at the appropriate dilution in blocking solution and incubated overnight at 4 degrees. After the incubation the membrane was washed 3 times in PBS tween. The secondary antibody was then applied at the appropriate dilution, made up in blocking buffer, and incubated for 1 hr at room temp with gentle agitation. The membrane was washed 3 times at 5-minute intervals with PBS TWEEN.

To visualise the protein bands a chemiluminescent detection agent was applied to the blot for one minute with constant agitation. The horseradish peroxidase catalyses the oxidation of luminol into a product that emits light when it decays. The amount and location of light that HRP catalyses, is directly correlated with the location and amount of protein on the membrane. After the one-minute the membrane was careful placed into a cassette where it was positioned on top of clear acetate, with another one over it and sellotaped into place. A sensitive sheet of photographic film was placed against the membrane, for a specific amount of time. The exposure to the light from the reaction creates an image of the antibodies bound to the blot. The developing process was carried out in a dark room.

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3.11 Real Time- Polymerase Chain Reaction

There are several types of RT-PCR established to date; TaqMan was the chosen method for this experiment. In this study, the technique RT-PCR was used to determine tTG mRNA in monocyte derived dendritic cells.

3.11.1 RNA extraction

Before carrying out the RNA extraction pipettes and worktop were cleaned with RNase zap to prevent RNase contamination. Cells were harvested and centrifuged for 5 minutes at 300g and the supernatants were aspirated. The RNeasy mini-kit (Qiagen) was used to extract the RNA. Cells were disrupted in 350μl of buffer RLT and homogenised. The lysate was pipetted into a QIAshredder spin column and centrifuged at full speed for 2 min. An equal volume of 70 per cent ethanol was added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample was well mixed by pipetting up and down and was then applied to the RNeasy spin column and spun in a microcentrifuge at 10,000rpm for 15 seconds. The total RNA binds to the column and the supernatant was collected and discarded. The spin column was then washed 3 times to remove any contaminants. First 700μl of buffer RW1 was added to the spin column and centrifuged for 15 s at 8000g. The column was then washed twice with 500μl buffer RPE for 15 s at 8000g and then for 2 min at 8000xg. The spin column was placed in a new collection tube and spun at full speed for 1 minute to eliminate any carryover of RPE buffer. The spin column was placed in a new 1.5ml collection tube and 40μl of RNase free water was added to the column. The tube was centrifuged for 1 minute at 8,000xg to elute the RNA. Samples were stored at -80°C until further analysis.

A sample of RNA was taken to measure the yield and purity of the RNA using an 8 sample spectrophotometer ND-800 (Nanodrop). Spectrophotometry was carried out in duplicate to achieve accurate results. Following RNA extraction, spectrophotometry revealed that the RNA yields were sufficient. RNA purity was also tested. Pure preparations of RNA have $\text{OD}_{260}/\text{OD}_{280}$ values of greater or equal to 2. If there was contamination with proteins such as nucleases, for instance, the ratio would be less than 2. RNase contamination was the primary concern but contamination with any proteins...
would prevent accurate quantification of RNA. Similarly, pure preparations of RNA have 
\( \text{OD}_{260}/\text{OD}_{230} \) values of 2. If there was contamination with phenolate ions or other organic 
compounds then, this ratio would be less than 2, and as with protein contamination, 
accurate quantification of RNA would not be possible. The \( \text{OD}_{260}/\text{OD}_{280} \) and \( \text{OD}_{260}/\text{OD}_{230} \) 
values were greater than but close to 2 for all the RNA samples of interest. Once the 
yield had been determined, the appropriate amount of RNA could be reverse 
transcribed to give 1000 ng of cDNA. This was the amount of cDNA needed to perform 
RT-PCR for tTG and house-keeping gene GAPDH.

### 3.11.2 Reverse transcription

Before PCR could be performed the RNA samples required reverse transcription into 
DNA. The work area, pipettes and gloved hands were swabbed with RNaseZap to ensure 
the area was RNA free. All kit components were thawed on ice. First 500ng from each 
RNA sample was aliquoted and made up to a final volume of 6μl with RNase free water 
and then 2μl of d(T)23 was added to the diluted RNA. 10μl of M-MuLV reaction mix 
(dNTPs and optimised buffer) and 2μl of M-MuLV Enzyme mix (reverse transcriptase and 
Murine RNase inhibitor) were added to the tubes. The cDNA synthesis reaction was 
incubated at 42°C for 1 hour and then heated to 80°C for 5 minutes to inactivate the 
enzyme. To detect if DNA contamination was present a negative control, all components 
were added except the enzyme, was included.

### 3.11.3 Polymerase Chain Reaction (PCR)

Once the cDNA was obtained, PCR could take place. Assays in real-time PCR assays, you 
monitor the progress of the PCR as it occurs. Data are collected throughout the PCR 
process rather than at the end of the PCR process (end-point PCR). There are two types 
of quantitative real-time PCR: absolute and relative. For the purpose of this experiment 
relative quantification was chosen employing the Taqman probe principle (Figure 3.12).

### 3.11.4 Controls used for RT-PCR

Several experimental controls were used to ensure that the results of RT-PCR were both 
accurate and true. The first experimental control was the housekeeping gene, GAPDH. It 
was used as a marker of cDNA quality i.e. if the cDNA was of poor quality then the cycle
threshold \((C_t)\) values for the housekeeping gene would be out of range i.e. significantly less than 15 or greater than 18. The third control was the non-template control (NTC). It contains the PCR reaction mixture for tTG, with RNase-free water substituting the cDNA. It is used to control for contamination and due to the intentional omission of cDNA, it should yield an undetermined \(C_t\) value unless, the PCR reaction mixture is contaminated. The final control was the assignment of triplicate wells for all standards and samples of interest. This reduces the potential for experimental error and maximises accuracy by ensuring that the \(C_t\) values for each duplicate are within 1-2 cycles of each other.

![TaqMan probe chemistry mechanism](image)

**Figure 3.12: TaqMan probe chemistry mechanism**
Taqman probe-based chemistry was employed for the detection and measurement of nucleic acid. It incorporates a fluorogenic probe to allow the detection of a specific PCR product as it accumulates during the PCR reaction. Each reaction contains a specific primer and a fluorescently labeled probe. The oligonucleotide probe has a 5' reporter dye and a 3' quencher dye and is designed to anneal and target the sequence between the forward and reverse primers. The close proximity of quencher greatly suppresses the fluorescence of the reporter dye. During amplification the Taq DNA polymerase cleaves the probe, separating the reporter dye from the quencher dye, increasing the reporter signal and allowing extension to continue. The increase in fluorescence only occurs if the target sequence is amplified, thus preventing detection of non-specific amplification. Within the exponential phase, the amount of product, and hence fluorescent signal, is directly proportional to the amount of amplicon present.

3.11.5 Analysis of PCR Data

The Ct values are the cycle at which fluorescence from a sample crosses the threshold of background. The quantity of DNA doubles every cycle during the exponential phase and relative amounts of DNA can be calculated. For example, a sample whose Ct value is 3 cycles earlier than that of another sample has $2^3$ times more template.

Relative quantification describes the change in expression of the target gene in a test sample relative to the calibrator. The calculation method used for relative quantification was comparative Ct method, employing the formula $2^{-\Delta\Delta CT}$. This method compares the Ct value of one target gene to another.

To perform the comparative Ct method for relative quantification summarised in table 3.7.

1. Average the tTG and GAPDH values.
2. Subtract the tTG average by the GAPDH average.
3. Designate the calibrator (monocyte).
4. Subtract the averaged sample value by the averaged calibrator value

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<table>
<thead>
<tr>
<th>$C_{T,tTG}$</th>
<th>$C_{T,\text{gapdh}}$</th>
<th>$\Delta C_T$</th>
<th>$\Delta C_T$</th>
<th>$2^{\Delta \Delta C_t}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_T(\text{target}) - C_T(\text{endogenous control})$</td>
<td>$\Delta C_T(\text{target}) - \Delta C_T(\text{calibrator})$</td>
<td>Amount of tTG relative to the calibrator mRNA,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7: Table summaries the comparative $C_T$ Method

### 3.12 Statistical Calculations

The Mann-Whitney U test was used to ascertain whether there were significant differences between the frequencies of cells in patients and control subjects. P-values lower than 0.05 were considered as significant, while those less than 0.005 and 0.0005 were considered to be highly significant. *, ** and *** represent $p<0.05$, $p<0.01$ and $p\leq0.001$ respectively.
Chapter 4 The Characterisation of Antigen Presenting cells in Coeliac Disease

4.1 Introduction

It is well known that gliadin reactive T cells play a central role in the pathogenesis of coeliac disease (Molberg et al., 1997), however, the mechanisms that lead to the generation of these cells are not fully understood. *Ex-vivo* experiments involving gluten exposure to coeliac duodenal biopsies have been shown to induce T cell activation within the lamina propria suggesting that antigen presenting cells present in the mucosa are capable of gliadin presentation (Molberg et al., 2000). This is further supported by the increase in the number of HLA-DQ2 positive cells found in the lamina propria of the small intestine of untreated coeliac patients (Melinda Ráki et al., 2006). Since oral tolerance and the type of adaptive immune response elicited is maintained in part by antigen presenting cells (APCs) (Goubier et al., 2008; Gregori, 2011; Hadeiba et al., 2012; Walker & Abbas, 2002), we hypothesise that dysregulation of the different APCs in coeliac patients may contribute to the loss of tolerance to gliadin and the immune cascade that ensues. Moreover, since CD is considered to be a systemic disease and circulating immune cells are believed to reflect the state of the immune system within the gut we propose to investigate the different APCs in both tissue and the peripheral blood in healthy subjects and coeliac patients and determine if modifications of the immune phenotype exist in the latter.

To date there have been several studies published investigating the different antigen presenting cells present in the coeliac mucosa. Both Raki *et al* and Sabatino *et al*, explored DC subsets in the coeliac mucosa, and produced conflicting results (Beitnes et al., 2011; Beitnes et al., 2012; Di Sabatino et al., 2007; Ráki et al., 2013; Ráki et al., 2006). Raki *et al* showed that the CD11c+ myeloid DC subset accumulated in the coeliac lesion and employing *ex-vivo* experiments demonstrated that isolated lamina propria myeloid DCs cells were capable of presenting the 33-mer-gliadin peptide to gluten reactive T cells (Melinda Ráki et al., 2006). Raki did not identify the second major DC subset, the plasmacytoid DC, which are characterised by their expression of CD123 and lack of CD11c (M Ráki et al., 2013; Melinda Ráki et al., 2006). In contrast, Sabatino *et al*
concluded that the CD123+ plasmacytoid DCs were the dominant subset found in the coeliac mucosa. The same group found higher levels of IFN-α mRNA in biopsies from untreated coeliac patients and employing blocking experiments against IFN-α, an inhibition of IFN-γ transcripts was observed in ex-vivo organ cultures of coeliac biopsies challenged with gluten (Di Sabatino et al., 2007). This suggests the plasmacytoid DC subset, which is a potent producer of IFN-alpha, may play a role in the pathogenesis of coeliac disease. However, due to the minimal published data and the inconsistency in results additional studies are warranted to define the major DC subsets found in healthy and coeliac mucosa.

Less is known about the prevalence of the major members of the peripheral innate immune system in CD. To date the majority of research has focused their attention on the frequency of innate T cells in the periphery, such as NK, NKT and iNKT cells, finding a decrease in all cell populations (Bernardo et al., 2008; Olaussen et al., 2007; Calleja et al., 2011; Cseh et al., 2011; Grose et al., 2008; Grose et al., 2007). However, information is limited with regard to the prevalence of circulating antigen presenting cells especially dendritic cells and monocytes. Several studies examining the peripheral APC subsets have been carried out in both paediatric (Cseh et al., 2011; Vuckovic et al., 2007) and adult coeliac subjects (R Ciccocioppo, Ricci, & Rovati, 2007). To date only one study has been conducted in an adult population with the aim of characterizing peripheral APCs. Ciccocioppo et al observed a decrease in the entire DC population, mainly the CD123+ plasmacytoid DC subset, in both treated and untreated coeliac patients. Two additional studies performed in a paediatric population have also reported opposing results. The first study carried out by Vuckovic and colleagues demonstrated no difference in the frequency of both myeloid and plasmacytoid DCs (Vuckovic et al., 2007). However, when comparing the absolute numbers a decrease in the myeloid subset was noted while the plasmacytoid subset remained unchanged. The second study carried out by Cseh et al found an increase in the absolute numbers of myeloid DCs in newly diagnosed children and a slight decrease, though insignificant, in the plasmacytoid subset (Cseh et al., 2011). Due the limited published data regarding APC frequencies and the conflict in results no strong conclusion can be drawn with regard to peripheral DCs and the potential role they may play in the pathogenesis of coeliac disease. Thus it is important
to further investigate these immune cells in the peripheral blood.
In this chapter we wish to define the key APCs in the coeliac mucosa and to investigate 
frequencies and phenotype of the main APC in the peripheral blood in both a paediatric 
and adult population. This will help us to further understand the role of APC \textit{in-vivo} in 
coeliac disease.

4.2 Objectives

1. Determine the main APC subsets in duodenal biopsy tissue taken from healthy 
   adults and to compare then treated and untreated coeliac adults employing 
   immunofluorescence confocal microscopy.
2. To investigate the main DC subsets freshly isolated from duodenal biopsies taken 
   from a paediatric population using flow cytometry.
3. Phenotype peripheral APCs employing flow cytometry in coeliac subjects from 
   both paediatric and adult population and aged matched controls.
4.3 RESULTS

Multicolor Immunofluorescence Tissue Staining

4.3.1 Antigen presenting cells in the Normal Duodenal Mucosa

In normal tissue, CD11c, a marker often used to identify myeloid DCs, recognised a minor cell population when compared to the predominant CD68+ macrophage (Figure 4.1). The CD11c+ cells were found widely distributed in the villi and in the deeper parts of the lamina propria (Figure 4.2). In the mucosa CD11c positive cells exhibited the classical characteristics of dendritic cells, displaying a non-uniform shape with surface projections as shown in figure 4.3.

Figure 4.1: Distribution of CD68 in paraffin sections from normal duodenal tissue from different patients displaying CD68 in the villi and lamina propria (A) & (B). Original magnification x 40
Figure 4.2: Distribution of CD11c in the normal duodenal mucosa. (A) and (B) shows CD11c (green) staining of a villus and the lamina propria from a healthy control, respectively. Original magnification x 400

Figure 4.3: Example of CD11c (green) dendritic cell morphology from normal duodenal mucosa. Original magnification x 400. (B) Is a zoom in of the area drawn around image A, demonstrating dendritic cell morphology.
4.3.2 Antigen presenting cells in the coeliac duodenal mucosa

Quantitative analysis was performed on all images taken from each section for all healthy controls, treated and untreated coeliac patients stained for CD11c (Figure 4.6 A) and CD68 (Figure 4.6 B). In summary, using Imaris analysis software, the surface area (um²) for CD11c and CD68 was determined and expressed as a percentage of the total lamina propria area measured.

A significant increase in CD11c expression in untreated coeliac biopsies was observed when compared to both treated and healthy mucosa (p 0.0002). The increased intestinal CD11c+ expression was particularly prominent in the subepithelial compartment under the basement membrane (Figure 4.4 A). In biopsies from treated coeliac patients, CD11c+ expression was similar to that of normal controls. No difference in CD68 expression was observed in untreated coeliac patients when compared to treated patients and healthy controls (Figure 4.6 B). Figure 4.5 shows CD68 staining of duodenal biopsy taken from an untreated coeliac patient displaying CD68 positive cells beneath the basement membrane and the deeper parts of the lamina propria.
Figure 4.4: Example of CD11c Distribution in a duodenal biopsy taken from an untreated coeliac. (A) and (B) shows CD11c (green) staining under the basement membrane and the deeper parts of the lamina propria, respectively. Note the prominent expression of CD11c at basement membrane (A). Original magnification x400.
Figure 4.5: Example of CD68 distribution in a duodenal biopsy taken from an untreated coeliac (A) and (B) shows CD68 (green) staining under the basement membrane and the deeper parts of the lamina propria, respectively. Original magnification x400.
Figure 4.6: Quantitative evaluation of CD11c (A) and CD68 (B) surface area expression in normal, TCD and UCD. Surface expression of CD11c and CD68 in sections from duodenal mucosa of healthy controls (n=13), treated (n=6), and untreated coeliac patients (n=9).
4.3.3 Absence of plasmacytoid DCs in the intestinal mucosa

In peripheral blood, plasmacytoid DCs are identified as lineage negative CD123+CD11c- and are a prominent DC subset found in the peripheral blood. This phenotype was not detected in the duodenal mucosa from both healthy and subjects with coeliac disease. To ensure suitability of monoclonal antibody with paraffin sections, a positive control was run parallel with each experiment. Positive staining was observed on lymph node sections on all occasions while no staining was observed in duodenal sections.

Investigation of freshly isolated lamina propria DCs employing flow cytometry

4.3.4 CD123+ plasmacytoid DCs are undetectable from freshly isolated lamina propria cells.

To help support our observations made in the immunofluorescence studies we investigated whether myeloid DC and plasmacytoid DC subsets could be identified in lamina propria cells isolated from duodenal tissue employing flow cytometry. Biopsies from newly diagnosed children with coeliac disease and aged matched controls were analysed. Fresh biopsies were prepared as described in chapter 3. Briefly, small intestinal epithelial and lamina propria cells were released from biopsies by a combination of agitation followed by collagenase digestion, respectively. Agitation removed the epithelial layer of cells, leaving the lamina propria attached to the basement membrane whereas collagenase destroys the extracellular matrix releasing the lamina propria cells. The remaining cell suspension was counted and stained with the appropriate DC markers.

Dendritic cells were identified from total lamina propria cells by gating on the total live cell population plotted on a forward scatter versus side scatter cytometric dot plot shown in figure 4.7a. The total antigen presenting cell population were identified within the HLA-DR versus CD45 cytometric dot plot. Gating on the double positive cells and regating this population against a lineage negative cocktail monoclonal antibody against HLA-DR, identified DCs as HLA-DR-positive, lineage-negative as shown in figure 4.7a.
4.7b. The lineage negative antibody recognises and eliminates T cells, B cells, monocytes, natural killer cells, and neutrophils.

No difference in the frequency of total DCs was observed between newly diagnosed coeliac patients and healthy subjects (figure 4.8). Both healthy controls and coeliac patients expressed similar levels of CD11c+ myeloid cells when expressed as a percentage of the HLA-DR+ lineage negative subset (Figure 4.8b). In support of our immunofluorescence studies plasmacytoid DCs were virtually undetectable in all patients studied, with a mean of 0.52% compared to myeloid DCs (mean 24.98%) (figure 4.8c). Table 4.1 summaries the frequencies of freshly isolated lamina propria DCs from duodenal biopsies.

Figure 4.7: Flow cytometric analysis for the identification of lamina propria dendritic cell freshly isolated from duodenal paediatric samples. Cells highlighted on the side scatter forward scatter dot plot (A) were gated against HLA-DR vs CD45 to identify the total antigen presenting cell population (B). This population was visualised against HLA-DR vs lineage negative cocktail, identifying the DC population highlighted in C.
Figure 4.8: Flow cytometric analysis of lamina propria dendritic cell subsets isolated from duodenal paediatric samples. Representative flow cytometric plots of lamina propria DCs from a healthy subject (A), and newly diagnosed paediatric patient (B). (C) Scatter plots for the frequency of total dendritic cells (top), and CD11c+ MDC (middle) and CD123+ PDC (bottom) expressed as a percentage of the total DC gate. FMO (fluorescence minus-one) controls were used to determine gates. Values in the top right/left corner of cytometric dot plots represent percentages of cells of interest. Medians are indicated by horizontal lines.
<table>
<thead>
<tr>
<th></th>
<th>Aged match controls</th>
<th>Newly diagnosed CD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total dendritic cells</strong></td>
<td>15.71% (1.67-27.3%)</td>
<td>18.98 (8.81-27.6%)</td>
</tr>
<tr>
<td><strong>Mean %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lin-HLA-DR+CD11c+</strong></td>
<td>24.98% (13.7-49.6%)</td>
<td>25.56% (10.4-65.1%)</td>
</tr>
<tr>
<td><strong>Mean %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lin-HLA-DR+CD123+</strong></td>
<td>0.52 (0.17-0.7)</td>
<td>0.47 (0.00-1)</td>
</tr>
</tbody>
</table>

Table 4.1: Frequency of dendritic cells isolated from paediatric duodenal tissue

* Lin-HLA-DR+CD11c+ cells were expressed as a percentage of the HLA-DR+ Lineage negative gate

The investigation of peripheral blood dendritic cells employing flow cytometry

4.3.5 Dendritic cell populations in adult human peripheral blood

To help understand the significant high expression of CD11c+ MDC found in the mucosa from active coeliac patients, enumeration of the different dendritic cell populations in human peripheral blood was determined in untreated coeliac patients and healthy controls for both an adult and paediatric cohort by flow cytometric analysis.

Circulating dendritic cells are a rare population of blood leukocytes (<1% of PBMC), which can be characterised as Lineage negative, HLA-DR positive cells shown in figure 4.9. In order to limit sample manipulation and the introduction of artifact whole blood samples from patients were stained and analysed, as described in chapter 2, as opposed to peripheral blood mononuclear cells.

Whole blood co stained with lineage negative antibody and HLA-DR identified the total dendritic cell population with a mean of 0.394% of total leukocytes. Within this gate, myeloid or plasmacytoid DCs were distinguished based on their expression of CD11c or CD123. The CD11c+ myeloid DC subset was found at a higher frequency.
compared to the CD123+ plasmacytoid subset and this trend was observed in all healthy donors, with MDC expression always dominating the PDC subset (Figure 4.10). Co-staining of these cells showed that the majority of these cells were two distinct populations (Figure 4.9 a). Data from these donors are summarised in Figure 4.10 b.

Figure 4.9: Gating strategy for the identification of total dendritic cells. Dendritic cells were identified by drawing a gate around the total live cell population (a) flow plot gating on the total live cell population (B) DCs were identified as an HLA-DR-positive, lineage-negative population highlighted within the gate. (C) Dotplot showing CD11c vs CD123, identifying two non-overlapping DC subsets.
Figure 4.10: (A) Myeloid and plasmacytoid DC subsets were studied for 15 healthy adults and expressed as a percentage of the total DC population identified as HLA-DR+ lineage negative cells. (B) Summary of peripheral dendritic cell subset data from healthy subjects

<table>
<thead>
<tr>
<th></th>
<th>Total Dendritic cells</th>
<th>CD11c DC</th>
<th>Plasmacytoid DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>0.39*</td>
<td>53.33</td>
<td>33.6</td>
</tr>
<tr>
<td>(Range %)</td>
<td>0.205-0.60*</td>
<td>(40-72)</td>
<td>18.6-42.9</td>
</tr>
</tbody>
</table>

Figure 4.10: (A) Myeloid and plasmacytoid DC subsets were studied for 15 healthy adults and expressed as a percentage of the total DC population identified as HLA-DR+ lineage negative cells. (B) Summary of peripheral dendritic cell subset data from healthy subjects

* Dendritic cells are expressed as a percentage of total dendritic cells

4.3.6 **CD11c+ myeloid DCs are found at a lower frequency in adult coeliac patients.**

Total dendritic cells, identified as HLA-DR+ lineage negative, expressed as a percentage of total leucocytes revealed similar frequencies in untreated and treated coeliac patients and healthy controls (figure 4.11a). Flow cytometry results revealed a significant reduction in the myeloid CD11c+ DC subset expressed as a percentage of total DC in untreated coeliac patients when compared to treated coeliac patients and healthy controls (p=0.0059) (Figure 4.12). In contrast, no difference in the plasmacytoid CD123+ population was observed between treated and untreated coeliac patients and controls. Representative dotplots for CD11c+ myeloid DC and CD123+ plasmacytoid DC subsets for a healthy control and an untreated and treated coeliac patient are illustrated in Figure 4.12. Table 4.2 summaries dendritic cell subset data for all adult donors.

Previously we noted that myeloid DCs were the major subset in the periphery in all Louise Elliott
healthy adults analysed. However, this trend was not as evident in untreated coeliac patients as several of the patients expressed a higher frequency of CD123+ plasmacytoid DC compared to the myeloid DC subset (Figure 4.13) resulting in an overall decreased myeloid/plasmacytoid ratio, with a mean of 1.3 compared to healthy controls with a mean ratio of 1.8.

Figure 4.11: Gating strategy for the identification of DC subsets. Representative flow cytometric analysis of whole blood from a healthy subject, (top) treated patient (middle panel), and an untreated coeliac (bottom) patient. The percentage of blood MDC (middle panel) and PDC (right panel) were expressed as a percentage of total DC obtained by plotting lineage cocktail against HLA-DR (left panel). Within this gate, myeloid or plasmacytoid DCs were identified based on the expression of CD11c or CD123. Values in the top right corner of cytometric dot plots represent percentages of identified DC subset.

Louise Elliott
Figure 4.12: Frequency of dendritic cells in adult coeliac patients. Data are represented as scatter plots for Healthy controls (n = 14), treated (n = 17) and untreated coeliac n (=14) patients for total dendritic cells (top), CD11c+ myeloid DC (middle) and CD123+ plasmacytoid DC.
Figure 4.13: Myeloid and plasmacytoid DC subsets were studied for 14 untreated coeliac adults and expressed as a percentage of the total DC population identified.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls</th>
<th>Treated Coeliac patients</th>
<th>Untreated Coeliac patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Dendritic Cells</strong></td>
<td>0.39</td>
<td>0.350</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Mean range</strong></td>
<td>0.205-0.600</td>
<td>0.130-0.575</td>
<td>0.035-0.65</td>
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<tr>
<td><strong>CD11c+ Myeloid</strong></td>
<td>53.8</td>
<td>51.2</td>
<td>43.94</td>
</tr>
<tr>
<td><strong>Mean range</strong></td>
<td>40-72</td>
<td>38-65.8</td>
<td>33.4-55.9</td>
</tr>
<tr>
<td><strong>CD123+ Plasmacytoid DC</strong></td>
<td>33.6</td>
<td>34.79</td>
<td>37.2</td>
</tr>
<tr>
<td><strong>Mean range</strong></td>
<td>18.6-42.9</td>
<td>16.3-56.7</td>
<td>22-56.2</td>
</tr>
<tr>
<td><strong>CD11c: CD123 ratio</strong></td>
<td>1.76</td>
<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Mean range</strong></td>
<td>1.0-2.9</td>
<td>0.6-2.9</td>
<td>0.6-2.1</td>
</tr>
</tbody>
</table>

Table 4.2: Summary of peripheral dendritic cell subset data for untreated adult coeliac patients
Expressed as a percentage of total leucocytes

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4.3.7 Comparison of myeloid and plasmacytoid DC in children with newly diagnosed coeliac disease and controls.

Our results revealed that peripheral dendritic cells from healthy paediatrics are found at a slightly higher frequency compared to healthy adults with a mean 0.5% of total leucocytes. In contrast to our observations made in adults, myeloid DCs were not the predominant DC subset in the periphery, as 50% of our controls presented with higher PDC frequencies than the MDC subset (Figure 4.14). A similar trend was observed among children newly diagnosed with coeliac disease, as 64% expressed higher plasmacytoid DC levels (Figure 4.14). Both coeliac children and controls displayed a similar MDC/PDC ratio, both with a mean of 1.2. When total dendritic cells expressed as a percentage of the total leukocyte population were analysed in newly diagnosed children and aged matched controls no difference was observed. Furthermore, similar frequencies of MDC and PDC within the total dendritic cell population were revealed in untreated children and healthy controls as shown in figure 4.16. Similar to percentages no significant difference in the absolute numbers of total DC, MDC and PDC in coeliac subjects was observed when compared with controls (data not shown). Table 4.3 summarises results for DC subsets in children newly diagnosed with coeliac disease.

Figure 4.14: Myeloid and plasmacytoid DC subsets were studied for 25 healthy controls and 17 children newly diagnosed with coeliac disease and expressed as a percentage of the total DC population.
<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls</th>
<th>Newly diagnosed coeliac</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Dendritic Cells</strong></td>
<td>0.55 (0.2-0.7)</td>
<td>0.53 (0.270-1.2)</td>
</tr>
<tr>
<td><strong>mean % (range %)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD11c+ mDC</strong></td>
<td>45.5 (31-60.9)</td>
<td>43.6 (24-67)</td>
</tr>
<tr>
<td><strong>mean % (range %)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD123+ PDC</strong></td>
<td>42.1 (18.4-58.4)</td>
<td>38.9 (12.8-58.9)</td>
</tr>
<tr>
<td><strong>mean % (range %)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD11c : CD123 ratio</strong></td>
<td>1.2 0.4-3.03</td>
<td>1.2 0.5-2.95</td>
</tr>
</tbody>
</table>

Table 4.3: Summary of peripheral dendritic cell subset percentages for newly diagnosed children
Figure 4.15: Numbers of dendritic cells in newly diagnosed children. Scatter plots demonstrate data for DC percentages and numbers for total dendritic cells (top), CD11c+ myeloid DCs (middle) and CD123+ plasmacytoid DCs (right). Blue and red circles represent results for healthy and coeliac subjects respectively.
The investigation of peripheral blood monocyte cells

4.3.8 Monocyte cell populations in adult human peripheral blood

Monocytes are the main antigen-presenting cell present in the peripheral blood and are believed to be precursors to tissue macrophages and a subset of mucosal dendritic cells, a phenomenon driven under inflammatory conditions. We investigated whether there was a difference in the frequency of each subset in healthy controls and coeliac patients in both an adult and paediatric population.

Monocyte subsets were defined as CD14++CD16- (classical subset), CD14++CD16+ (non-classical) and CD14+loCD16+ (intermediate) as shown in figure 3.16. The classical subset defined as CD14+CD16- comprise on average 76 percent of the total monocytes. The remaining two subsets, the intermediate CD14++CD16+ and the CD14+loCD16+ constitute the remainder.
Figure 4.16: Gating strategy for monocyte identification. Shown is an example of a staining for the identification of monocyte subsets using antibodies directed to CD14 and CD16 in a fresh blood sample taken from a healthy volunteer. Cells are visualised on FSC vs SSC and a gate was drawn around the monocyte subset to exclude debris, neutrophils and lymphocytes. These cells were then viewed on a HLA-DR vs FSC lin plot to exclude CD16+ NK cells. Finally CD14 was plotted against CD16 to identify the, Classical subset (CD14++ CD16-), intermediate (CD14++CD16+) and non-classical (CD14+lo CD16+) subsets. Three distinct monocyte subpopulations are delineated by squares that were drawn based on CD14+ and CD16+ FMO (fluorescence-minus-one) controls.
No difference in peripheral monocyte subset percentages in adults

No difference in the frequency of total peripheral blood monocytes in untreated coeliac patients (n=12) when compared to treated subjects (n=18) and healthy controls (n=25) was observed. On comparing the different monocyte subsets; classical, intermediate, and non-classical, similar levels were found among all untreated, and treated coeliac patients, and healthy controls as shown in figure 4.17. Table 4.4 summarises results for peripheral blood monocyte subsets in adult coeliac patients and control.

Figure 4.17: Monocyte numbers in adult coeliac patients. Scatter plots of the percentage of monocyte subsets, CD14++CD16- (top), CD14++CD16+ (middle), and CD14+loCD16+ (bottom) in the total monocyte HLA-DR positive gate. Black lines represent mean ± SEM. Healthy controls (HC), blue; treated coeliac disease (TCD), orange; untreated coeliac disease (UCD), red.

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4.3.10 No difference in the distribution of peripheral blood monocyte subsets in newly diagnosed children compared to aged matched controls

Next the distributions of CD14++CD16-, CD14++CD16+, and CD14+lo CD16+ monocytes from fresh whole blood from children newly diagnosed with coeliac disease (n=19) and aged matched controls (n=25) were compared. No difference in the frequency and absolute numbers of the classical, intermediate and non-classical monocyte subsets between coeliac subjects and aged matched healthy controls was observed (figure 4.18).

![Figure 4.18: Distribution of peripheral blood monocytes from children with newly diagnosed coeliac disease. Dot plots demonstrating the frequency (left) and absolute numbers (right) for each monocyte subset, CD14++CD16- (top), CD14++CD16+ (middle), and CD14+loCD16+ (bottom) for healthy and newly diagnosed paediatrics.](image-url)
Phenotypic Investigation of Peripheral Blood dendritic cells

4.3.11 HLA-DR expression on peripheral DCs is not altered in untreated coeliac patients.

Myeloid DC expressed higher levels of HLA-DR compared to the plasmacytoid DC in all patients studied. Figure 4.19 shows a flow cytometric histogram comparing the HLA-DR expression for plasmacytoid DC and myeloid DC taken from a healthy control. Myeloid DC and plasmacytoid DCs from both adult and paediatric coeliac patients displayed a similar HLA-DR surface expression as the healthy controls (figure 4.20).

![Figure 4.19: HLA-DR expression of DC subsets. Flow cytometry histogram plots of HLA-DR expression of CD11c+ MDC (red) and CD123+ PDC (blue). Values in histograms are for MFI (mean fluorescence intensity) of HLA-DR expression. Myeloid DCs consistently expressed higher levels of HLA-DR than the plasmacytoid subset.](image)
Figure 4.20: Distribution of HLA-DR expression for peripheral dendritic subsets. Histogram plots comparing HLA-DR expression for CD11c+ myeloid DC (top) and CD123+ plasmacytoid DC (bottom). Accumulative HLA-DR surface expression for both a paediatric (left) HC=25 CD=19; and adult (right) HC=25, TCD=12 UCD=15

Louise Elliott
4.3.12 An increase in the frequency of CD83 positive myeloid DCs are found in untreated children

We investigated the APC markers CD86 and CD80, co-stimulatory molecules that interact with CD28 or CTLA-4 on the surface of T cells. Upon dendritic cell activation these markers can be detected at increased levels on their surface compared to immature DCs. In addition we assessed CD83 expression, a further activation marker for human DC.

Of the three surface markers examined, CD86 was the most abundantly expressed on myeloid DCs in healthy controls, with a mean of 94.67%. CD80 and CD83 positive myeloid DC cells were almost undetectable in the periphery with a mean of 4.3% and 1.92%, respectively (Figure 4.21). When CD86 distribution from healthy controls was compared with untreated coeliac no difference in the percentage of CD86 positive cells and MFI expression was determined. A higher frequency (p = 0.0016) of CD83 positive myeloid DCs was found in newly diagnosed coeliac children compared to aged matched controls although similar levels of surface CD83 was revealed in both controls and coeliac subjects. Similar to CD86, no difference in both CD80 positive cells and MFI surface expression was found in active coeliac. The results are summarised in figure 4.22.
Figure 4.21: Gating strategy used to determine percentage and MFI expression for CD83, CD86, and CD80. (A) and (B) Represents flow cytometric plots for fluorescence minus control (FMO) and staining with antibodies CD83 (top), CD86 (middle), CD80, (bottom). (C) Histogram overlays represent staining with antibody (blue) against FMO control (green). Values in the top right corner represent percentage of positively stained cells. Values in histograms represent MFI results for corresponding surface antigen.
Figure 4.22: Phenotype profile of myeloid dendritic cells in newly diagnosed coeliac children. (A) Scatter plot showing the percentage of CD11c+ myeloid DC positive for the surface marker CD83 (top), CD86 (middle), and CD80 (bottom). (B) Histograms represent the mean fluorescence intensity (MFI) for CD86, CD83, and CD80 surface expression. Healthy controls (HC) and coeliac patients are shown in blue and red, respectively. Means are indicated by horizontal lines.
4.3.13 MDCs express higher levels of CCR6 in untreated patients compared to healthy controls

In this experiment we examined the distribution of chemokine receptor CCR6 on peripheral myeloid DCs in untreated coeliac patients and healthy controls.

CCR6 is involved in regulating several aspects of mucosal immunity, including the ability to recruit immature DCs to the sites of inflammation. It also plays a role in homing DCs to the gut mucosal lymphoid tissue. CCR6 binds chemokine CCL20 which is strongly upregulated by pro-inflammatory signals.

CCR6 surface expression determined by mean fluorescence intensity was found to be upregulated on myeloid DCs from untreated patients compared to healthy controls (p = 0.0120) shown in figure 4.23 a. CCR6 positive Myeloid DCs found in the peripheral blood were found at a higher frequency in untreated patients compared to healthy controls, although did not reach statistical significance (Figure 4.23 b).

Figure 4.23: CCR6 distribution on peripheral MDC. (A) Representative flow cytometry overlay histogram and bar chart comparing CCR6 surface expression (MFI) on myeloid DC from a HC (blue) (n=9) and untreated coeliac (red) (n=5)). (B) Representative dot plots of CCR6+ myeloid DC from a healthy control (left) and untreated coeliac (middle). Histogram plots represent accumulative data for CCR6+ myeloid DCs from HC and UCD patients.
Phenotypic Investigation of peripheral Blood Monocytes

4.3.14 Monocyte HLA-DR expression

As the MHC-class II molecule HLA-DR reflects the activation state of these cells (Tsakalidou, 2001), the expression of HLA-DR was determined on all monocyte subsets.

The CD14++CD16+ (intermediate subset) expressed the highest levels of surface HLA-DR while the classical (CD14++CD16-) and the non-classical (CD14+loCD16+) subsets expressed low and moderate levels, respectively (Figure 4.24).

Similar levels of HLA-DR expression for all three monocyte subsets were detected in healthy children and children newly diagnosed with coeliac disease. Comparable findings were found in the adult population with no difference in HLA-DR expression found among all monocyte subsets between untreated and treated coeliac patients and controls (Figure 4.25).

![Figure 4.24: HLA-DR distribution on monocyte subsets.](image-url) Flow cytometric histogram demonstrating HLA-DR surface expression for CD14++CD16- (green), CD14++CD16+ (blue), and CD14+loCD16+ (red) monocytes. Values represent MFI for surface HLA-DR. (B) Shows accumulative data for HLA-DR expression for healthy subjects.
Figure 4.25 HLA-DR surface expression of the identified monocyte subsets in both paediatric and adult populations. Accumulative data for HLA-DR Mean fluorescence intensities are shown for both adults, HC (n=13), TCD (n=12), UCD (n=13) (right) and children, HC (n=33) and CD (n=20) (left). A, B, and C represent data for CD14++CD16-, CD14++CD16+, and CD14+loCD16+ monocytes, respectively.
Intracellular cytokine production in peripheral blood monocytes

4.3.15 Cytokine production by blood monocytes

Next we analysed monocytes for the secretion of pro-inflammatory and anti-inflammatory cytokines either spontaneously or after stimulation with TLR ligand LPS alone or with IFN-γ by intracellular cytokine staining. The expression of TNF-alpha, IL-10, and IL-12/23 were studied in HLA-DR+ CD14+ peripheral blood monocytes from healthy controls, and patients with and treated and untreated coeliac disease, and untreated coeliac patients from an adult cohort only. It’s important to note, on analysing the results two distinct populations of monocytes were identified based on their differential expression of CD14. Therefore it was decided to analyse cytokine production from total monocytes and CD14+lo and CD14+hi subsets separately.

Expression of TNF-alpha in peripheral monocytes

Minimum levels of spontaneous TNF-α production was detected in Healthy controls and untreated patients while moderate levels were detected in treated patients. Upon stimulation with LPS a large proportion of total monocytes produced TNF-alpha with the highest levels detected in treated and untreated subjects compared to healthy controls. A similar difference in controls and coeliac patients was detected for the different monocyte subsets. No difference in TNF-alpha production was detected between CD14+lo and CD14+hi LPS stimulated monocytes.

Monocytes produced higher amounts of TNF-alpha when stimulated with LPS & IFN-γ compared to LPS alone. The biggest difference was observed among the monocyte subsets with the CD14++ monocytes producing higher amounts compared to the CD14+lo subsets, with almost a 2 fold in the difference. Similar to LPS stimulation monocytes taken from treated and untreated patients with coeliac disease produced higher levels of TNF-alpha compared to HC. A bigger fold increase in cytokine production was observed in UCD (16 fold) compared to TCD (3.4 fold) and HC (fold 9.3)

Examples of TNF alpha expression in peripheral blood monocytes stimulated with LPS&IFN-γ from a healthy control are illustrated in dot plots in Figure 4.26 a. Table 4.5 and figure 4.26b summaries the expression of TNF alpha from untreated, and treated CD patients and controls.

Louise Elliott
Table 4.5: Summary of intracellular TNF-alpha expression in CD14 positive peripheral monocytes in patients with coeliac disease and controls

<table>
<thead>
<tr>
<th></th>
<th>unstimulated</th>
<th>LPS</th>
<th>LPS &amp; IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC</td>
<td>TCD</td>
<td>UCD</td>
</tr>
<tr>
<td>Total Monocytes</td>
<td>7.4</td>
<td>20</td>
<td>4.4</td>
</tr>
<tr>
<td>CD14⁺⁺⁺</td>
<td>6.7</td>
<td>22.5</td>
<td>3.7</td>
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<tr>
<td>CD14⁺⁺</td>
<td>8.2</td>
<td>37</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Figure 4.26: Intracellular TNF-alpha production of monocytes in coeliac adults and control donors. (A) Flow cytometric dot plots showing the intracellular production of TNF-alpha in monocytes stimulated with LPS & IFN-γ. The cytokine profiles for total monocytes (left), CD14⁺⁺⁺ monocytes (middle), and CD14⁺⁺ monocytes (right) were determined. (B) Histogram plots representing the accumulative data for intracellular expression of TNF-α in blood monocytes in the presence of medium alone, lipopolysaccharide (LPS) and LPS/IFN-gamma. Intracellular cytokine expression was analysed in 7 healthy controls, 10 treated coeliac patients, and 8 untreated coeliac patients.

Louise Elliott
Expression of IL-12/23 in peripheral monocytes

Spontaneous IL-12/23 production was detected in 4 out of the 6 untreated patients, 3 out of 10 treated patients and one out of 8 controls.

An increase in IL-12/23 production was observed in all monocytes stimulated with LPS. Cytokine production was further elevated when monocytes were stimulated with LPS & IFN-gamma. In the presence of LPS alone and LPS & IFN-γ, the CD14++ monocytes produced higher amounts of IL-12/23 in all patient groups compared to the CD14+° population with the greatest difference observed among LPS & IFN-γ activated monocytes.

Monocytes stimulated with LPS and LPS & IFN-γ from healthy controls produced the highest amounts of IL-12/23 compared TCD and UCD patients. Unstimulated monocytes from untreated patients produced comparable levels of IL-12/23 as monocytes stimulated with LPS, with cytokine production only induced in the presence of both IFN-γ and LPS.

Examples of IL-12/23 expression in CD14+ monocytes from one healthy control are illustrated in dot plots in Figure 4.27 a. Table 4.6 and Figure 4.27 b summaries the expression of IL-12/23 from untreated, and treated CD patients and controls.

<table>
<thead>
<tr>
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<th>LPS</th>
<th>LPS &amp; IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC</td>
<td>TCD</td>
<td>UCD</td>
</tr>
<tr>
<td>Total Monocyte</td>
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<td>(% of positive cells)</td>
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<td>2.9</td>
<td>8.7</td>
</tr>
<tr>
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<tr>
<td>(% of positive cells)</td>
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<td>9.4</td>
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<tr>
<td>CD14+++</td>
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<tr>
<td>(% of positive cells)</td>
<td>6.3</td>
<td>5.7</td>
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</table>

Table 4.6: Summary of intracellular IL-12/23 expression in CD14 positive peripheral monocytes in patients with coeliac disease and controls. Values represent mean percentage. N= 5
Figure 4.27: Intracellular IL-12/23 production of monocytes in coeliac adults and control donors. (A) An example of flow cytometric dot plot for intracellular production of IL-12/23 in monocytes stimulated with LPS & IFN-γ from a HC. The cytokine profiles for total monocytes (left), CD14⁺°monocytes (middle), and CD14⁺⁺hi monocytes (right) were determined. (B) Histogram plots representing the accumulative data for intracellular expression of IL-10 in blood monocytes in the presence of medium alone, lipopolysaccharide (LPS) and LPS/IFN-gamma. Intracellular cytokine expression was analysed in 7 healthy controls, 10 treated coeliac patients, and 8 untreated coeliac patients.

Expression of IL-10 in peripheral blood monocytes

IL-10 production was only analysed in monocytes stimulated with LPS as IFN-gamma inhibits IL-10 cytokine production compared to IL-12/23 which is enhanced by the presence of IFN-gamma.

IL-10 production was detected in all patients analysed. The CD14⁺⁺ hi monocytes were a more potent IL-10 producer compared to the CD14⁺° lo subset.

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Elevated levels of spontaneous IL-10 production was detected in untreated patients compared to healthy controls and treated coeliac patients. A similar trend was observed for both CD14+ and CD14++ monocytes from untreated subjects.

On comparing total monocytes stimulated with LPS, monocytes from untreated patients produced the highest amounts of cytokine with an almost 2 fold increase in cytokine production compared to healthy controls. However, when the different monocyte subsets were analysed both unstimulated and stimulated monocytes produced similar levels of cytokine. While similar levels of IL-10 production was induced in stimulated monocytes from both healthy controls and patients with treated coeliac disease.

Examples of IL-10 expression in CD14+ monocytes from one healthy control are illustrated in dot plots in Figure 4.28. Table 4. and figure 4.28 b summarises the expression of IL-10 from untreated, and treated CD patients and controls.

<table>
<thead>
<tr>
<th></th>
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<tr>
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<td>TCD</td>
</tr>
<tr>
<td>Total Monocytes</td>
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<td>(% of positive cells)</td>
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</tr>
<tr>
<td>(% of positive cells)</td>
<td></td>
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</tr>
</tbody>
</table>

Table 4.7: Summary of intracellular IL-10 expression in CD14 positive peripheral monocytes in patients with coeliac disease and controls. Values represent mean percentage of positive cells n=5.
Figure 4.28: Intracellular IL-10 production of monocytes in coeliac adults and control donors. (A) Flow cytometric dot plots representative of IL-10 intracellular staining in a HC. The cytokine profiles for total monocytes (left), CD14<sup>+</sup> monocytes (middle), and CD14<sup>++</sup> monocytes (right) were determined. (B) Histogram plots representing the accumulative data for intracellular expression of IL-10 in blood monocytes in the presence of medium alone, lipopolysaccharide (LPS) and LPS/IFN-gamma. Intracellular cytokine expression was analysed in 7 healthy controls, 10 treated coeliac patients, and 8 untreated coeliac patient. Results are shown as percentages of monocytes expressing cytokine (mean ± SEM).
4.4 Discussion

Our examination of human duodenal mucosa and peripheral blood resulted in several major findings. First based on the identification of myeloid DCs as CD11c+ and plasmacytoid DCs as CD123+, only the myeloid DC subset was confirmed to be present in the duodenal mucosa employing in-situ staining. This was further confirmed when assessed by flow cytometry, which found PDCs to represent less than 1 percent of HLA-DR+ lineage negative cells in both healthy and CD duodenal mucosa. Results obtained from our immunofluorescence staining, showed the macrophage to be the dominant cell population in the duodenal mucosa when compared to the CD11c+ DC subset. In agreement with past studies (Melinda Ráki et al., 2006) we found the myeloid DC was significantly increased in the untreated coeliac lesion. Interestingly, in the peripheral blood the myeloid DC subset was significantly decreased in untreated coeliac patients compared to healthy controls while the plasmacytoid DC remained unchanged. Notably the mDC subset was nearly twice as numerous as pDCs in the peripheral blood in adults only. No difference in the frequency of peripheral blood monocytes was observed in both adults and children with coeliac disease when compared to age matched healthy controls. Upon phenotypic analysis, myeloid DCs were shown to express significantly higher levels of co-stimulatory molecule CD86 in both healthy controls and coeliac patients when compared to markers CD80 and CD83. A higher percentage of CD83 positive myeloid DCs were observed in untreated patients when compared to healthy and treated controls. Unstimulated and stimulated monocytes from untreated coeliac patients produced similar amounts of IL-10 and IL-12/23. Whereas unstimulated monocytes from healthy controls and treated coeliac patients produced insignificant amounts of cytokine compared stimulated monocytes.

The characterisation of antigen presenting cells in the duodenal mucosa

It is well reported that gluten reactive T cells in the duodenal mucosa play a pivotal role in CD pathogenesis (Kagnoff, 2007; Stepniak & Koning, 2006; Wal et al., 2000). However, the mechanisms that regulate the activation of these T cells have yet to be deciphered. Antigen presenting cells are crucial in self/non-self discrimination, and are likely to be
instrumental in loss of tolerance to gliadin resulting in coeliac disease. In this study we have used a multicolour Immunofluorescence study to investigate the prevalence of macrophages and dendritic cells in the duodenal mucosa of healthy controls and coeliac patients. In contrast with other authors who assessed immunofluorescence staining in a semi quantitative and subjective manner by determining cell densities manually, we took a novel approach and employed imaging analysis software. This software allowed us to objectively determine the surface area expression for each cell marker employed in the study.

The majority of cells present in the mucosa expressed CD68, a well-defined macrophage marker. Under homeostatic conditions, most of the macrophages were strategically located in the subepithelial mucosa and scattered through out the lamina propria. The minor subset was identified as CD11c positive and was referred to as myeloid DCs. Interestingly the plasmacytoid DC subset was not detected in both healthy and coeliac duodenal tissue. In sections taken from untreated patients with coeliac disease a significant increase in CD11c expression was observed when compared to treated coeliac patients and healthy controls. No difference in the expression of the macrophages marker, CD68, was found in untreated coeliac patients when compared to treated and healthy controls.

Our data suggest that the myeloid DC may potentially play a role in the activation and maintenance of gluten reactive T cells as demonstrated by the accumulation of DCs in the coeliac lesion. A similar increase in CD11c+ DCs in the untreated coeliac lesion has been previously reported by Raki et al (2006). He also found that isolated mucosal CD11c+ cells from coeliac biopsies were capable of activating gluten specific T cells. In agreement with our study, Raki and colleagues did not identify the CD123+ plasmacytoid subset in both healthy and coeliac mucosa. In contrast, Di Sabantio et al (2007) found that the increase in DCs in the untreated coeliac lesion was largely due to the plasmacytoid subset with a contaminant increase in myeloid DCs.

It is well reported that DCs are critical for immune regulation in the gut (Rescigno & Di Sabatino, 2009; Rimoldi et al., 2005; Weiner et al., 2011) and changes in DC function have been shown to be associated with an array of inflammatory diseases (Baumgart et al., 2011; Cavanagh et al., 2005; Niess, 2008; Rutella & Locatelli, 2011). Coeliac disease

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is driven by a Th1 response (Fina et al., 2008), which is normally initiated and maintained by IL-12, however, IL-12 is undetectable in the coeliac mucosa (Leon, 2006). Therefore an important issue that has yet to be fully established is what cytokine is responsible for driving this immune response. Interestingly a close relationship between IFN alpha and coeliac disease has been proposed. Di. Sabatino et al (2007) demonstrated the upregulation of IFN alpha in the coeliac mucosa and found that blocking IFN alpha in mucosal biopsies inhibited IFN-γ mRNA. Additional evidence suggesting that IFN alpha may play a potential role in disease development, is the trigger of gluten sensitivity in patients with hepatitis C on IFN α treatment (Lim, 2010) as well the increased incidence of coeliac disease among Down syndrome individuals (Nisihara, 2005). The exact mechanism responsible for the high prevalence of CD in individuals with Down syndrome is not well understood but it may be associated with the additional chromosome 21, the location of the IFN-alpha gene (Langer et al., 1990). This extra chromosome may be responsible in driving the over production of IFN-α resulting in the manifestation of coeliac disease in genetically susceptible individuals. However, our findings are not in keeping with this hypothesis considering we did not identify the plasmacytoid subset in the duodenal mucosa. Thus we propose that IFN alpha may only play a potential role in the pathogenesis of coeliac disease under unusual circumstances, were the immune system is already dysfunctional individuals with additional comorbidities.

To confirm the findings obtained from our confocal analysis we employed an additional method, flow cytometry, to evaluate the different DC subsets freshly isolated from duodenal biopsies. Similar to our previous observations we could only detect the presence of the myeloid DC subset and not the plasmacytoid subset when assessing total lineage negative HLA-DR positive cells. Our results confirms those made by Raki et al (2013) who also found plasmacytoid DCs to be scarcely present in the gut mucosa. Thus, our data lead us to believe that plasmacytoid DCs have stricter migration patterns compared to the CD11c+ myeloid DC and has no functional role in the pathogenesis of CD.
The effects of ageing on peripheral blood antigen presenting cells

In the present study, we evaluated peripheral blood DCs and monocytes in patients with coeliac disease compared to healthy controls. We used flow cytometry methods that allowed the enumeration and phenotypical characterisation of DCs directly in-vivo. Moreover, to the best of our knowledge, this is the first study ever conducted in both a paediatric and adult population investigating the prevalence of peripheral blood antigen presenting cells in healthy and subjects with coeliac disease. This allowed us to make direct comparisons between the two populations eliminating the introduction of laboratory variables caused by user technique, flow antibodies, and instrument set up.

First, on comparing the frequency of dendritic cell subsets in the peripheral blood between healthy children and adults we noted the possible effects age may have on innate immune cells. We noted a significant increase (p<0.0001) in the frequency of total peripheral blood DCs in our children cohort when compared to adults. When we further evaluated the different DC subsets, we found a higher prevalence of plasmacytoid DCs in children while adults exhibited higher frequencies of myeloid DCs. Several studies have reported the effects of age on the innate immune system and similar to our observations, mDCs has been reported to increase with age although no change in the plasmacytoid DC was obtained (Della Bella et al., 2007). However, in agreement with our study, Shodell & Siegal (2002) reported an age dependent decrease in plasmacytoid DCs.

The plasmacytoid DC are a specialised subset of cells that produce large amounts of IFN-alpha in response to viral invasion. Therefore it is not surprising that children display higher frequencies of these cells in the blood considering children are more frequently infected by certain viral mediated diseases, such as chicken pox, measles, mumps, rubella, and the common cold. The myeloid subset expresses a wider range of PRRs and is central in establishing and maintaining adaptive T -cell responses. The finding of a significant decrease in mDCs (p 0.042) in healthy children compared to adults may relate to the reduced ability of younger people to generate an effective cell mediated response, as their immune system has yet to fully mature.

In this study, we assessed the frequencies of three monocytes subsets identified based on their differential expression for CD14 and CD16, referred to as classical

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(CD14++CD16−), intermediate (CD14++CD16+), and non-classical (CD14+CD16+). When evaluating the difference in monocytes subsets between healthy children and adults several differences were observed. In adults, a significant increase (p 0.0003) in the classical subset was found whereas a higher frequency of intermediate monocytes was noted in healthy children. To the best of our knowledge, these findings have never been reported previously, although in keeping with our observations, Della Bella et al did describe an increase in the absolute numbers of monocytes in older patients. Overall these results seem suggest an overall increase in the production of myeloid cells in older people.

Prevalence of antigen presenting cells in coeliac subjects

In this study we investigated the prevalence of interacting cells of innate immunity at the diagnosis of CD to determine if an immune alteration exists in coeliac patients. In adults, we demonstrated a significant reduction in the percentage of CD11c+ myeloid DCs in coeliac patients compared to healthy individuals while no change was observed in the CD123+ plasmacytoid subset. The latter finding is in disagreement with observations made by Ciccocioppo et al (2007) who reported that, the decrease in absolute number of total DCs was primarily due to the plasmacytoid DC. A possible reason for this conflict in result may be due to a difference in methodology used in the study. Another question that has yet to be addressed is whether the decrease in the CD11c+ myeloid DCs observed in the circulation is due to the recruitment of these cells to the site of inflammation. Although our data supports the notion that the lower than normal CD11c+ myeloid DC in the peripheral blood may be, at least partly, due to their accumulation in the mucosa, matched biopsy and blood samples would further help establish this hypothesis.

In this study we demonstrated similar frequencies of both peripheral myeloid and plasmacytoid DCs in children. In contrast, Cseh et al (2011) observed an increase in the absolute numbers of myeloid DCs whereas Vuckovic and colleagues (2007) reported a decrease in absolute numbers. Conversely, we did observe a trend in that an increase in absolute myeloid DCs was revealed in newly diagnosed children although it did not reach statistical significance possibly due to the small sample number used in our study. However, in support of our findings, both studies found no alteration in the frequency of
plasmacytoid DCs.

Similar to blood dendritic cells (Baumgart et al., 2011; Chan et al., 2012; Gerl et al., 2010), modifications in the different monocyte subsets have been reported in several inflammatory disorders, suggesting a pro-inflammatory role for these cells (Ahmad, 2007; Carvalheiro et al., 2012; Grip et al., 2007; Moniuszko et al., 2009; Rossol, Kraus, Pierer, Baerwald, & Wagner, 2012; Scherberich & Nockher, 1999). It is believed that half of circulating monocytes leave the peripheral blood in a steady state and migrate to the tissue where they differentiate into macrophages (Auffray, Sieweke, & Geissmann, 2009). In-vitro, it is well described that monocytes can differentiate into both macrophages (Rey-Giraud, Hafner, & Ries, 2012) and dendritic cells (Andersson et al., 2012), however, in-vivo the differentiation of monocytes into dendritic cells in humans is scarce and is mostly speculation, this phenomenon has been well described in mice (Domínguez & Ardavin, 2010; Varol et al., 2007).

Resembling previous reports (Yona & Jung, 2010), we found the classical monocyte (CD14++CD16+) to be the dominant subset in the blood, while the intermediate (CD14++CD16+) and non-classical (CD14+CD16+) subset made up the remaining 10-15 percent of circulating monocytes. It has been proposed, a developmental relationship exists between the three monocyte subsets; classical, intermediate and non-classical. Ex-vivo in the absence of a stimulus the classical subset differentiates into the non-classical subset (Fung et al, 2010). Therefore when undertaking this study, it was ensured to much to our ability, that all samples were processed within four hours from the time the sample was taken, which has been reported to be the optimum time frame to prevent sample manipulation (Fung et al., 2010). We observed no alteration in the distribution of all monocyte subsets in both adults and children newly diagnosed with coeliac when compared to healthy aged matched controls. Although no other studies have investigated the prevalence of monocyte subsets in neither children nor adults newly diagnosed with coeliac disease, one previous study carried out children found no significant difference in the percentage of total circulating monocytes when compared to healthy controls.

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Phenotypical analysis of dendritic cells

We further proceeded to the immunophenotypic analysis of myeloid DCs by analysing the expression of co-stimulatory molecules CD80 and CD86. Both these molecules are crucial in determining whether T cell immunity or tolerance is induced. DC activation markers HLA-DR and CD83 were also assessed. From our evaluation we found an increase in CD83 positive myeloid DCs in the peripheral blood in untreated coeliac patients compared to healthy controls. CD83 is upregulated on DCs after the exposure to an activating stimulus, potentially derived from the coeliac lesion. On comparing the remaining surface markers no significant differences were found compared to healthy controls. The difference in activated myeloid DC prevalence values indicate a systemic inflammation in CD and supports a notion of a general alteration of innate immunity in patients with untreated coeliac disease.

On comparing CCR6 expression on myeloid DCs we found higher levels, determined by mean fluorescence intensity, along with an increased percentage in CCR6 positive cells compared to healthy controls. CCR6 is upregulated on the surface of blood dendritic cells promoting their migration to mucosal sites (Ito et al., 2011; Williams, 2006). Upon uptake of antigen in the tissue, DCs down regulate CCR6 and upregulate CCR7 where they home to the lymph nodes to prime naive T cells (Ito et al., 2006). The upregulation of CCR6 on peripheral dendritic cells signifies their migration to the site of inflammation in response to the chemokine CCL20 (Greaves et al., 1997; Ito et al., 2011) Chemokines are key mediators in recruiting leucocytes through out the body under physiological and inflammatory conditions (Rot & von Andrian, 2003). An increased expression of chemokine receptors on APCs in the periphery could facilitate their recruitment into the tissue and potential involvement in disease exacerbation. Thus the increase in CCR6 may support our previous observation that a decreased frequency in peripheral myeloid DCs is accompanied with the subsequent accumulation of CD11c positive cells in the coeliac lesion.

Cytokine profiling

We evaluated the monocyte expression for IL-12/23, IL-10, and TNF-alpha, both in basal (unstimulated) and stimulated conditions. We used both LPS alone and LPS in combination with IFN-gamma because both have shown to be the optimal stimulus for Louise Elliott
the analysis of cytokine expression by monocytes (Schuerwegh et al., 2001). Only LPS alone was used to stimulate monocytes when investigating IL-10 production, as IFN-gamma has been shown to inhibit IL-10 production (Chomarat et al., 1993).

Although, the data obtained is from peripheral monocyte, relevance to the gut can be made since monocytes are precursors to tissue macrophages a phenomenon augmented during inflammation.

As expected, in vitro stimulation of blood monocytes with LPS with or without IFN-gamma significantly enhanced the production of IL-12/23 and TNF-alpha by monocytes from healthy and coeliac patients, whereas LPS alone drove IL-10 production. Attempts were made to analyse monocytes based on their differential expression for CD14 and CD16; however, the upregulation of CD16 on the majority monocytes during incubation with LPS prevented us from doing so. Therefore, we analysed the monocytes as a whole population and based upon their expression for CD14, dividing them into two separate groups; CD14 high and CD14 low.

On comparing their cytokine profiles, we found that the CD14 high monocyte expressed higher levels of IL-12/23 when stimulated with LPS with IFN-y in healthy controls whereas no difference was observed with IL-10 and TNF-alpha. The increased production of IL-12/23 by the CD14 high subset may suggest this subset represents a more pro-inflammatory cell.

Interestingly, when we evaluated LPS stimulated monocytes, we observed no difference in IL-10 and IL-12/23 production in our untreated coeliac patients when compared to unstimulated monocytes. This observation was more pronounced when the monocytes were divided into CD14 low and CD14 high. We hypothesise the no change in the cytokine production between unstimulated and LPS stimulated monocytes in untreated coeliac patients, maybe due to a prior stimulation encountered in-vivo, that has left them partially desensitized. Thus, this finding indicates the existence of an in vivo activation of human monocytes in untreated coeliac patients, with a lower capacity to respond to LPS ex-vivo.

The increased production of IL12/23 by blood monocytes from untreated patients found in the present study could also be related to priming of a T-helper (Th)-1 response characteristic of the coeliac disease. Although the cytokine IL-12 is undetectable in the

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coeliac lesion, IL-23 has been implicated in the pathogenesis of CD (Castellanos-Rubio et al., 2009; K. M. Harris, Fasano, & Mann, 2008). Harris and colleagues reported PBMCs stimulated with gliadin induced significantly higher levels of IL-23 in CD patients compared with HLA-DQ2(+) healthy controls. IL-23, a member of the IL-12 family, is involved in the generation of Th17 cells which are now known to be pivotal in driving autoimmunity (Costa, Mattana, & Da Silva, 2010; Croxford, Mair, & Becher, 2012; Kim et al., 2013; Wu, Zepp, & Li, 2012) and more importantly have been recently incriminated in the pathogenesis of coeliac disease where high levels of IL-17A mRNA was detected in the mucosa from untreated coeliac patients (Castellanos-Rubio et al., 2009).

4.5 Conclusion

In conclusion, we have demonstrated that dendritic cells accumulate in the coeliac lesion compared healthy mucosa suggesting a role for mucosal DCs in the pathogenesis of coeliac disease. Complementary to our observations made in the mucosa we found that peripheral myeloid DCs were decreased in untreated coeliac adults when compared to normal subjects. However, further work is required to determine whether the increased pool of mucosal DCs is derived directly from blood DCs by employing a more in depth phenotypic analysis of dendritic cells in untreated coeliac patients and healthy controls.

In contrast, no difference in the frequency of peripheral blood dendritic cells was observed in children newly diagnosed with coeliac disease. This may be a reflection of the paediatric immune system, which may be in a state of developmental flux. Thus changes in cell populations may not be as apparent in circulation compared to when cell homeostasis has been established. This is further supported by the multiple differences in cell frequencies found between healthy adults and children.

Finally, we found no difference in CD68 expression in untreated coeliac mucosa when compared to healthy tissue. This finding was accompanied by an unchanged frequency in peripheral blood monocyte populations in untreated coeliac patients. Therefore we propose that monocytes are not recruited to the inflammatory lesion at a higher rate compared to monocyte migration during homeostasis. This also suggests that the macrophage involvement in the progression of the coeliac lesion is not due to Louise Elliott
an increase macrophage numbers but more likely due to a change in function that is further highlighted by the spontaneous production of IL-12/23 and IL-10 production from monocytes taken from untreated coeliac patients compared to healthy monocytes.
Chapter 5  The Investigation of CD163 Expression on Antigen Presenting Cells

5.1 Introduction

CD163, expressed on monocytes and macrophages, is a member of the class B scavenger receptor cysteine-rich superfamily (Fabriek, Dijkstra, & van den Berg, 2005). It was first identified in 1987 and was previously known as the RM3/1 antigen (Högger et al., 1998). It is a 130 KD type 1 transmembrane protein and four different isoforms have been described, which all differ in the structure of their cytoplasmic domain. It is composed of nine group B scavenger receptor rich cysteine (SRCR) domains that are linked to a short transmembrane segment and a short cytoplasmic tail (Etzerodt & Moestrup, 2012). The short intracellular tail contains sequences for phosphorylation with protein kinase C and creatine kinase and these phosphorylation events are linked to cytokine production induced by CD163 cross-linking (Ritter et al., 2001). Its gene has been mapped to region p13 on chromosome 12 and consists of 17 exons (Akila et al., 2012; Madsen et al., 2004; Onofre et al., 2009).

5.1.1 Function in inflammation

CD163 is a scavenger receptor mostly known for its role in the clearance and endocytosis of Haemoglobin/Haptoglobin (Hb/Hp) complexes (Kristiansen et al., 2001). Haemoglobin is one of the most abundant proteins found in circulation sequestered within red blood cells. Under normal circumstances haemoglobin is cleared from the circulation by extravascular haemolysis, where macrophages located in the spleen and bone marrow phagocytose damaged or old red blood cells. A small proportion of red cells undergo intravascular haemolysis most commonly seen in individuals with inherited red cell defects and autoimmune disorders (Schaer et al., 2013). Free haemoglobin is toxic and if not cleared in an efficient manner it can precipitate in the tissue mediating oxidative damage (Na et al., 2005). The haemoglobin released directly into the circulation immediately binds to haptoglobin, a serum glycoprotein, forming a stable Hb/Hp complex. The formation of these complexes results in a conformational change exposing a new epitope (Andersen et al., 2012). The binding site for the Hb/Hp complex

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occurs within the 3 SRCR domain and operates in a calcium and pH-dependent fashion (Kristiansen et al., 2001; Madsen et al., 2004). The ligation of the Hb/Hp complex with surface CD163 triggers intracellular signalling which induces the production of IL-10 (Philippidis et al., 2004). Once internalised, the Hp/Hp complex is delivered to the lysosomes where the heme undergoes intracellular degradation by the rate limiting enzyme heme oxygenase-1 (HO-1), giving rise to free iron, biliverdine, and carbon dioxide (Abraham & Drummond, 2006). Upon binding of Hb/Hp complexes, IL-10 and CO are released by macrophages which can exert strong anti-inflammatory responses thus the efficient clearance of Hb prevents activation of an inflammatory response by extracellular Hb (Etzerodt & Moestrup, 2012; Krzysztof Kowal et al., 2011; Moestrup & Møller, 2004; Zuwata-Jagietto, 2006).

Other more recently recognised functions of CD163 include its ability to act as a scavenger receptor for the TNF-like weak inducer of apoptosis (TWEAK) (Bover et al., 2007). TWEAK is a member of the TNF superfamily that is largely produced as a soluble cytokine by leukocytes and acts via binding to a cell surface receptor named Fn14 (fibroblast growth factor-inducible 14). Fn14 is found on fibroblasts, endothelial cells, and epithelial cells which all secrete a vast array of chemokines, cytokines, and MMPs in response to stimulation by TWEAK. The TWEAK/Fn14 pathway plays a role in tissue repair and regeneration as well as contributing to pathological tissue remodeling. In contrast to TNF-alpha, the related protein TWEAK has been shown to attenuate or dampen down proinflammatory immune responses. Maecker and colleagues demonstrated that TWEAK deficient mice had overabundant natural killer (NK) cells and displayed hypersensitivity to bacterial endotoxin, with their innate immune cells producing excess IFN-gamma and IL-12 (Maecker et al., 2005).

Thus, TWEAK curbs production of IFN-gamma and IL-12, suppressing the immune response that drives CD4+ cells towards Th1 immunity. Likewise, inhibition of TWEAK by monoclonal antibodies resulted in an increase in IFN-gamma and IL-12 production by human peripheral blood nuclear cells. Functional studies have shown that the interaction of TWEAK with surface CD163 on monocytes leads to neutralisation of both molecules, which may skew the immune response by promoting a Th1 response (Bover et al., 2007; Krzysztof Kowal et al., 2011; Onofre et al., 2009; Van Gorp, Delputte, &
It has also been reported that CD163 can behave as an immune sensor for both Gram positive and negative bacteria (Fabriek et al., 2009). The binding motif has been located within the second SRCR domain of CD163. Recognition of bacteria by surface CD163 results in the production of pro-inflammatory and anti-inflammatory cytokines, including, IL-1 beta, IL-6, and TNF-alpha, a response similar to that of TLR stimulation (Akila et al., 2012; Kowal et al., 2011). However, the exact role of CD163 during bacterial recognition needs to be elucidated. Thus further studies are warranted to establish the nature of the bacterial ligands and the intracellular signaling pathways involved in this process.

Overall studies clearly demonstrate that surface CD163 can act as a receptors for a vast array of ligands, thus engaging in the initiation and/or maintenance of the immune response. It is most likely that the outcome of the immune response is dependent on the location of the interaction of the ligand with the binding motif on the extracellular domain of CD163.

5.1.2 CD163 Expression and regulation

CD163 expression is restricted to cells of the myeloid lineage and is tightly regulated by various factors. In general anti-inflammatory stimuli tend to induce its synthesis while pro-inflammatory mediators down regulate its expression (Buechler et al., 2000). Original reports demonstrated low expression of CD163 on peripheral blood monocytes, while more recent studies have shown CD163 on the majority of monocytes. In contrast it was always thought that peripheral blood dendritic cells lack CD163 expression until Maniecki and colleagues found CD163 expressed on a small proportion of myeloid dendritic cells (Maniecki et al., 2006).

CD163 mRNA and protein expression is upregulated during monocyte macrophage-colony stimulating factor (M-CSF) dependent differentiation into macrophages in-vitro. Generally, peripheral monocytes express lower levels of surface CD163 compared to their tissue counterparts suggesting that CD163 could be a marker of macrophage differentiation (Fabriek et al., 2005). Conversely, it has been reported that monocyte differentiation into DCs in the presence of IL-4 and GM-CSF results in the
down-regulation of CD163 expression (Lehtonen et al., 2007; Van den Heuvel et al., 1999). *In vitro*, pro-inflammatory stimulation of monocytes with LPS, TNF-α, IFN-γ, IL-4 and IL-13 suppresses CD163 mRNA and protein while CD163 expression is upregulated by anti-inflammatory cytokines IL-10, IL-6 and glucocorticoids (Buechler et al., 2000; Van Gorp et al., 2010). Moreover activation via TLR-2, TLR-4, and TLR-5, can lead to rapid down regulation of surface CD163 by monocytes/macrophages (Weaver et al., 2007).

Tissue macrophages that exhibit high levels of CD163 include Kupffer cells in the liver, red pulp macrophages in the spleen, cortical macrophages of the thymus and mature bone marrow macrophages (Fabriek et al., 2005). Macrophages can be subdivided into two subtypes with regard to their cytokine production and function. The classical activated macrophage (M1) and the alternatively activated macrophage (M2) display pro-inflammatory and anti-inflammatory phenotypes, respectively (Allavena & Mantovani, 2012). The M1 macrophage phenotype is generated in response to microbial products or IFN-γ and produces IL-1β, IL-6 and TNF-α. In contrast, the M2 macrophage produces low IL-12, IL-23 and high IL-10 and TGF-β and displays high expression of CD163 (Rey-Giraud et al., 2012). It has been shown that expression of CD163 on macrophages increases during the wound healing phase (Buechler et al., 2000; Higashi-Kuwata, Makino, Inoue, Takeya, & Ihn, 2009).

Tumour associated macrophages exhibit M2 phenotype, whose presence has been associated with poor prognosis in various tumours (Allavena & Mantovani, 2012; Edin et al., 2012). It has been suggested that CD163 expressing tumour macrophages are associated with a more advanced histological grade, a higher occurrence of distant metastasis and reduced patient survival. The expression of high levels of CD163 in patients with bladder cancer (Maniecki et al., 2012), colorectal cancer (Edin et al., 2012; Herrera et al., 2013), and ovarian cancer (Lan et al., 2012) was shown to be associated with poor prognosis and patient outcome. Additionally, CD163 was found expressed on blast cells from patients with acute myeloid leukaemia M4/M5 but not on normal CD34+ haematopoietic stem cells (Ba, Schaer, & Walter, 2006).
5.1.3 Soluble CD163 (sCD163)

In addition to surface CD163, a 130kDa soluble form of CD163 can be detected in plasma and other bodily fluids (Møller, 2012). CD163 is cleaved from the cell membrane as soluble CD163 via ectodomain shedding in response to pro-inflammatory stimuli such as LPS or PMA (Møller et al., 2010). Soluble CD163 can be detected at levels of 0.7-3.9mg/L in healthy individuals (Møller, 2012). A study carried out by Möller and co-workers (2003) reported that the biological variation of serum sCD163 levels between subjects was high, although comparable to the variation seen in immunoglobulins and complement levels. The study also reported little difference between age and sex suggesting that only one reference range is required (Møller et al., 2003).

At least two enzymes have been implicated in the shedding of CD163 from the cell surface: matrix metalloproteinase-9 (MMP-9) (K. A. Hintz et al., 2002) and tumor necrosis factor alpha-converting enzyme (TACE/ADAM17) (Etzerodt, Maniecki, Møller, Møller, & Moestrup, 2010). The ADAM17 enzyme is mainly involved in the shedding of TNF-α from the cell surface in response to various stimuli (Black, 2002) whereas MMP9 involved in tissue remodelling in both normal physiology processes and pathological conditions (Verma & Hansch, 2007).

5.1.4 Function of soluble CD163

The induction of surface CD163 by anti-inflammatory mediators and its quick release in response to TLR activation implicates a role for soluble CD163 in inflammation. However, to date its exact functional role has not been elucidated. It has been proposed that soluble CD163 may exhibit anti-inflammatory properties as it has been shown that purified sCD163 can inhibit phorbol ester-induced T lymphocyte activation in a dose dependent manner (Högger & Sorg, 2001). The interaction between soluble CD163 and lymphocytes is speculated to occur through the binding of sCD163 with non-muscle myosin heavy chain in the lymphocyte (Timmermann, Buck, Sorg, & Högger, 2004). However, how this interaction modulates lymphocyte activation is not known and thus warrants further investigation.

The association of T cell proliferation and CD163 also came from in vivo studies in Louise Elliott
which elevated tissue expression of CD163 is found in locations with attenuated T cell}
proliferation. Baeten and colleagues (2004) reported an inverse correlation between
soluble CD163 and CD69+ activated lymphocytes in the synovium from patients with
spondylarthropathy synovitic compared to rheumatoid arthritis patients (Baeten et al.,
2004). This finding may indicate that T cell activation is impaired due to the presence of
high levels of soluble CD163 in the synovium. In addition to this observation they also
reported increased numbers of CD163+ macrophages in the spondylarthropathy
synovium that correlated with soluble CD163 production suggesting that macrophages
are the likely source of soluble CD163. Similarly in asthmatic patients who presented
with prolonged airway inflammation associated with activation of allergen specific T
cells a decrease in both CD163 monocyte expression and plasma soluble CD163 were
established. Whereas in patients who did not develop late asthmatic responses, an
increase in soluble CD163 and monocyte CD163 surface expression were found (K
Kowal, Møller, Dubuske, Moestrup, & Bodzenta-Lukaszyk, 2006).

Overall the literature to date strongly suggests a role for CD163 in regulating T cell
proliferation and in the resolution of inflammation. Therefore it would be of great
interest to determine whether soluble CD163 does exhibit anti-inflammatory properties,
making it a potential target for therapeutic intervention. Figure 5.1 shows the regulation
of CD163 on the surface of the cell.
Macrophage

Figure 5.1: Overview of the generation CD163 pathway. Anti-inflammatory mediators IL-6, IL-10 and glucocorticoids induce an upregulation of surface CD163 on the macrophage. Pro-inflammatory stimulus induced by LPS (lipopolysaccharide) or PMA (phorbol myristate acetate) promotes proteolytic shedding of surface CD163 generating soluble CD163. The suggested anti-inflammatory properties of sCD163 require further investigation in order to determine a mechanism of action. Image taken from Moestrup & Møller, (2004)

5.1.5 Soluble CD163 and Inflammatory Disorders

Serum levels of soluble CD163 have shown to be elevated in several inflammatory disorders including autoimmune disorders (Lories & Baeten, 2009), hematological malignancies (Ba et al., 2006) (Hj et al., 2004), malaria (Kusi et al., 2008; Mendonça et al., 2012), and bacterial, but not viral meningitis. Previously, our group published work demonstrating that levels of soluble CD163 detected in the serum from coeliac patients positively correlated with lesion severity (Daly et al., 2006). This finding has also been reported in other inflammatory disorders such as rheumatoid arthritis where the amount of serum sCD163 correlates with disease activity (Greisen et al., 2011.) Furthermore, the levels of sCD163 were higher in the synovial fluid than in paired serum samples, implying that sCD163 is shed by local pro-inflammatory macrophages.

An additional autoimmune disorder associated with increased concentration of
soluble CD163 is scleroderma (SSC). This study demonstrated that serum sCD163 was significantly higher in patients with scleroderma compared to healthy controls. Although the study did not find a correlation between CD163 serum levels and skin sclerosis, it did report a correlation with renal damage (Higashi-Kuwata et al., 2009; Shimizu & Ogawa, 2012).

In HIV, an increase in soluble CD163 was found in patients with chronic HIV infection compared to HIV seronegative individuals. Moreover, patients on effective anti-retroviral therapy (ART) were found to have lower levels of soluble CD163 that paralleled with HIV RNA, demonstrating the utility of monocyte/macrophage derived soluble CD163 as a marker of HIV activity (Burdo et al., 2011).

Increased concentrations of sCD163 were found in overweight and lean individuals at risk of developing of type 2 diabetes suggesting that sCD163 may be useful a useful marker for predicting insulin resistance. It also suggests that monocyte/macrophage activation may be an important determinant of insulin resistance in obesity (Holger J Møller, Frikke-Schmidt, Moestrup, Nordestgaard, & Tybjærg-Hansen, 2011; Parkner et al., 2012)

Finally, serum soluble CD163 was shown to be a valuable marker in the diagnosis and prognosis for sepsis. Patients with sepsis admitted to the intensive care unit had significantly higher levels of soluble CD163 compared to patients with systemic inflammatory response syndrome (SIRS) and was also shown to be superior biomarker to procalcitonon and C-reactive protein (CRP) in the diagnosis of sepsis. Moreover, the utility of soluble CD163 proved to be a useful tool in predicting disease outcome by demonstrating among patients with sepsis that soluble CD163 levels in the non-surviving group were significantly higher compared to patients that survived (Feng et al., 2012; Su et al., 2012). An additional study detected the presence of soluble CD163 in the urine of patients diagnosed with sepsis but failed to detect sCD163 in the urine of normal controls. Moreover, sepsis patients that presented with acute kidney injury exhibited higher urine soluble CD163 levels when compared to those with non-kidney acute injury (Su et al., 2012). Thus urine soluble CD163 levels may indicate renal function, which may be useful for the assessment of patient outcome.

The above studies clearly demonstrate the potential for sCD163 as a diagnostic
marker for a vast array of clinical diseases, as well as its utility in monitoring monocyte/macrophage activation in inflammatory disorders. Considering the shedding of CD163 from the cell surface reflects increased macrophage activation it is possible that the soluble form may also participate in anti-inflammatory signalling. Furthermore sCD163 does not correlate with CRP, suggesting that sCD163 does not reflect the acute phase response but rather chronic inflammation (Moestrup & Møller, 2004; H J Møller et al., 2002).

5.1.6 CD163 and Coeliac Disease

Soluble CD163 has been reported to be elevated in the serum in a vast array or pro-inflammatory disorders. Interestingly, high levels of sCD163 were detected in the serum of patients with untreated coeliac disease and was shown to correlate with the inflammatory lesion (Daly et al., 2006). Unlike many other inflammatory disorders coeliac disease cannot be monitored using conventional inflammatory markers such as C-reactive protein or erythrocyte sedimentation rate. Instead tissue transglutaminase and endomysial antibody tests are employed in the diagnosis of coeliac disease, as well as to monitor dietary compliance but are not always reflect the degree of inflammation in the small intestine.

A small percentage of coeliac patients do not respond to a gluten free diet and are considered to have refractory coeliac disease (Malamut et al., 2012). These patients present with a negative anti-tTG test despite persistent mucosal inflammation, thus making soluble CD163 a potential biomarker for monitoring intestinal involvement. Presently patients must undergo invasive, time consuming and expensive biopsy tests in order to assess the status of the intestinal lesion.

The previous study carried out in our lab determined high soluble CD163 by establishing a cut off range using optical density values from healthy controls. However, new reagents for the determination of absolute concentrations of soluble CD163, opposed to relative quantification, needs to be optimised that will help provide more sensitive, accurate and comparable results. Although there are well-established kits available for the quantification of serum soluble CD163, these kits are expensive and not feasible for the quantification of CD163 in large patient cohorts. Therefore, we aim to optimise a commercial kit used for the quantification of soluble CD163 in supernatants

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Elevated levels of soluble CD163 were observed in children with systemic juvenile idiopathic arthritis (Behrens, Beukelman, Paessler, & Cron, 2007) and to date this is the only study published with regard to CD163 serum levels and children with pro-inflammatory disorders. Therefore, it would be of interest to establish whether children newly diagnosed with coeliac disease also present with high levels of serum soluble CD163.

CD163 is a known monocyte/macrophage marker although to what extent it is expressed on different monocyte subsets is still controversial (Maniecki, Etzerodt, Moestrup, Møller, & Graversen, 2011). Moreover, only one study has shown CD163 to be expressed on a small proportion of peripheral myeloid DCs while no one has investigated plasmacytoid DC CD163 expression.

Given the nature of coeliac disease, it is highly likely that the small intestine is the site from which the elevated levels of sCD163 originate with mucosal macrophages being the main source. The pro-inflammatory milieu characterised by high levels of IFN-γ and TNF-α are known mediators capable of inducing ectodomain shedding of CD163 from the cell surface. As previously discussed macrophages can be classified into two different entities dependent on their surface expression of CD163. It would be interesting to examine whether there is a shift in the ratio of these macrophage subsets predicting a higher prevalence of CD163 positive macrophage in the normal intestine compared to the coeliac lesion. This skewed ratio would account for the high soluble CD163 in the serum but also the chronic inflammation associated with coeliac disease.

Despite the high concentrations found in the serum the functional relevance of soluble CD163 has not yet been established. It has been hypothesised to exhibit immunoregulatory functions, due to its ability to negatively regulate phorbol ester-induced human T lymphocyte activation in vitro although others have not yet confirmed this observation. To further support this hypothesis, the CD163+ macrophages have been shown exhibit anti-inflammatory properties and to play a role in the healing process.

There are several limitations associated with the study mentioned above mainly associated with the T cell stimulant used, PMA (Frings, Dreier, & Sorg, 2002).
PMA is usually used in conjunction with ionomycin to stimulate cells and PMA alone is not sufficient to induce T cell proliferation. Hence the functional properties of soluble CD163 warrants further investigation employing an *in-vitro* model that better represents a physiological response.

### 5.2 Objectives

The overall objective of this chapter is to gain a better understanding of CD163 expression in the gut and peripheral blood. To determine the potential source of soluble CD163 found elevated in the serum of adult coeliac subjects and to examine its potential use as a biomarker for disease activity in a paediatric population. Finally, evaluate soluble CD163 immunological functional properties.

- Quantify CD163 expression in the duodenal mucosa from untreated and treated coeliac subjects and compare to normal duodenal mucosa
- Determine CD163 co-expressing APCs (macrophages and dendritic cells) in the gut
- Quantify CD163 and CD68 co-localisation in coeliac and normal mucosa
- Characterise the main CD163 positive antigen presenting cells in the peripheral blood.
- Investigate whether soluble CD163 can modify T cell responses *in-vitro*
5.3 Results

Confocal Tissue Immunofluorescence Staining

5.3.1 CD163 is highly expressed in healthy and coeliac disease duodenal mucosa

CD163 expression was widely distributed throughout the lamina propria in both normal and coeliac mucosa. CD163+ macrophages were densely located under the subepithelial and throughout the lamina propria, displaying variable morphology in both normal and disease mucosa illustrated in Figure 5.1 and 5.2. To establish whether a difference in CD163 expression existed between healthy subjects and treated and untreated coeliac patients, quantitative analysis was carried out employing Imaris imaging analysis software. In summary, using Imaris analysis software, the surface area (um²) for CD163 was determined and expressed as a percentage of the total lamina propria area measured. Upon analysis a significant decrease (p=0.0176) in CD163 expression was observed in the untreated coeliac lesion compared to treated mucosa and healthy mucosa, a difference that could not be distinguished visually (Figure 5.3 & Table 5.4).

Figure 5.1: Confocal immunofluorescence image of CD163 staining in the normal duodenal mucosa. (A) and (B) shows CD163 (RED) staining of a villus and the lamina propria from a healthy patient, respectively. Original magnification x 400
Figure 5.2: Confocal immunofluorescence image of CD163 staining in the untreated coeliac. (A) and (B) shows CD163 (RED) staining of a villi and the lamina propria from an untreated coeliac patient. Original magnification x 400

Figure 5.3 Quantitative evaluation of CD163 surface area expression in the duodenal mucosa. Surface area expression of CD163 expressed as a percentage of the total lamina propria area measured in sections from duodenal mucosa of healthy controls (n=13), treated (n=6), and untreated coeliac patients (n=9). Kruskal-Wallis non-parametric test was used to analyse the difference between the groups. Asterisks indicate significant differences between individual groups (indicated by bars) using post hoc Dunn’s multiple comparison tests; *p<0.05.
Table 5.1: Summaries CD163 surface area expression compiled using Imaris Bitplane analysis software.

<table>
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<th>Healthy Control</th>
<th>Treated Coeliac</th>
<th>Untreated Coeliac</th>
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<tr>
<td>CD163 (μm²) Median</td>
<td>9,578 (4562.4-12,775)</td>
<td>7843.4 (281.8-12840.9)</td>
<td>10,160 (6221.70-12552.5)</td>
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5.3.2 CD68+ macrophages co-express CD163

Dual immunofluorescence staining of CD68 and CD163 was carried out on normal duodenal mucosa to determine the specificity of CD163 for mucosal macrophages. CD68 is a well characterised pan macrophage marker that is predominantly used for the identification of macrophages. For this study we wanted to determine if both markers were comparable when staining for macrophages. Co-staining experiments showed that CD68+ and CD163+ cells were almost complete overlapping populations in healthy controls (Figure 5.4).
Figure 5.4: CD68 and CD163 expression in the small intestine. CD68 (Green) and CD163 (RED) both identify mucosal macrophages, evident in the merged image (bottom) were both CD163 and CD68 are overlapping populations emphasised by the presence of yellow. (Left) Images of biopsy specimen from one representative of a healthy control. (Right) Images of biopsy specimen from one representative of an untreated coeliac patient.

5.3.3 A decrease in surface area of CD163 and CD68 co-expression in untreated coeliac duodenal mucosa

Considering macrophages are a prominent cell in the intestinal mucosa and little is known about their activation state within the coeliac lesion. We next established, employing CD163, if a skew in the macrophage phenotype exists in the coeliac lesion. It is known that the use of a CD163 marker can help differentiate between the classically (M1) and alternative activated (M2) macrophage phenotype differentiated by their pro-inflammatory and anti-inflammatory cytokine profile, respectively. This was achieved using Imaris imaging co-localisation software, which allowed us to determine the percentage of CD68 colocalised with CD163 and whether a significant difference in the surface area co-expression existed between healthy controls and untreated coeliac patients. Upon analysis a significant decrease (p= 0.0264) in the surface area of CD163 and CD68 co-localised was determined in untreated coeliac patients when compared to treated coeliac patients and healthy controls (Figure 5.5). Although no significant difference in the percentage of CD68 co-localised with CD163 was observed and strong trend in favour of a decrease in the percentage of CD68 co-expressed in untreated coeliac patients was found when compared to both treated coeliac patients and healthy controls.
Figure 5.5: CD68 and CD163 co-localisation. (A) Quantitative analysis of CD68 and CD163 co-expression in the duodenal mucosa from healthy controls (n=13), treated (n=6) and untreated coeliac patients (n=9) obtained from Bitplane Imaris analysis software. The left graph displays results for the percentage of CD68 co-localised with CD163. The right graph displays results for the co-localisation surface area expressed as a percentage of the total area measured. Kruskal-Wallis non-parametric test was used to analyse the difference between the groups. Asterisks indicate significant differences between individual groups (indicated by bars) using post hoc Dunn’s multiple comparison tests; *p<0.05 (B) Visual co-localisation (right) of CD68 (left) and CD163 (middle) determined by Bitplane analysis software.
5.3.4 CD11c positive DCs do not co-express CD163 in the duodenal mucosa

As illustrated in the previous chapter dendritic cells can be identified in the duodenal mucosa based on their expression for CD11c. Next we aimed to determine whether CD11c+ dendritic cells in the mucosa co-express CD163, a marker thought to be restricted to monocytes and macrophages.

Thus to establish if both CD163 and CD11c positive cells are two separate entities in the mucosa, double immunofluorescence studies for both markers were carried out on healthy and coeliac duodenal sections. We found that the majority of CD163+ and CD11c+ cells were expressed on distinct, nonoverlapping cell populations (Figure 5.6). However, consistent with previous reports we did identify a minor subset of cells that did co-express both CD163 and CD11c. These double positive cells were detected in both normal and coeliac mucosa at a very low frequency and often expressed lower levels of CD163 or CD11c compared to single positive cells (Figure 5.7). Due to their extremely low frequency in both healthy and coeliac mucosa, it was decided there was no necessity in quantifying their presence. An additional observation made when carrying out image analysis, is that CD163+ macrophages and CD11c+ dendritic cells were found in close proximity in the lamina propria (Figure 5.8).
Figure 5.6: CD11c and CD163 expression in the small intestine. CD11c (green) and CD163 (red) staining in a healthy control and untreated coeliac mucosa, respectively. No significant co-localisation of CD11c and CD163 was observed in normal and coeliac mucosa. Data are representative of staining performed in healthy controls (n=13), treated (n=6), and untreated coeliac patients (n=9).
Figure 5.7: CD11c and CD163 co-localisation. A magnified image of a double positive cell, identified in the top image with arrow head, expressing both CD11c and CD163. Note the dendritic cell morphology. Original magnification x40. The dotted white line highlights the basement membrane.
Figure 5.8: Dual staining for CD163 and CD11c. This image displays that both CD11c and CD163 are found in close proximity to each other, depicted by the white arrow.
CD163 characterisation of peripheral blood antigen presenting cells employing flow cytometry

5.3.5 CD163 surface expression on human peripheral blood monocytes determined by different monoclonal antibodies

To determine which CD163 mAB clone would be selected for subsequent experiments, two clones RM3/1 and GHI/6 were compared employing two different anti-coagulants, EDTA and citrate. Freshly drawn EDTA anti-coagulated blood was stained with specific monoclonal antibodies identifying monocytes on the basis of their CD14 and CD16 expression. Monocyte surface CD163 expression was determined with different CD163 mAbs. The mAB RM3/1, which binds to domain 9 of CD163 did not recognise CD163 surface expression on peripheral monocytes. In contrast clone GHI/61, which binds to domain 7, identified a significantly larger fraction of peripheral monocytes with a higher CD163 density. In contrast to EDTA, whole blood samples stabilised with citrate recognized a significantly lower percentage of CD163+ monocytes employing mAB GHI/61 compared to mAB RM3/1 (data not shown). It was decided that mAB GHI/6 and anti-coagulant EDTA would be used for all flow cytometry results.

**Figure 5.9:** CD163 expression in fresh whole blood samples as measured by flow cytometry using different CD163 mAbs. Flow cytometric dot plots for peripheral blood monocyte CD163 staining with monoclonal antibody RM3/1 (left column) GH1/6 (right column) with whole blood anti-coagulated with EDTA.
5.3.6 CD163 expression by peripheral antigen presenting cell subsets

In the peripheral blood, CD163 is believed to be restricted to monocytes (Buechler et al., 2000; Maniecki et al., 2011) with one study reporting CD163 expression on myeloid DCs (Maniecki et al., 2006). Thus to confirm these previous findings we aimed to establish CD163 expression on all circulating antigen presenting cells.

The expression of CD163 was investigated by flow cytometry by co-staining CD163 with CD14 and CD16 (monocytes), CD11c (myeloid DCs), CD123 (Plasmacytoid DCs) and CD19 (B cells) employing a whole fresh blood assay (chapter 3).

CD163 was undetectable on the surface of peripheral blood B cells shown in figure 5.10.

![Flow cytometric scatter plots demonstrating CD163 staining for CD19+ B lymphocytes. A represents the fluorescence minus one control for CD163 while B shows cells stained in the presence of CD163.](image)

**Figure 5.10: Frequency of CD163 staining on CD19+ B lymphocytes.** Flow cytometric scatter plots demonstrating CD163 staining for CD19+ B lymphocytes. **A** represents the fluorescence minus one control for CD163 while **B** shows cells stained in the presence of CD163.
5.3.7 Majority of CD11c+ myeloid DC express CD163

As depicted from our previous results, mucosa CD163 expression was not strongly associated with mucosal DCs (Section 5.3.4). Next we investigated whether if the main peripheral DC subsets expressed CD163, a marker mostly associated with monocytes. In contrast to our observations made in the mucosa, we found that approximately 70% of the CD11c+ myeloid DCs expressed CD163 while the plasmacytoid DC subset did not. Representative flow cytometric dot plots for CD163 distribution on peripheral blood dendritic cells are shown in Figure 5.11

Subsequently we examined the percentage of CD163+ myeloid DCs and surface expression in coeliac patients from a paediatric and adult population. We found no significant difference in the frequency of CD163+ myeloid DCs in healthy controls compared to coeliac subjects. However, a trend was observed among untreated adults, in which there was a moderate decrease in the frequency of CD163+ myeloid DCs (figure 5.12 (top)).

In children both healthy controls and untreated coeliac subjects expressed comparable levels of surface CD163 determined using mean fluorescence intensity. In adults a significant decrease (p=0.0413) in surface CD163 expression in untreated patients was revealed when compared to treated coeliac patients and healthy controls. Treated coeliac subjects and healthy controls expressed similar levels of surface CD163. Results are summarised in figure 5.12 (bottom).
Figure 5.11: Frequency of CD163 on peripheral blood dendritic cells. Flow cytometric density plots characteristic for healthy (top) and coeliac patients (bottom) for CD11c+ myeloid DC (left) and CD123+ plasmacytoid DC (right). Values in top right corner of density plots are for percentages of CD163 positive monocytes. Gates were determined using FMO (fluorescence —minus-one) controls.
Figure 5.12: Distribution of CD163 expression for peripheral Blood CD11c+ myeloid DCs in both a paediatric (left column) and adult population (right column). A represents the summary of analysis for CD11c+ myeloid DC for healthy (n=20) and coeliac (n=18) paediatric subjects while B shows the accumulative data for healthy controls (n=14), and treated (n=12) and untreated adult coeliac (n=10) subjects. The top row shows the percentage of positive myeloid DCs. Data is depicted as scatter plots. Means are noted by black lines. The bottom row represents results for mean fluorescence intensity (MFI) for CD163 surface expression. Data depicted as column bar graphs. Medians are noted by bars while whiskers represent standard deviation. Mann-Whitney and Kruskal-Wallis non-parametric tests were used to analyse the difference between the groups. Significance indicated by asterisks.
5.3.8 CD163 is differentially expressed on monocyte subsets

Monocyte are known to express CD163 (Maniecki, 2006) but to what extent has not yet been fully established due to the conflicting reports in the literature (Maniecki et al., 2011). Therefore we examined the percentage of CD163+ circulating monocytes in whole blood and their corresponding surface expression. As previously described in the chapter 3, monocytes can be sub-divided into 3 subsets based on their expression for CD14 and CD16. The major subset, known as the classical subset expresses high CD14 but no CD16 while the remaining two minor subsets, referred to as the intermediate and non-classical subset, can be defined as CD14hiCD16+ and CD14loCD16+, respectively.

We found that both CD14++CD16- and CD14++CD16+ monocyte subsets expressed similar CD163 surface levels, as determined by MFI, while the CD14+loCD16+ monocytes exhibited significantly lower levels compared to the other two subsets (Figure 5.13). Likewise, the majority of the CD14++CD16- and CD14++CD16+ monocytes were CD163 positive. Whereas only a small proportion of the CD14+loCD16+ subset were positive for CD163 (Figure 5.15).

![Figure 5.13: Expression of CD163 on peripheral blood monocytes in healthy children.](image)

(A) Flow cytometry histogram plots of CD163 expression by different monocyte subsets. Values in histograms are for MFI (mean fluorescence intensity) of CD163 expression. Green, blue and red line represents staining of CD14++ CD16-, CD14++CD16+, and CD14+lo CD16+ monocytes, respectively. (B) The summary of analysis of CD163 expression by monocyte subsets in healthy subjects (n=19). Kruskal–Wallis ANOVA nonparametric tests was used to analyse differences among subsets. Asterisks indicate statistically significant differences.
5.3.9 Monocyte derived dendritic cells to not express CD163

In order to determine the dynamics of CD163 expression during dendritic cell differentiation and to establish whether ex-vivo generated DCs bare any resemblance to peripheral blood DCs, we examined CD163 expression on monocyte derived DCs (moDC). The DC was generated ex-vivo by incubating IL-4 and GM-CSF for 6 days. While CD163 is expressed on the surface of freshly isolated monocytes at day 0 the expression is lost from the cell surface upon differentiation into moDC at day 6 (figure 5.14).

![Flow cytometry histogram plots of CD163 expression comparing freshly isolated monocytes (left) and monocyte dendritic cells (moDC) (right), generated from monocytes cultured in the presence if IL-4 and GM-CSF for 6 days. Values in histograms are for MFI (mean fluorescence intensity) CD163 expression. Data shown is a representative of 3 experiments.]

Figure 5.14: Differential CD163 expression between monocytes and monocyte derived dendritic cells. Flow cytometry histogram plots of CD163 expression comparing freshly isolated monocytes (left) and monocyte dendritic cells (moDC) (right), generated from monocytes cultured in the presence if IL-4 and GM-CSF for 6 days. Values in histograms are for MFI (mean fluorescence intensity) CD163 expression. Data shown is a representative of 3 experiments.
5.3.10 CD163 distribution on peripheral blood monocytes from newly diagnosed children

On comparing the CD163 mean fluorescence intensity for each monocyte subset in the paediatric population no significant difference was found. Although we found a trend towards increased surface expression of CD163 in the CD14+CD16+ subset in newly diagnosed children (Figure 5.16 A). Likewise no difference in the frequency of CD163+ monocytes was observed between the healthy controls and coeliac subjects, although a slight increase in the percentage of CD163+ CD14+CD16+ subset was detected in the untreated population (Figure 5.16 B).

Figure 5.15: Frequency of CD163 positive peripheral blood monocytes in a healthy control and newly diagnosed paediatric. (A) Flow cytometric density plots characteristic for healthy (top) and coeliac patients (bottom) for CD14++CD16- (left), CD14++CD16+ (middle), and CD14+CD16+ (right) monocytes. Values in top right corner of density plots are for percentages of CD163 positive monocytes. Gates were determined using FMO (fluorescence —minus-one) controls.
Figure 5.16: CD163 distribution comparing HC (n=15) and newly diagnosed coeliac patients (n=18) from a paediatric population (A) Summary of results for CD163 mean fluorescence intensity and (B) percentage of CD163+ cells for each monocyte subset; CD14++CD16- (left column), CD14++CD16+ (middle column) and CD14+CD16+. Data are depicted as scatter plots. Black lines note medians.

5.3.11 CD163 distribution on peripheral blood monocytes from untreated coeliac adults

On comparing the frequency of CD163 positive cells for each monocyte subset no significant difference was found. While statistical significance was not reached, a decrease in the frequency of CD163 positive cells was observed in untreated coeliac subjects compared to treated coeliac patients and healthy controls. All three monocyte subsets expressed similar levels of CD163 measured using mean fluorescence intensity (MFI). Results are summarised in Figure 5.17.

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Figure 5.17: Monocyte CD163 distribution comparing untreated (n=12) and treated (n=11) adult coeliac patients with healthy controls (n=19). (A) Percentage of CD163+ cells (B) Summary of results for CD163 mean fluorescence intensity for each monocyte subset; CD14++CD16+ (middle column) and CD14+lo CD16+. Data are depicted as scatter plots. Black lines note medians.
5.3.12 Increased levels of serum Soluble CD163 found in children with untreated coeliac disease

Next using an ELISA the levels of soluble CD163 in serum samples taken from our paediatric subjects was investigated. On analysis, a significant increase (p=0.0039) in soluble CD163 was found in untreated children when compared to aged matched controls. Detectable levels of CD163 in serum from healthy controls ranged from 20.097-890.97 ng/ml with a median of 250.13ng/ml while levels in children with CD ranged from 130.14-1110.8ng/ml with a median of 460.74ng/ml.

![Figure 5.18](image)

Figure 5.18: Serum levels of soluble CD163 in newly diagnosed children (n=17) and healthy paediatric subjects (n=17). Scatter plot showing the accumulative data for soluble CD163 levels in the serum detected employing an ELISA. Black horizontal lines note medians. Asterisks signify statistical significance (p=0.0039).

5.3.13 Increased levels of serum Soluble CD163 found in adults with untreated coeliac disease

A significant increase (p=0.0001) in soluble CD163 in serum from untreated and partially treated adults was revealed when compared to both treated coeliac patients and healthy controls. A slight elevation in soluble CD163 was detected in untreated coeliac patient when compared to partially treated coeliac patients but did not reach statistical significance. Similar to the paediatric population, soluble CD163 detected in the serum of healthy adults ranged from 70.4-1000.5ng/ml, with a median of 230.5ng/ml and comparable levels of soluble CD163 was detected in treated coeliac patients.

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Meanwhile, partially treated coeliac patients presented with similar levels to that of untreated children, with a median of 500.50ng/ml while levels detected in untreated adults ranged from 190-4370μg/ml, with a median of 850.90μg/ml. Figure 5.19 summarises soluble CD163 ELISA results.

![Figure 5.19](image)

**Figure 5.19:** Serum levels of soluble CD163 in untreated (n=16), treated (n=12), partial treated (n=13) coeliac subjects and healthy adults (n=16). Scatter plot showing the accumulative data for soluble CD163 levels in the serum detected employing an ELISA. Black horizontal lines note medians. Asterisks signify statistical significance. Data was analysed using Kruskal-Wallis test, p < 0.05.

### 5.3.14 Soluble CD163 inversely correlates with surface CD163 expression on monocytes

Using Spearman Rank correlation analysis we found a strong significant, inverse correlation between serum soluble CD163 and surface CD163 expression levels on monocyte subsets CD14++CD16- and CD14++CD16+ in children with newly diagnosed coeliac disease compared to aged matched controls shown in **figure 5.19**. The spearman correlation Co-efficient for the latter two are r=-0.789 (p=0.0004) and r=-0.7286 (p=0.0021), respectively. No correlation between soluble CD153 and surface CD163 was revealed for the CD14+ and CD16+ subset for both healthy controls and coeliac subjects.

Due the small sample number in adults with both matched serum and blood samples correlation studies could not be performed.
Figure 5.19: Relationship between soluble CD163 and monocyte subset CD163 surface expression. Correlation graphs showing the relationship between mean fluorescence surface (MFI) CD163 surface expression for each monocyte subset CD14++CD16- (top), CD14++CD16+ (middle), and CD14+CD16+ (bottom) and serum soluble CD163 for healthy controls (n=17) (Left) and Newly diagnosed children (n=17) (Right). Correlation was determined using Spearman r (r).
5.3.15 No correlation between monocyte frequency number and soluble CD163

When correlation analysis was used to determine a relationship between the percentage of CD163+ monocytes subsets and soluble CD163 no relationship was revealed for healthy controls and coeliac subjects. Figure 5.20 summarises the correlation between soluble CD163 and frequency of CD163+ monocytes.

Figure 5.20: Relationship between soluble CD163 and percentage of CD163+ monocytes. Correlation graphs showing the relationship between the CD163 % for each monocyte subset CD14++CD16- (top), CD14++CD16+ (middle), and CD14+CD16+ (bottom) and serum soluble CD163 for healthy controls (n=17) (Left) and Newly diagnosed children (n=17) (Right).
5.3.16 Recombinant CD163 has no effect on T cell proliferation

Previously it was reported by Hogger & Sorg (2001) that soluble CD163 was capable of mediating anti-inflammatory effects by inhibiting phorbol ester – induced human T lymphocyte activation. This finding suggests that soluble CD163 possesses functional properties capable of attenuating the adaptive immune response. In order to investigate this hypothesis experiments were designed to determine the effects of soluble CD163 on T cell proliferation and cytokine production.

CD4+ lymphocytes were freshly isolated and stained with cell trace violet. Stained cells were incubated overnight alone or in the presence of 2ug/ml or 10ug/ml of recombinant CD163. T cells were then stimulated with different stimuli including PMA, PMA & Ionomycin, and anti-CD3 and anti-CD28 for 5 days to induce T cell proliferation. The violet peaks correspond to a round of T cell division. The parent population has the highest intensity of violet and this is reduced each round of cell division, resulting in populations of decreasing violet intensities.

Histogram analyses of a representative cell trace proliferation assay are shown in figure 5.22. T cells were incubated with treatments as per histogram headings. CD4+ lymphocytes stimulated with anti-CD3 and anti-CD28 showed significant evidence of cell division compared to lymphocytes incubated alone or stimulated with PMA. PMA & Ionomycin induced T cell proliferation but not to the same extent as anti-CD3/CD28. On comparing the effects of rhCD163 on T cell proliferation with each stimulus no difference was observed.
Figure 5.22: rhCD163 has no effect on CD4+ proliferation. CD4+ T cells enriched from PBMCs were stained with cell trace violet and stimulated with PMA alone (A), PMA and Ionomycin (B), or anti-CD3 & CD28 (C) and cultured for 5 days in the absence or the presence of 2ug/ml or 10ug/ml of rhCD163. (N=4)
5.3.17 Recombinant CD163 has no effect on T cell cytokine production

While soluble CD163 had no apparent effect on T cell proliferation, the possibility remained that it may influence cytokine production by activated CD4+ T cells. T cells were freshly isolated and incubated overnight in the absence of rhCD163 or in the presence of 2ug/ml or 10ug/ml of rhCD163. T cells were then stimulated with different stimuli including PMA, PMA & Ionomycin, and anti-CD3 and CD28 for 5 days in culture. Cells were then re-stimulated on day 5 using PMA & Ionomycin and intracellular cytokine production was investigated. Analysis of cytokine production on day 6 is presented in figure 5.23.

In PMA & Ionomycin stimulated T cells the presence of rhCD163 resulted in a slight increase in IL-10 and IL-4 production. In anti-CD3/CD28 stimulated T cells, the inclusion of rhCD163 in the culture resulted in a slight decline in IL-4, IFN-γ, and IL-10. The addition of 10μg/ml of rhCD163 had the greatest effect. In PMA stimulated T cells a slight increase in both IL-4 and IL-17 production was observed in the presence of 10ug/ml of rhCD163.
Figure 5.23 rhCD163 has minimal affect on T cell cytokine production CD4+ cells were isolated from PBMCs and incubated overnight alone or in the presence of rhCD163 at 2ug/ml or 10ug/ml. Next the cells were stimulated with PMA alone, PMA & ionomycin, or anti-CD3/28 for 5 days. Lymphocytes were re-stimulated with PMA and ionomycin overnight in the presence of BFA on the last day of culture. Cells were stained for IL-4 (A), IFN-γ (B), IL-10 (C), IL-17 (D). (N=4)
5.4 Discussion

This study investigated the differential expression of CD163 on antigen presenting cells in the duodenal mucosa and peripheral blood. We also established soluble CD163 levels in serum from patients newly diagnosed with coeliac disease. CD163 was highly expressed in both healthy and coeliac duodenal mucosa and a high degree of co-localisation was observed with the classical macrophage marker CD68. A marked decrease in CD163 expression was found in the untreated coeliac lesion, which was accompanied with a decrease in the co-expression of CD68 with CD163. In contrast CD11c+ mucosal DCs lacked CD163 expression. Interestingly peripheral blood myeloid DCs demonstrated high levels of surface CD163 while plasmacytoid DCs and B cells did not. No difference in the frequency of CD163+ CD11c DCs was revealed between healthy controls and untreated coeliac adults, although a significant decrease in CD163 surface expression was noted in myeloid DCs from untreated coeliac adults. The monocyte subsets analysed in chapter 3 exhibited varying levels of surface CD163. However, no difference in both the frequency and CD163 MFI for all three subsets was obtained when comparing healthy controls and newly diagnosed coeliac patients. Soluble CD163 was significantly elevated in the serum from both children and adults newly diagnosed with coeliac disease. This increase in soluble CD163 was not due to an increase in circulating monocytes but was due to the shedding of surface CD163 from their cell surface. Finally, recombinant CD163 did not exhibit any functional properties that may have the potential to modulate the adaptive immune response.

CD163 expressing antigen presenting cells in the duodenal mucosa

In the duodenal mucosa, CD163 was highly expressed in both normal and coeliac mucosa. As previously reported (Beitnes et al., 2011; Beitnes et al., 2012) CD163 exhibited a significant overlap with the classical macrophage marker CD68. Similar to CD68, CD163 was strategically located under the basement membrane and throughout the lamina propria. Using quantitative analysis we found a significant decrease in CD163 mucosal expression in the untreated coeliac lesion when compared to healthy and treated mucosa. In agreement with our observations is that made by Beitnes et al, 2011, who also reported a decrease in the number of CD163 positive macrophages in the
coeliac lesion. Next, using quantitative co-localisation analysis software, we investigated whether a difference in co-expression of CD68 and CD163 in the untreated coeliac lesion differed from healthy and treated mucosa. We found a significant decrease in the co-localisation of the two antigens in the untreated coeliac lesion. Since CD163 has been used to differentiate between the M2 macrophage (CD163+) and M1 (CD163-) macrophage, our results indicate that there is a skew in the macrophage phenotype in the coeliac lesion from an anti-inflammatory M2 phenotype to a pro-inflammatory M1 phenotype. These two subsets are interchangeable depending on their microenvironment, thus it is not surprising that the M1 subset is more prominent in the coeliac lesion. High levels of IFN-gamma are characteristic of the coeliac lesion, a cytokine known to support the generation of the classically activated M1 subset. This subset is maintained by the Th1 proinflammatory milieu but in return contributes to IFN-gamma and TNF-alpha production thus helping to sustain the inflammatory lesion.

In contrast to the macrophage, CD11c+ mucosal DCs did not display any significant CD163 expression and therefore were identified as two separate cell populations. Occasionally, cells expressing both CD11c and CD163 were identified but they were too infrequent to allow any quantitative analysis. In contrast to our observations, Beitnes et al (2011) found these double positive cells increased within the coeliac mucosa. While those results are not repeated here, the difference in results obtained may reflect the different techniques used. Beitnes et al used standard microscopy compared to confocal microscopy employed in our study, which is far superior in preventing spectral bleed and artifact interference such as autofluorescence. In addition, they did not use the DAPI nuclear counterstain, which is advantageous when distinguishing between autofluorescence and true staining.

The origin of these double positive cells is not known but one could speculate that they may be newly recruited monocytes that have yet to down regulate CD11c expression and to differentiate into a macrophage. A further possibility is that this cell subset represents a newly recruited myeloid DC from the peripheral blood that has yet to shed CD163. The Beitnes study referred to these cells as a subset of dendritic cells due to their CD11c expression. However, CD11c is highly expressed on monocytes and considering this subset was also shown to express CD14 (Beitnes, 2011) it more than
likely this population are monocytes newly recruited from the peripheral blood.

**CD163 expressing antigen presenting cells in the peripheral blood**

While we found that myeloid DCs in the mucosa did not co-express CD163, it was noted that a large proportion of CD11c+ myeloid DCs in the peripheral blood co-expressed CD163. In contrast, the second major blood DC subset, the CD123+ plasmacytoid DC, displayed no CD163 expression. Likewise B cells also professional antigen presenting cells, did not express CD163. To date only one other study has characterised CD163+myeloid DCs. However, Maniecki et al (2006) reported a much lower percentage of CD163 positive myeloid DCs while using the same monoclonal antibody for detection (10% in comparison to 70% in this study). This discrepancy is probably due to the different sample preparation and staining procedures employed. Maniecki et al performed all staining on DCs isolated by magnetic bead separation from PBMCs and defined DCs as CD14- ILT3+HLA-DR+. In comparison, this study performed all staining on fresh whole blood to prevent sample manipulation as it has been reported that sample preparation influences CD163 expression (Maniecki, 2011). In addition to this, we have shown along with Maniecki et al, that that detection of surface CD163 on fresh cells is dependent on the presence or absence Ca^{2+}, therefore careful consideration should be given to the anticoagulant and clone of antibody used in combination.

It is well acknowledged that DCs are a heterogeneous population of cells (Geissmann et al., 2010; Liu & Nussenzweig, 2010) and that functional differences among the different phenotypes are expected. Therefore, the significance of CD163 on myeloid DCs needs to be clarified. One could hypothesise that the CD163+ subset may represent an anti-inflammatory, or immature subset (Amodio & Gregori, 2012). This is supported by the knowledge that CD163 is upregulated by anti-inflammatory mediators such as glucocorticoids while pro-inflammatory mediators induce CD163 shedding from the cell surface (Buechler et al., 2000; Krzysztof Kowal et al., 2011). Surface CD163 may not simply represent a distinct subset but may also serve a function. Considering CD163 possesses many domains (Van Gorp et al., 2010) and has been shown to bind to bacteria (Fabriek et al., 2009), this molecule may also function as a receptor for other antigens.
promoting their uptake into the cell for antigen processing and presentation, thus promoting the main functional characteristic of dendritic cells.

The importance in the varying expression of CD163 between myeloid DCs and plasmacytoid DCs has yet to be established but may simply reflect their distinct functional properties. The former playing a central role in regular immunity and the latter in viral immunity (Swiecki & Colonna, 2010).

Blood monocytes can be divided into three main subsets and all have been shown to express varying levels of CD163. Previous reports regarding monocyte CD163 expression have reported ranges from undetectable to 100% (Davis & Zarev, 2005; Högger et al., 1998; Maniecki et al., 2011; Philippidis et al., 2004; Sulahian et al., 2000). Data from this study along with, Tippet al (2011), found CD163 expressed predominantly on the “classical” subset (CD14hiCD16-) with minimum expression found on the non-classical subset (CD14loCD16+). However, while Tippet et al found only moderate levels of CD163 on the intermediate subset (CD14hiCD16+), our experiments revealed that the majority of these cells express CD163.

The differential expression of CD163 between subsets may reflect distinct monocyte subset functions or changes in a developmental pathway from an immature to mature monocyte. For example the non-classical subset may represent an activated or mature monocyte originally derived from the main classical subset in the blood. Thus in response to a stimulus the immature classical monocyte (CD14hiCD16-) becomes activated inducing phenotypic changes such as CD16 upregulation and CD163 shedding, transforming into a non-classical monocyte. This is further supported by previous reports that have found the non-classical subset expanded in several proinflammatory conditions (Grip et al., 2007; Scherberich & Nockher, 1999; Shantsila et al., 2011). Moreover, cytokine analysis carried out on the different monocyte subsets found the non-classical subset capable of producing the highest levels of TNF-alpha and minimal IL-10 when compared to the intermediate and classical subsets (Yona & Jung, 2010).

We showed that when monocytes differentiate into monocyte derived dendritic cells, CD163 expression is dramatically down regulated, in agreement with previous observations (Buechler et al., 2000). It is not known whether CD163 is down regulated via endocytosis or due to ectodomain shedding. This phenotype is in complete contrast
to peripheral blood myeloid DCs suggesting that these two cells are very separate entities that potentially possess different functional properties. The blood subset may represent an immature or anti-inflammatory phenotype while the moDC constitutes a more pro-inflammatory cell type. Therefore this finding may support the opinion that monocyte derived DCs are not a suitable *in-vitro* model for investigating dendritic cell function *in-vivo*. Furthermore the lack of CD163 on mucosal DCs and moDCs allows us to propose that peripheral blood monocytes may indeed be potential precursors to mucosal dendritic cells, a theory that is center of much debate in the literature (Bogunovic et al., 2009; Merad et al., 2013) However, one cannot ignore the possibility that peripheral blood dendritic cells are the source of mucosal DCs, which lose CD163 in response to the environmental milieu in the mucosa.

In experiments investigating CD163 expression on monocytes and dendritic cells, both the adult and paediatric cohort exhibited no significant differences among the cell populations when comparing untreated coeliac patients with healthy controls. In the adult cohort a smaller proportion of peripheral blood DCs expressed CD163, although the same was not obtained for newly diagnosed paediatric patients. Interestingly, untreated coeliac patients from our adult cohort showed a trend towards a lower frequency of CD163+ monocytes in all subsets. The relevance of this finding is not known but may simply represent shedding of surface CD163 from the cell in response to inflammatory mediators derived from the coeliac lesion.

**Increased levels of soluble CD163 in the serum from untreated coeliac patients**

This study is the first to report increased levels of soluble CD163 in serum from children with newly diagnosed coeliac disease. In a previous study carried out by Daly and colleagues it was first established that adults with untreated coeliac disease had significantly higher levels of soluble CD163 compared to healthy controls (Daly et al., 2006). Moreover, the same study found that high levels of soluble CD163 correlated with lesion severity. Unfortunately detailed histology reports were not available for our paediatric cohort thus we were unable make the same correlation. However, considering all of the paediatric patients were newly diagnosed and had very high tTG levels one could infer that the majority of patients had a high degree of intestinal

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involvement.

To confirm previous observations made in the adult population, a new adult cohort was studied and in agreement with previous work, we established that adults with untreated coeliac disease do present with significantly higher levels of soluble CD163 in their serum. Partially treated patients also exhibited higher levels of CD163 but at lower levels than untreated patients. This difference in soluble CD163 levels may reflect resolution of inflammation in the intestinal mucosa thus adherence to a gluten free diet.

As previously discussed, elevated levels of soluble CD163 in the serum has been associated with a diverse range of inflammatory disorders which all report-varying values. Initially, Moller first reported a reference range of 0.89-3.95mg/L employing an in-house assay (Møller, 2012). However, in recent years commercially available assays tailor-made for detecting soluble CD163 in serum have been introduced. These assays have been shown to measure lower levels of soluble CD163 compared to in-house assays. Although there is a significant difference in results achieved by the two assays, a high correlation between the in-house and commercial assays exists (Møller, Rejnmark, & Moestrup, 2003). For this study a commercial assay not previously optimised for measurement of soluble CD163 in serum was used. It was found that soluble CD163 levels detected in our study were similar to results obtained by other studies that have used commercially available assays from R&D and IQ optimised for serum soluble CD163 (Burdo et al., 2011; Feng et al., 2012; Hasegawa, Matsushige, Inoue, & Takahara, 2012; Jude., 2012; Parkner et al., 2012; Shimizu & Ogawa, 2012). However, due to the lack of standardisation and the difference in levels obtained from different assays a thorough standardisation that uses internationally accepted calibrators is much needed in order to establish a universal recognised reference range.

The increased levels of soluble CD163 in the serum may reflect the loss in macrophage hyporesponsiveness characteristic of healthy tissue. In pro-inflammatory conditions, the macrophage may shift in phenotype from the “alternative” activated M2 macrophage towards an immunogenic responsive “classically” activated M1 cell promoted by the proinflammatory milieu. Thus CD163 may simply be a biomarker of macrophage activity with no functional properties. Interestingly, we found a significant
decrease in the co-localisation of CD68 and CD163 in the duodenal mucosa of untreated coeliac patients. Thus the elevated CD163 serum levels associated with untreated coeliac disease are most likely derived from intestinal macrophages that have switched phenotype from a M2 to M1 macrophage.

Another interesting observation made in our study, was the strong inverse relationship between soluble CD163 and CD163 surface expression for both classical (r= -0.7989, p=0.0004) and intermediate monocyte subsets (r= -0.7286, p=0.0021) in children newly diagnosed with coeliac disease whereas healthy controls exhibited no such correlation. This finding indicates that a portion of soluble CD163 levels is directly derived from the shedding of CD163 from the surface of peripheral blood monocytes. This study found no correlation between the percentage of CD163 positive monocytes and soluble CD163 suggesting the increased levels of soluble CD163 found in patients reflects a monocyte functional response rather than an increase in the frequency of monocytes. These findings lead us to postulate that disease severity is accompanied by activation of circulating monocytes in response to inflammatory mediators released into the blood from the inflamed tissue. Such as the study carried out by Manavalan et al (2010), which reported a 5-fold increase in serum IFN-gamma in untreated coeliac patients when compared to healthy controls, which may be responsible for the shedding of CD163 from the surface of monocytes.

As mentioned above we have shown that approximately 70% of circulating myeloid DCs expresses CD163, while the majority of CD11c+ cells in the normal and coeliac mucosa do not co-express CD163. Therefore, we could hypothesise that newly recruited myeloid DCs migrating from the blood shed CD163 from its surface upon entry into the mucosa contributing to the elevated pool of soluble CD163 observed in untreated coeliac patients. However, due to the small number in the frequency of myeloid DCs found in the peripheral blood it is doubtful dendritic cells are the main source of soluble CD163 and that monocytes and mucosal macrophages are the likely source due to their higher frequency and high expression of CD163.

Additional work is needed to explore the extent to which tissue macrophages within the pro-inflammatory coeliac lesion contributes to CD163 levels in the serum found elevated in our paediatric population, similar to the study carried out in our adult

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patients, which determined CD163 and CD68 co-expression in duodenal biopsies.

Considering the potential diagnostic utility of soluble CD163 as a monitor for monocyte/macrophage pro-inflammatory conditions this project is now ongoing in the routine Immunology laboratory where they plan to investigate soluble CD163 in a vast array of inflammatory disorders.

**Functional potential of soluble CD163**

It has been hypothesised that soluble CD163 may have a direct effect on peripheral T cells, as it has been shown to inhibit T cell proliferation stimulated by PMA (Högger & Sorg, 2001). Interestingly, in children with newly diagnosed with CD a decrease in CD69L, an early activation marker on the surface of peripheral lymphocytes was detected (Csecht et al, 2010). The reason for this decreased expression is not known but one could postulate a role for soluble CD163. This hypothesis is further supported by observations made in SpA patients were an inverse relationship between CD69 and soluble CD163 in the synovial fluid was found (Baeten et al., 2004).

One study has suggested that soluble CD163 may have the functional capacity to regulate the adaptive immune response, particularly the down regulation of T cell proliferation (Högger & Sorg, 2001). However, there are several drawbacks associated with this study. The main issue being the stimulus, PMA, used to induce T cell proliferation. It is unusual to use PMA alone as a stimulus considering it is recommended that it be used in conjunction with ionomycin to trigger T cell activation (Chatila et al., 1989). In fact, it has been shown that PMA alone is not sufficient to drive T proliferation (Davis & Lipsky., 1985; Thompson et al., 1989), therefore the assay used was not suitable for evaluating the effects of soluble CD163 on T cell proliferation. In an attempt to investigate whether soluble CD163 could modulate the adaptive immune response, recombinant CD163 was added to lymphocytes stimulated with various stimuli.

From our results it was established that recombinant CD163 had no effect on T cell proliferation or cytokine production, which implies soluble CD163 does not possess the ability to alter T cell responses. However, several factors may have contributed to the negative results observed in this study. Recombinant CD163 may lack functional qualities that its natural occurring counterpart may possess. Recombinant CD163 was
generated in a mouse myeloma cell line with a predicted molecular weight of 110kDa, whereas soluble CD163 has a molecular weight of 130kDa, thus depending on the domain affected this difference may potentially account for the lack of function exhibited by the recombinant protein. Likewise an antigen specific T cell proliferation assay may have been more appropriate compared to the artificial stimulus of the T cell receptor complex employed in our experiment. This stimulus may have been too strong to allow rhCD163 to exert any effect on T cell responses (Gunnlaugsdottir, Maggadottir, & Ludviksson, 2004). Therefore, additional assays are required using soluble CD163 along with a more physiological stimulus to establish whether CD163 has the capacity to modulate T cell responses.

5.5 Conclusion

- We have demonstrated a significant decrease in the co-expression of CD68 and CD163 in the untreated coeliac mucosa. This decrease in co-expression most likely represents a shift in the macrophage phenotype from an anti-inflammatory M2 phenotype to a pro-inflammatory M1 phenotype. The M1 macrophage helps maintain the inflammatory cascade via the production of pro-inflammatory cytokines contributing to the development of the coeliac lesion.
- We have shown that CD163 is highly expressed on peripheral blood CD11c+ myeloid DCs while mucosal myeloid DCs lacked CD163 expression suggesting that mucosal DCs are of different origin to circulating DCs or they shed CD163 upon arrival into the tissue.
- Peripheral blood myeloid DC express CD163, whereas plasmacytoid DC lack CD163 expression. Reinforcing the idea that these cells are two separate entities with different functional capacities reflected in their distinct expression for CD163.
- We demonstrated significantly increased levels of soluble CD163 in the serum of patients newly diagnosed with coeliac.
- We have shown a strong inverse correlation of soluble CD163 and monocyte membrane bound CD163 that is independent of monocyte frequency.
- We recommend that both soluble CD163 and surface CD163 be investigated
when monitoring monocyte/macrophage activation.

- Our limited functional experiments were unable to establish the exact function of sCD163. However, the enhanced sCD163 levels in individuals with untreated coeliac disease may signify the presence of a chronic macrophage activation state attempting to control T cell responses through the inhibition of T-cell activation and proliferation.
Chapter 6 The Investigation of Tissue Transglutaminase Expression in the Gut and in Monocyte derived Dendritic Cells

6.1 Introduction

Heinrich Waelsch and co-workers first used the term “transglutaminase” in 1959 to describe the calcium dependent transamidating activity present in the liver and some other organs of the guinea pig (Mycek, et al, 1959). Transglutaminases are a group of widely distributed Ca2+ dependent enzymes that catalyse the post-translational modification of proteins by the formation of isopeptide bonds (Griffin, Casadio, & Bergamini, 2002). All different human transglutaminases exhibit differences in substrate specificity, expression pattern and function (Table 6.1).

<table>
<thead>
<tr>
<th>NAME</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood coagulation Factor XIII</td>
<td>Coagulation cascade. Wound healing and stabilisation of the fibrin clot (Richardson, Cordell, Standeven, &amp; Carter, 2013).</td>
</tr>
<tr>
<td>Transglutaminase 1 (or keratinocyte transglutaminase)</td>
<td>Differentiation of keratinocytes (Mehta, 2005).</td>
</tr>
<tr>
<td>Transglutaminase 2 Tissue transglutaminase (tTG)</td>
<td>Main autoantigen in coeliac disease; Stabilisation and remodelling of the extracellular matrix; regulation of cell death; differentiation and apoptosis (Klöck, Diraimondo, &amp; Khosla, 2012)</td>
</tr>
<tr>
<td>Transglutaminase 3 (or epidermal/hair transglutaminase)</td>
<td>Terminal differentiation of keratinocytes (John et al., 2012; A. Yamane et al., 2010)</td>
</tr>
<tr>
<td>Transglutaminase 4 (or prostatic secretory TG)</td>
<td>Seminal vesicle coagulation (Jiang &amp; Ablin, 2011)</td>
</tr>
<tr>
<td>Transglutaminase 5 (or TG X)</td>
<td>Epidermal differentiation (Candi et al., 2002)</td>
</tr>
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Table 6.1 : The human transglutaminase family
6.1.1 Tissue Transglutaminase

Tissue transglutaminase (tTG) or TG2, a 85kD protein, is a multifunctional calcium dependent ubiquitous enzyme with recognised roles in apoptosis (M Griffin & Verderio, 2000; Nurminskaya & Belkin, 2012) wound healing (Verderio, Johnson, & Griffin, 2004), cell growth, cell signalling (Nurminskaya & Belkin, 2012), metastatic functions in tumour cells (Chhabra, Verma, & Mehta, 2009; Mehta, Kumar, & Kim, 2010), and extracellular matrix (ECM) interactions (Belkin, 2011). It is widely distributed and can be found in the extracellular matrix or intracellularly (mostly the cytoplasm but can be detected in the nucleus (Kuo, Tatsukawa, & Kojima, 2011) and the cell surface (Nurminskaya & Belkin, 2012).

6.1.2 Tissue transglutaminase structure

The genes coding for this family of enzymes are clustered over five different chromosomes, with the gene for tTG found on the long arm of chromosome 20, (locus 20q11-12). The 5′ promoter region of the tTG gene contains response elements to transcriptional activators such as TGF-β (Jung et al., 2007; Quan et al., 2005), retinoids (Cai, Ben, & De Luca, 1991), and steroid hormones, which all up-regulate expression of tTG in a tissue-dependent manner.

TtTG is composed of 4 domains; a N-terminal beta sandwich, a core domain (containing the catalytic triad and the regulatory sites), and C-terminal beta barrels 1 and 2. Typically, domains 1-4 span amino acids 1-139, 140-454, 479-585 and 586-687, respectively with different secondary structures. Domains 1, 3 and 4 are folded into a beta-structures and domain 2 presents an alpha-helical secondary structure (Wang & Griffin, 2012). The active site consists of a catalytic triad built by cysteine 277, histidine 335, and asparatic acid 358 and performs the crosslinking reaction (Griffin et al., 2002; Klöck et al., 2012). The 3D structure and some of the functional domains are shown in Figure 6.1
Figure 6.1: Structure and functional domains of tissue. The 3D structure of tTG. (Image adapted from (Casadio, et al. 1999) (b) Domain organisation of tTG. The guanine nucleotide binding sites are shown in green. Fibronectin binding, catalytic residues (C277, H335, D358), putative Ca2+ binding sites (N398, D400), α1B-adrenergic receptor and phospholipase C, delta 1 (PLCδ1) binding domains are respectively shown in grey, orange, red, blue and purple. (Image adapted from Mhaouty-Kodja, 2004).
In 2007 Pinkas and colleagues identified the active conformation of tTG revealing a fundamental difference between the active and inactive conformation. The complete C-terminal beta 1- and beta2 – barrels, which cover the N terminal core domain in the catalytically inactive form, are in an extended conformation in the active form. This large conformational shift results in the exposure of the catalytic triad responsible for the transamidation and deamidation of its target proteins (Figure 6.2).

Figure 6.2: Structure of Tissue Transglutaminase. The N-terminal β-sandwich is shown in blue (N), the catalytic domain (Core) in green, the C-terminal β-barrels (β1 and β2) in yellow and red, respectively. (Image adapted by Pinkas et al., 2007)

6.1.3 Function of tissue transglutaminase

tTG possesses the ability to catalyse the covalent and irreversible crosslinking of a protein with a glutamine residue (glutamine donor) to another protein with a lysine residue (glutamine acceptor), resulting in the formation of an ε – (γ – glutamyl )-lysine (isopeptidyl) bond (Király, Demény, & Fésüs, 2011). Under certain conditions, such as in the absence of suitable amine groups and in the presence of low pH, tTG can deamidate glutamine residues to glutamic acid a process central to CD (Klöck et al., 2012; Qiao et al., 2012; Quarsten, Molberg, Fugger, McAdam, & Sollid, 1999; Sjöström et al., 1998; van de Wal et al., 1998)
tTG is only active in the presence of high concentration of calcium ions. In the absence of calcium ions, the enzyme assumes a latent conformation and the activity of Cys277 is decreased either by hydrogen-bonding with the phenolic hydroxyl group of Tyr516 or by formation of a disulphide bond with the neighbouring Cys336 (Noguchi et al., 2001).

The enzyme also exhibits guanosine triphosphate (GTP)ase activity where binding of GTP inhibits substrate binding to the catalytic site (Lismaa et al., 1997). Considering the concentration of calcium ions in the cytosol, it is thought that the transamidating activity of tTG is absent intracellularly (Király et al., 2011). However, in circumstances associated with calcium influx, such as apoptosis, the cross-linking activity of tTG is thought to be activated (Autuori et al., 1998).

More recently, tTG has been shown to play a role in the activation of TGF-β, a cytokine known to possess anti-inflammatory properties as well as playing a role in the prevention of autoimmunity (Shweke et al., 2008; Telci et al., 2009). This suggests that tTG may a play a role in regulation and maintenance of immune tolerance.

6.1.4 Tissue transglutaminase and the Extracellular matrix

In addition, to its cytoplasmic and nuclear localisation, tTG is also found on the cell surface and the extracellular matrix. The mechanism by which tTG is translocated to the surface and subsequent release into the extracellular matrix is not yet known. However, it is though to be dependent on the active site conformation and an intact N-terminal β sandwich domain (Belkin, 2011; Gaudry et al., 1999). This translocation is believed to occur in response to cell trauma and stress (Lentile et al., 2007) and may play an important role in regulating the inflammatory response, and the remodelling and stabilisation of the matrix proteins.

Once externalised from the cell, tTG binds to extracellular proteins where it is believed to have many physiological roles in the extracellular matrix (Wang & Griffin, 2012). In addition, to matrix stabilisation through protein crosslinking (Belkin, 2011), tTG may act as an integrin binding adhesion co-receptor for fibronectin (Akimov et al., 2000), a function that may facilitate in cell adhesion and migration (Akimov, 2001; Wang & Griffin, 2012). tTG acting as integrin binding co-receptor is independent of its crosslinking activity and depends on its close association with cell surface integrins β1 Louise Elliott
6.1.5 Tissue transglutaminase and Coeliac disease

The identification of tissue transglutaminase (tTG) as the predominant autoantigen has been the most important finding in the area of coeliac disease in recent time. Its discovery allowed for the introduction of reliable and sensitive diagnostic tests for coeliac disease as well as unveiling another piece of the puzzle with regard to the immune mechanisms that drive coeliac disease (Gujral et al., 2012). tTG plays a fundamental role in the pathogenesis of CD via the deamidation of specific glutamine residues in wheat gliadin peptides which significantly enhances their binding to HLA-DQ2 or HLA-DQ8 molecules, thus increasing their immunogenicity (Fleckenstein et al., 2002; Gjertsen et al., 1994; Klöck et al., 2012; Lundin et al., 1989; Molberg, McAdam, & Sollid, 2000).

Considering tTG plays a pivotal role in both the diagnosis and pathogenesis of coeliac disease, it is no surprise that the coeliac mucosa has some specific characteristics of tTG expression. There is a substantial amount of evidence that clearly demonstrate that tTG is over expressed in the duodenal mucosa (Brusco et al., 1999; Carla Esposito et al., 2003; Gorgun et al., 2009; Hansson et al., 2002; Sakly et al., 2005; Skovbjerg et al., 2004; Villanacci et al., 2009) of untreated coeliac patients although the data regarding its distribution pattern is controversial. The majority of studies that have examined tTG distribution in the coeliac mucosa employed immunohistochemistry techniques whereas only one study opted for immunofluorescence (Ciccocioppo et al., 2003). What’s more, all studies except for one (Gorgun et al., 2009), assessed immunohistochemical and immunofluorescent staining using qualitatively or semi-quantitative methods as opposed to quantitative analysis.

Most studies have reported an increase in tTG expression in the coeliac mucosa when compared to healthy controls (Brusco et al., 1999; Esposito et al., 2003; Gorgun et al., 2009; Sakly et al., 2005; Skovbjerg et al., 2004). In the normal mucosa, tTG is predominantly expressed in the mucosa muscularis and lamina propria while the untreated coeliac lesion is characterised by tTG over expression with the greatest difference found at the basement membrane, and the lamina propria. In disagreement with these observations, some studies failed to reveal any difference in tTG expression...
between CD patients and normal controls (Mercan & Celiac, 2003; Villanacci et al., 2009). On examining the crypt and superficial epithelium, some investigators revealed epithelial tTG expression in normal duodenal mucosa (Villanacci et al., 2009) whereas some believe tTG expression within the epithelial compartments is specific to coeliac disease only (Gorgun et al., 2009). Such differences in the literature is probably due to the different methods used in these studies reinforcing the need for a more sensitive quantitative assay for examining tTG tissue expression.

Several studies have reported tTG staining near the crypt epithelium displaying stellate elongated morphology similar to that of myofibroblasts (Ciccocioppo et al., 2003). However, no studies have confirmed the identity of these cells postulated to be myofibroblasts. Sub epithelial intestinal myofibroblasts play a fundamental role in orchestrating many diverse functions in the intestine. They are found in close proximity to the intestine crypts and have strongly been implicated in the regulation of the proliferation, differentiation and function of the epithelium (Hinz et al., 2007; Mifflin et al., 2011). The myofibroblasts that stain for smooth muscle alpha actin secrete growth factors such as TGF-beta, which are necessary for epithelial cell differentiation and proliferation as well as maintaining the crypts and villous of the small intestine (Mifflin et al., 2011). Since villous atrophy and crypt hyperplasia are features of untreated coeliac disease, changes in pericryptal myofibroblasts may have an influence on lesion formation (Crivellato et al., 2006). Thus the preferential localisation of tTG expression in the lamina propria and near the cryptal epithelium may reflect the involvement of tTG in the development of mucosal atrophy in CD.

Another controversial issue that is still at much debate is whether anti-tTG antibodies possess the ability to affect tTG function (Caputo et al., 2009; Esposito et al., 2002), thus playing a pathogenic role in coeliac disease. Although if this was the case, anti-tTG antibodies would also have the ability to block the role of tTG in driving the adaptive immune response via glutamine deamidation. One interesting study that used T84 crypt epithelial cell differentiation in a fibroblast co-culture model found that the TGF-beta dependent differentiation of crypt epithelial cells was blocked by the addition of a blocking antibody to TGF-beta or tTG IgA autoantibodies (Halttunen & Mäki, 1999). Thus, the local production of autoantibodies within the mucosa has been suggested to

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contribute to the mucosal transformation observed in the coeliac lesion.

In the coeliac mucosa gliadin-tTG complexes can be found mainly in the epithelial and sub epithelial areas in untreated coeliac patients while in controls their co-localisation is restricted to the lamina propria area (R Ciccocioppo et al., 2003). In the coeliac mucosa the enterocytes also exhibit increased tTG activity. Thus on this basis, it was suggested that tTG could come in contact with gliadin peptides within the enterocytes where they are deaminated and subsequently transported to the extracellular matrix for presentation to T cells.

A number of inflammatory cytokines and growth factors can also induce tTG expression, including TGF-beta, interleukins IL-6, TNF-α, and IFN-γ, which are thought to upregulate tTG via activation of the nuclear factor κB (NF-κB). Recently, Bayardo et al (2012) demonstrated that TNF-α and IFN-γ in combination induced the strongest tTG expression in intestinal tissue. Thus suggesting that IFN-γ, the dominant cytokine found in the mucosa of untreated coeliac patients can induce tTG expression, and synergism with TNF-α can further enhance tTG expression promoting the immune response to gliadin.

Within the lamina propria, tTG can be found in the extracellular matrix or as cellular tTG (Wang & Griffin, 2012), which can be differentiated by employing stains such as dapi for immunofluorescence or haematoxylin for immunohistochemistry that stain the nuclei of cells. This allows the researcher to identify tTG positive cells and distinguish these cells from extracellular tTG that has been externalised into the matrix. The over-expression of tTG within specific areas and the presence of individual tTG positive lamina propria cells suggests that the enzyme could be translocated from the intracellular to the extracellular matrix in the lamina propria via these unidentified cells in response to cellular stress (lentile et al., 2007). However, no studies have been carried out to determine the identity of these cells in situ. Therefore, to further understand the pathogenesis of coeliac disease, it is important to learn the specification of these cells. It has been speculated that these cells are APCs (Ráki et al., 2007), cells key to the induction and maintenance of the adaptive immune response (Rescigno & Sabatino, 2009), which is further supported by the finding that both dendritic cells and macrophages are found increased in the coeliac mucosa compared to healthy controls.
(Beitnes et al., 2011; Ann-Christin Røberg Beitnes et al., 2012; Di Sabatino et al., 2007; Räki et al., 2006) and that cell surface tTG has been found on macrophages and dendritic cells generated in vitro from monocytes cultured in the presence of M-CSF or IL-4 and GM-CSF, respectively (Hodrea et al., 2010).

Our previous finding that CD11c positive dendritic cells are found increased in the untreated coeliac lesion, along with published that work have demonstrated similar findings with one study demonstrating their ability to present gliadin to T-cells (Melinda Räki et al., 2006) has led us and other researchers to hypothesise that the presence of surface tTG on antigen presenting cells may participate in deamidation of gliadin peptides and facilitate their uptake and subsequent presentation to naïve T cells (Hodrea et al., 2010; Räki et al., 2007). It has been suggested that tTG-catalysed gliadin deamidation may occur within the endocytic compartment of antigen presenting cells. The pH of this compartment would facilitate the deamidation reaction, which is preferentially carried out by tTG in acidic conditions (Figure 6.3).

Moreover, it has been shown by previous studies that moDCs express tTG, with one group claiming that tTG on the cell surface was capable of being endocytosed from the cell surface (M Räki et al., 2007). However, this group has since published evidence that the monoclonal antibody used in this study does not bind tTG, but rather CD44. Therefore, further studies are warranted to determine the relationship between tTG and dendritic cells and the potential involvement of surface tTG in coeliac disease. Unfortunately, the lack of monoclonal antibodies that binds surface tTG in flow cytometry assays has hampered the investigation of this issue. Fortunately, a collaborator of ours (Fernando Chirdo) has raised 2 monoclonal antibodies against tTG, which have been shown to bind surface tTG employing a FACs assays with Jurkat T cells (pilot work) and will be tested further in this study.
6.2 Objective

- To grow monocyte derived DCs and establish a maturation system.

- To culture monocyte-derived dendritic cells in order to quantify and locate expression of tTG in immature and mature moDCs using flow cytometry, quantitative reverse transcriptase PCR, and Western blotting.

- To examine expression of tTG on dendritic cells and macropahges in coeliac disease small intestinal biopsies using triple-colour immunofluorescence and high content screening methods

- To quantify mucosal tTG expression in duodenal sections from untreated, and treated coeliac patients and healthy controls using Bitplane quantitative analysis software.
6.3 RESULTS

6.3.1 Generation of monocyte derived dendritic cells

DC comprises a heterogeneous population of cells that comprise less than 1% of circulating blood cells. Due to their scarcity, DC must be derived from more abundant precursors in order to facilitate in vitro work. In the studies described here, a widely used approach for the generation of monocyte derived DC (moDC) was employed, involving the culture of monocytes with the cytokines interleukin-4 (IL-4) and granulocyte/monocyte colony-stimulating factor (GM-CSF) (Sallusto & Lanzavecchia, 1994). Sterility was essential in order to avoid spontaneous DC maturation thus low endotoxin foetal calf serum (HyClone) was used in all buffers and media throughout the experiments.

Blood packs were used as a source of monocytes, kindly provided by the Irish Blood Transfusion Service (IBTS), and monocytes were magnetically separated from PBMCs using CD14+ magnetic beads as described in chapter 3. An aliquot of the CD14+ fraction was taken for flow cytometry to determine cell purity (Figure 6.1). Monocytes were then incubated in complete medium (with IL-4 and GM-CSF) for 6 days to generate monocyte derived DCs or in medium alone to serve as controls. On day 6 cells were harvested and the phenotype of cells was determined.

The cell yields from the 8 donors are summarised in table 6.2. The mean purity of monocyte-gated cells was 91.87%. Employing 18x10^6 monocytes on day 0, a mean of 6.8x10^6 moDC was obtained.

Phenotypic analysis of monocytes freshly isolated on day 0 and monocytes cultured for 6 days in medium alone (control) showed distinct differences from cells treated with IL-4 and GM-CSF for 6 days (the moDC population). On comparing CD14 expression on monocytes in culture for 6 days with freshly isolated monocytes, CD14 surface expression was retained along with their homogenous shape determined from the cytometric forward scatter side scatter position [Fig 6.2a]. In contrast CD14 expression on moDCs was greatly reduced, in keeping with the differentiation of these cells into moDCs (Figure 6.2c). moDCs retained high CD11c (Fig 6.3) expression similar to
monocytes [fig 6.4]. Morphologic changes were also observed in cytospin preparations made of freshly isolated monocytes and moDCs. Following differentiation into moDC, monocytes lost their horseshoe shape nucleus and homogenous round shape, becoming more irregular in shape, developing a round nucleus and cellular hair like projections characteristic of DC (Figure 6.2b).

Figure 6.1. Flow cytometric analysis of CD14 expression by PBMC, CD14-depleted PBMC, and CD14+ enriched populations. (A) CD14 expression was determined in PBMCs prior to magnetic bead separation. (B) CD14+ expression in the negative fraction after magnetic bead separation. (C) The monocyte purity in the CD14+ enriched fraction. Percentages shown correspond to the percentage of total cells.
<table>
<thead>
<tr>
<th>Exp</th>
<th>Starting PBMC nos x10^6</th>
<th>CD14+ isolated nos x10^6</th>
<th>CD14^+ purity (%)</th>
<th>monocyte purity (of monocytes plated)</th>
<th>moDC yields x 10^6</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>95</td>
<td>87.1</td>
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</tr>
<tr>
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<td>89.3</td>
<td>13.2</td>
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<td>676</td>
<td>55</td>
<td>94.2</td>
<td>5.4</td>
<td></td>
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<td>62</td>
<td>91.87</td>
<td>6.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2 Data show yields and purities of CD14 magnetic bead enrichment of monocytes. PBMCs were enriched for CD14+ monocytes. Flow cytometric analysis was used to determine the purity of CD14+ fractions. Cell counts were carried out to determine cell yields.
Figure 6.2: CD14+ monocytes undergo a morphological and phenotypic change when cultured with GM-CSF and IL-4 (A) flow cytometric dot plots representative of a monocyte (left) and moDC (right). Note difference in side scatter/forward scatter profiles. (B) Cytospin preparation of monocyte (left) and moDC (right). Values represent diameter of cells highlighted [see box]. (C) Flow cytometric histogram plots displaying CD14 expression on monocytes (left) and moDC (right). Note shift in peak to the left for the moDCs, indicating loss of CD14 expression.
6.3.2 Generation of mature monocyte derived dendritic cells

moDC and moDC stimulated with LPS (mature moDC) were stained with a variety of DC maturation markers, namely HLA-DR, CD80, CD86, CD83, CD40 and were analysed employing flow cytometry (Figs 6.4 and 6.5). For comparison purposes, these experiments were also performed on freshly isolated monocytes. Lipopolysaccharide (LPS), an antigenic component found in the cell walls of Gram-negative bacteria, is known to trigger DC maturation into fully functioning APCs capable Th1 polarisation.

All maturation markers analysed were upregulated on both monocytes and moDC upon LPS stimulation when compared to cells cultured in medium alone. A representative experiment is shown (Fig 6.4) and the results of 7 experiments are given in Fig. 6.5. High levels of CD40 and HLA-DR were found on both cell populations. However, analysis of monocytes with moDC showed distinct differences between these cell populations. moDCs expressed a significantly higher amount of CD40 compared to monocytes and this expression was further enhanced by LPS stimulation. In contrast, HLA-DR was more strongly expressed by monocytes. While immature moDCs expressed varying levels of HLA-DR ranging from low to high, mature moDC exhibited homogenous levels as represented by a single peak in the flow histogram (Fig 6.4). Low levels of expression for CD83 and CD86 were noted on both immature monocytes and dendritic cells. Upon stimulation with LPS little change was observed in monocytes while LPS induced an upregulation of the two molecules in moDC. Finally, both monocytes and moDCs expressed high levels of CD11c, but upon activation with LPS, enhancement of expression was only noted in moDC (Fig. 6.4).

6.3.3 Cytokine production by monocytes and moDC

In response to stimulation with LPS, moDCs are known to rapidly produce a range of cytokines including IL-10 and IL-12p70, which allows them to be differentiated to their immature counterpart. The supernatants from monocytes and moDC incubated
overnight in medium alone or in the presence of LPS were removed for cytokine analysis by ELISA. Both unstimulated monocytes and moDCs expressed low levels of IL-12p70 and IL-10. LPS stimulation caused a marked enhancement of both these cytokines, with the greatest difference observed between moDC and mature moDC. LPS treated cells produced approximately 3 times more IL-12p70 and 18 times more IL-10 \((p=0.0140)\) than unstimulated moDC. Figure 6.6 and 6.7 show results for 4 experiments.

\[\text{Figure 6.3: Monocyte upregulate costimulatory, and antigen presentation markers in response to stimulation with TLR agonists LPS. Histogram peaks indicate marker expression intensity for DC treated with medium only (blue lines), LPS (green lines. Data shown is from a single donor, representative of 4-10 similar experiments.}\]
Figure 6.4: DC upregulate costimulatory, and antigen presentation markers in response to stimulation with TLR agonists LPS. Histogram peaks indicate marker expression intensity for DC treated with medium only (red lines), LPS (yellow lines). Data shown is from a single donor, representative of 4-10 similar experiments.
Figure 6.5: Monocytes and moDC upregulate costimulatory, and antigen presentation markers in response to stimulation with TLR agonists LPS. moDC were stained for cell surface markers HLA-DR, CD86, CD83, and CD40, and analysed by flow cytometry. Average MFI values are shown for each marker.
Figure 6.6: IL-12p70 content by ELISA. Monocytes and moDCs were incubated in the presence of medium and LPS. Data was analysed by t-test, p<0.05. No Data shown are of 4 similar experiments.

Figure 6.7: IL-10 content by ELISA. Monocytes and moDCs were incubated in the presence of medium and LPS. Data was analysed by t test, p<0.05. Data shown are of 4 similar experiments.
6.3.4 TG100 monoclonal antibody detects cell surface tTG on monocyte derived DCs while other monoclonal antibodies do not.

The importance of tTG-mediated deamidation of gliadin in the pathogenesis of coeliac disease has been well established. However it is not clear where this deamidation occurs or what cells may be potential involved in this process. Since we identified a marked increase in the number of CD11c+ cells in the mucosa of patients with active celiac disease (Chapter 4), we postulated that this cell population might be involved in tTG mediated deamidation.

As a starting point, we first investigated the expression of tTG on monocytes and moDC. A series of monoclonal antibodies were employed in flow cytometry experiments and comparisons were made between freshly isolated monocytes, moDC and LPS stimulated moDC (Fig 6.8). In the case of monocytes, all antibodies with the exception of Cub7402, appeared to detect surface tTG on the surface of these cells. However, the high background staining with the isotype control antibody raised questions about the specificity of this staining. In contrast, TG100 was the only monoclonal antibody to detect tTG on moDC and this expression was further enhanced by LPS stimulation.

An example of tTG expression on moDC, which is enhanced by LPS, is shown in Fig. 6.9.
Figure 6.8: tTG distribution on monocytes and moDC and moDC stimulated with LPS detected by a variety of antibodies employing flow cytometry. All were labeled with (A) a tTG antibody directly conjugated with FITC (A), 5G7G6 (B), 2G3H8 (C), TG100 (D), and Cub7402 (E) shown in red and an isotype antibody shown in blue. Overlays are representative of 6 experiments.
Figure 6.9: Increase in surface tTG expression is induced on moDC stimulated with LPS. Representative flow cytometric dot plots showing an upregulation of surface tTG on activated moDC (right) compared to moDC (middle).

6.3.5 All tTG monoclonal antibodies bind non-specifically to monocytes as determined by confocal microscopy

Because of questions concerning the results for tTG expression as examined by cytometry, further studies were performed employing confocal microscopy. Cytospin preparations of freshly isolated monocytes and moDC were indirectly stained with each of the monoclonal antibodies in parallel with an isotype antibody to control for non-specific binding. All images were acquired using confocal microscopy with representative images shown in Fig. 6.9.

All tTG monoclonal antibodies employed in the study appeared to bind non-specifically to monocytes and this finding was suggested by the presence of fluorescent green staining observed with the isotype control antibody. In contrast, only monoclonal antibodies TG100 and Cub7402 detected tTG on moDCs despite the finding that Cub7402 failed to recognise tTG in the flow cytometry experiments. No staining on moDC was found with the isotype control antibody. Positive staining on moDC demonstrated a more homogenous, diffuse staining pattern most prominent in the cytoplasm and potentially the plasma membrane compared to what was detected on monocytes. In agreement with our flow cytometry results monoclonal antibodies 2G3H8 and 5G7G6 did not detect tTG staining on moDC.

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Figure 6.9: Indirect fluorescent tTG (green) staining of monocytes (left panel) and moDC (right panel). Cells suspensions were prepared by cytospin, fixed, and stained and acquired by confocal microscopy. Both monocytes and moDCs were stained with an isotype antibody along with the following monoclonal antibodies, 2G3H8, 5G7G6, Cub7402, and TG100.

6.3.6 tTG mRNA quantification in monocytes and moDC

Total tTG mRNA was analysed employing RT-PCR in monocytes (Day 0), moDC (Day 6) and moDC stimulated with LPS (Day 7). For the purpose of this experiment relative quantification was used. In relative quantification, changes in gene expression were analysed in a given sample relative to another reference sample referred to as the calibrator. tTG mRNA was detectable at very low levels in freshly isolated monocytes therefore the monocyte was defined as the calibrator sample. The relative mRNA for tTG in monocyte derived DCs and stimulated moDCs were expressed as an n-fold difference relative to the calibrator (monocyte).

Upon analysis, a sharp increase in mRNA level was observed after one day of culture in the presence of IL-4 and GM-CSF (Fig 6.11). After day one, a sharp decline in tTG expression was noted and thereafter levels remained constant, through to day six. Following stimulation with LPS, a further increase in tTG mRNA was induced in moDC, in keeping with the flow cytometry results observed with monoclonal antibody TG100.
Figure 6.10: Relative mRNA quantification. Figure A shows mRNA levels for monocytes (day 0), monocyte derived DC (day 6) and LPS stimulated moDC (day 7) (n=6). Figure B represents mRNA levels from day 0-6 in culture with GM-CSF and IL-4 (n = 4)
6.3.7 Western blot analysis of tTG protein expression in monocytes and moDC.

To further support our observations made with RT-PCR, protein tTG was detected employing immunoblotting. Fresh monocytes (day 0), monocyte derived dendritic cells (day 6), and moDCs stimulated with LPS (day 7) were collected and washed and subsequently lysed in the presence of protease inhibitors. Samples were then prepared and loaded onto a SDS polyacrylamide gel. Next immunoblotting was carried out for the detection of tTG (as described in chapter 3).

tTG protein was undetectable in freshly isolated monocytes but was clearly expressed by moDC although no increase in moDC was apparent after stimulation with LPS (Figure 6.10 a).

Next membrane proteins were isolated from monocytes (day 0) and moDCs (Day6) following the MEM-PER Plus membrane protein extraction kit protocol described in chapter 3, to confirm the presence of tTG on the surface of monocyte derived dendritic cells. Similar to the previous results, tTG protein was undetectable in freshly isolated monocytes but was expressed by moDCs (Figure 6.10b).

![Western blot images of tTG protein expression](image)

**Figure 6.11:** (A) Human monocytes (day 0), monocyte derived dendritic cells (day 6) and LPS stimulated moDC (day 7) were collected and tTG protein expression was detected in cell lysates by western blotting with monoclonal antibody Cub7402. (n=3) (B) Westernblot analysis of protein isolated from the cell membrane of monocytes and monocyte derived DCs. A band appears at the molecular weight of tTG using monoclonal antibody Cub7402 in the moDC wells only. (n=2)
6.3.8 Investigation of tissue transglutaminase staining in normal and coeliac patients
duodenal mucosa

Immunofluorescence studies, employing the monoclonal antibody Cub7402, were then performed to investigate the expression of tTG in small intestinal biopsy specimens from healthy controls and celiac subjects. Samples from all samples showed tissue transglutaminase expression in the mucosa muscularis and the lamina propria. However, tTG expression in coeliac mucosa differed significantly from that in healthy controls (Figure 6.11). In celiac mucosa, marked staining of the superficial and crypt epithelium was evident whereas tTG expression was either absent or very weak in healthy subjects. A high proportion of tTG positive cells were identified under the basement membrane in both coeliac and control mucosa.
Figure 6.12: Tissue transglutaminase staining employing the monoclonal antibody Cub7402 of duodenal mucosa from a healthy control (left panel) and untreated coeliac patient (right panel) x 400. There is strong staining of the surface epithelium (A) and the lamina propria (B) in untreated coeliac mucosa when compared to healthy mucosa.
A quantitative analysis of tTG expression was then performed employing Imaris image analysis software. This allowed objective quantification of tTG expression, in comparison to previous reports which based their results on visual observation (Brusco et al., 1999; Mercan & Celiac, 2003; Sakly et al., 2005; Villanacci et al., 2009). Initially, the total surface area for mucosal tTG expression was objectively quantified for all image acquired from each section stained. The results are summarised in figure 6.12 and Table 6.3. A 2.5 fold increase in tTG expression was observed in untreated coeliac mucosa, in comparison to healthy mucosa (p = 0.0012) (Figure 6.12). Mucosa from treated coeliac patients expressed similar levels of tTG to healthy controls.

**Figure 6.12:** Quantitative evaluation of total tTG expression in normal controls and coeliac disease patients (p value 0.0012). HC (n=12), TCD (n= 9), and UCD (n= 15). Means are denoted by black horizontal lines.

<table>
<thead>
<tr>
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<th>Healthy Control</th>
<th>Treated Control</th>
<th>Untreated Control</th>
</tr>
</thead>
<tbody>
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<td><strong>Mean um^2</strong></td>
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<td>19,163.4</td>
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<td>0128.03 – 38232.04</td>
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</tr>
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</table>

**Table 6.3:** Quantitative results for total tTG surface area compiled using analysis software for healthy controls, treated and untreated coeliac patients

The software allowed quantification of positive tTG staining within selected...
compartments of the duodenal mucosa, in order to determine the level of expression in the epithelial and lamina propria regions. The results are summarised in figures 6.13 and table 6.4. The results are expressed as a percentage of the total area measured, i.e. the lamina propria or epithelium, and not in micrometers\(^2\) in contrast to the results in Fig 6.12 and Table 6.1. The level of tTG expression in the epithelium and lamina propria in the untreated coeliac mucosa was significantly greater than that found in healthy control mucosa (p= 0.0086 and p=0.007 respectively). The level of tTG expression in the mucosa of treated coeliac patients was also increased, but did not achieve significance. Minimal tTG staining of the epithelium was found in the healthy controls, whereas expression was clearly evident in the majority of the untreated coeliac patients.

<table>
<thead>
<tr>
<th>Lamina propria tTG</th>
<th>Epithelial tTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean(%)</td>
<td>Mean(%)</td>
</tr>
<tr>
<td>(range (%))</td>
<td>(range (%))</td>
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<tr>
<td>Healthy control</td>
<td></td>
</tr>
<tr>
<td>11.5 (2.4 – 22.0)</td>
<td>1.1 (0.05 - 2.7)</td>
</tr>
<tr>
<td>Treated coeliac</td>
<td></td>
</tr>
<tr>
<td>19.34 (10.7 – 30.6)</td>
<td>5.6 (0.08 – 23)</td>
</tr>
<tr>
<td>Untreated coeliac</td>
<td></td>
</tr>
<tr>
<td>29.3 (13.5 – 66.6)</td>
<td>12 (0.5 – 37.6)</td>
</tr>
</tbody>
</table>

**Table 6.4**: Quantitative results for tTG surface area expressed as a percentage of area measured, lamina propria or epithelial, compiled using analysis software for healthy controls, treated and untreated coeliac patients.
Figure 6.13: The quantitative results for tissue transglutaminase expressed in the different mucosal compartments for HC (n=12), TCD (n= 5), and UCD (n= 10). A & B are scatter plot that represent data for tissue transglutaminase staining within the lamina propria (P value 0.007) and epithelial compartments (p value 0.0086) expressed as a percentage of total area measured, respectively. Means are denoted by black horizontal lines.
6.3.9 Cell surface tTG expression on myeloid DC and macrophages in intestinal tissue.

In the previous section we described the presence of positive tTG lamina propria cells, typically found under the basement membrane in active coeliac mucosa, illustrated again in figure 6.14.

![Figure 6.14](image_url): Tissue transglutaminase staining (red), illustrating the presence of tTG positive cells beneath the basement membrane (broken white line) in a paraffin section from an untreated coeliac patient.

Since we have also shown an increase in myeloid dendritic cells, expressing CD11c, in untreated coeliac mucosa and found increased numbers under the basement membrane, experiments were performed to determine if these CD11c cells co-express tTG. The monoclonal anti-tTG antibodies CUB7402 and TG100 were employed in these studies. We found no co-expression of CD11c and tTG in neither healthy nor coeliac mucosa (Figure 6.15). Further studies were then performed to see if CD68+ macrophages co-expressed tTG in the coeliac mucosa. In these studies a rabbit
polyclonal tTG antibody was employed, since it was not possible to combine the anti-tTG monoclonal antibodies (CUB7402 or TG100) with the mouse CD68 monoclonal antibody. In the studies, double immunofluorescence staining was carried out and co-localisation of CD68 and tTG was found most prominently located in the untreated coeliac mucosa beneath the basement membrane compared to healthy mucosa (Figure 6.16).
Figure 6.15: Double Immunofluorescence staining of a small intestinal lamina propria showing tTG (red) and CD11c (green) staining in paraffin section from a normal duodenal mucosa and an untreated coeliac mucosa. Original magnification x 40
Figure 6.16: Immunofluorescence staining of a small intestinal lamina propria from an untreated coeliac patient with CD68 (green) and tTG (red). Co-localisation appears orange yellow in merged image, located most notable beneath the basement membrane (bottom image). Original magnification x 400
6.3.10 Expression of smooth muscle α-actin in duodenal biopsies

The pronounced staining of tTG in the pericryptal area of the lamina propria in biopsies from patients with untreated celiac disease was often located in cells with a stellate, elongated morphology. It was postulated that these cells might be myofibroblasts and to help identify these cells, tissue sections were stained with monoclonal antibody to smooth muscle alpha actin (SMα-actin) in immunofluorescence studies (figure 6.17). In both healthy and coeliac subjects intense fluorescence staining was observed in the mucosa muscularis and around the crypt epithelium. Positive cells displayed a stellate, elongated morphology, typical of myofibroblasts.

IgA autoantibodies directed against alpha actin have been detected in the serum of patients with newly diagnosed coeliac disease and have been shown to correlated lesion severity (Achour et al., 2010; Granito et al., 2004; Pedreira et al., 2005; Porcelli et al., 2013). Therefore further studies were then performed to see if the level of expression of SMα-actin differed between healthy controls and subjects with coeliac disease. A quantitative analysis was performed employing Imaris imaging analysis software. The surface area of SMα-actin staining in the lamina propria was measured and the results were expressed as a percentage of total lamina propria area. A summary of the quantitative analysis data for SMα-actin is shown in figures 6.18 and table 6.3. The level of expression was similar in both healthy subjects and patients with coeliac disease treated and untreated.
Figure 6.17: Immunofluorescence staining for smooth muscle alpha actin (green) from a healthy control (left) and untreated coeliac (right). Objective x 400

Figure 6.18: The quantitative results for the surface expression of smooth muscle alpha actin expressed as a percentage of total lamina propria area, in sections from the duodenal mucosa of untreated (n=8) and treated coeliac (n=5) patients and controls (n=7).
<table>
<thead>
<tr>
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<th>Healthy Control</th>
<th>Treated Coeliac</th>
<th>Untreated Coeliac</th>
</tr>
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<td>(3.1 – 14.8)</td>
</tr>
</tbody>
</table>

Table 6.3: Summary of quantitative data for the surface area of smooth muscle alpha actin expressed as a percentage of total lamina propria area measured.

6.3.11 Co-expression of tissue transglutaminase with smooth muscle alpha actin in duodenal biopsies

Using tissue transglutaminase and smooth muscle alpha actin antibodies triple immunofluorescence studies were carried out, to further study the co-expression of these two molecules. Employing overlaid images acquired from confocal microscopy, it was demonstrated that most of the tissue transglutaminase expression found in close proximity to the crypts co-localised with smooth muscle alpha actin positive myofibroblasts. Co-localisation of the two antigens appears yellow in the merged images illustrated in figure 6.18. From macroscopic analysis, co-localisation appears to be most prominent in the untreated coeliac lesion.

Quantitative co-localisation analysis was then performed on all images employing Imaris imaging software and the results are summarised in figures 6.19. Prior to co-localisation analysis, a threshold was set for each immunofluorescence channel that eliminated background interference, thus eliminating any staining below the set threshold from analysis. From the quantitative analysis, it was demonstrated that a larger percentage of smooth muscle alpha actin above the set threshold co-localised with tissue transglutaminase in untreated coeliac mucosa (p value 0.0046) compared to healthy and treated subjects (Figure 6.19 b). In addition, the total surface area of co-localisation of tissue transglutaminase and smooth muscle alpha actin was determined for each image and expressed as a percentage of total lamina propria area measured. A slight, but insignificant increase in the area of co-expression was observed in untreated coeliac mucosa when compared to treated coeliac and healthy subject mucosa (Figure 6.19 a).
Figure 6.18: Tissue transglutaminase and smooth muscle alpha actin co-localisation. Three colour immunofluorescence staining for tTG (red), smooth muscle alpha actin (green) and Dapi (blue) in paraffin sections from a healthy control (left panel) and untreated coeliac (right panel). Co-localisation appears orange yellow in merged image (bottom row). Original magnification x 40.
Figure 6.19: Quantitative analysis of tissue transglutaminase and smooth muscle alpha actin co-localisation healthy controls (n=7), treated coeliac patients (n=5), and untreated coeliac patients (n=8) (A) Surface area of tTG and SMα actin co-localised expressed as a percentage of total lamina propria area. (B) Represents the percentage of SMα actin co-localised with tTG above the set threshold.

<table>
<thead>
<tr>
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<th>Healthy Control</th>
<th>Treated coeliac</th>
<th>Untreated coeliac</th>
</tr>
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<tbody>
<tr>
<td>Mean %</td>
<td>4.6</td>
<td>5.24</td>
<td>6.3</td>
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<tr>
<td>(Range %)</td>
<td>(1.4 – 8.3)</td>
<td>(3.5 – 8.2)</td>
<td>(2.0 – 9)</td>
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</tbody>
</table>

Table 6.5: Summary of quantitative data for tissue transglutaminase and smooth muscle alpha actin co-localised area expressed as a percentage of lamina propria area measured.
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<thead>
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<th>Healthy Control</th>
<th>Treated coeliac</th>
<th>Untreated coeliac</th>
</tr>
</thead>
<tbody>
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<td><strong>Mean %</strong></td>
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<td>35.1</td>
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<td><strong>(Range %)</strong></td>
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<td>(24-39.5)</td>
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**Table 6.6:** Summary of quantitative data for the percentage of total smooth muscle alpha actin above the threshold co-localised with tissue transglutaminase.
6.4 Discussion

From the experiments described in this chapter several important observations were made. First we noted the presence of tTG on monocyte derived DCs generated in vitro employing the monoclonal antibody TG100. In contrast, we found that mucosal DCs lacked tTG expression in both healthy and coeliac duodenal biopsies. However, tTG expression was found on few mucosal macrophages located in the duodenal lamina propria. In agreement with previous studies we confirmed that tTG was significantly upregulated within the coeliac lesion compared to healthy mucosa. We also found that tTG was detectable at much higher levels in the epithelial compartments in untreated duodenal sections. We found no difference in smooth muscle alpha actin mucosal expression between healthy and untreated coeliac patients. However, we did find that myofibroblasts, identified as smooth muscle alpha actin positive cells, expressed a significantly higher amount of tTG in the untreated coeliac lesion when compared to healthy controls.

Expression of tTG on monocyte derived DCs

It is well accepted that tissue transglutaminase plays a fundamental role in the pathogenesis of coeliac disease via the deamidation of gliadin peptides (Di Sabatino et al., 2012). However, it is not known where this deamidation occurs or what cells are involved. We have previously (Chapter 3) shown that the CD11c myeloid DC subset accumulates in the coeliac lesion and these cells have been shown to be capable of presenting the 33-mer gliadin peptide to gluten specific T cells (Räki et al., 2006). Here, we aimed to determine if myeloid DCs expressed tTG, which would suggest a role for surface tTG in gliadin uptake and its subsequent deamidation within the DC compartment. This would further contribute to our understanding of the pathophysiology of coeliac disease.

Initially, monocyte derived dendritic cells were used as an in-vitro model for the determination and characterisation of tTG expression by DCs. After 6 days in culture with GM-CSF and IL-4, successful generation of monocyte derived DCs was associated with loss of CD14 expression and change in cell morphology. Monocyte derived DCs had a distinct morphology that could be easily distinguished from their progenitor with a pronounced display of cytoplasmic projections along with the loss of the monocytic

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horseshoe nucleus. Monocyte derived DCs also displayed enhanced HLA-DR and accessory molecules essential for cell-mediated immunity. Both IL-4 and GM-CSF are necessary for the generation of moDCs; IL-4 inhibits monocytes from differentiating into macrophages (Jansen et al., 1989) while GM-CSF is the driving force in production of all myeloid cells (Mayani et al., 1993). The addition of LPS was able to induce further maturation of moDCs with the upregulation of antigen presenting molecules and activation markers. We also observed that IL-12(p70) secretion could be induced by LPS and that IL-10 production was concomitantly increased. Thus our results confirmed previous reports that circulating monocytes may represent a pool of precursor cells capable of rapid differentiation into DCs (Berger et al., 2002; Dauer et al., 2003; Jarnjak-Jankovic et al., 2007; Kvistborg et al., 2009; Romani et al., 1994). When we studied the differential expression of tTG by moDCs and their precursor the monocyte, we found that differentiation from a monocyte to its DC counterpart was associated with the appearance of cell surface tTG. Employing flow cytometry, the presence of surface tTG on moDC was only detectable with the monoclonal antibody TG100 while the remaining antibodies failed to detect tTG. These observations are in agreement with Hodrea and colleagues (2010) who also confirmed the presence of tTG on the surface of moDC using the monoclonal antibody TG100. Upon analysing cell surface tTG on freshly isolated monocytes we observed varying degrees of tTG expression in the presence of high background staining exhibited by the isotype control, thus a further approach was used to confirm our flow cytometry results. Therefore confocal microscopy was performed on fresh cytospin preparations and stained with the same range of antibodies. These studies showed that all antibodies bound non-specifically to monocytes while TG100 and Cub7402 detected tTG on moDCs. These findings were supported by our Westernblot results, were minimum or undetectable levels of tTG were found in whole protein lysates from freshly isolated monocytes when compared moDCs. This was verified by the real time PCR assay established, which showed moDCs to express significantly higher levels of relative tTG mRNA levels when compared to monocytes. Our immunoblot results are consistent with that of Hodrea and colleagues (2011), which showed undetectable levels of tTG protein in monocyte lysates. Thus when all our results were examined in combination it was concluded that monocytes do not express tTG but
upregulate the enzyme upon differentiation into moDCs in the presence of IL-4 and GM-CSF.

**tTG antigenicity depends on conformational status**

It is believed that the great difficulty in finding an antibody that recognises cell surface tTG is attributable to the different conformation states that the enzyme adopts on the cell surface. The molecule is known to exist in two distinctive conformations, “open” and “closed” (Martin Griffin et al., 2002). The open conformation is the calcium bound catalytically active transglutaminase in which the four domains are aligned. The closed conformation is the GTP/GDP bound molecule in which the active site is inaccessible and the beta 1 and beta 2 domains are folded over the core domain (Pinkas et al., 2007). Thus with conformational change epitopes are exposed that are not accessible in the closed conformation. Epitopes of most of the antibodies used in this study have been identified. The monoclonal antibody Cub7402 epitope falls between 447-478 amino acids located within the calcium binding domain and the first barrel (Sblattero et al., 2002). Similar to Cub7402, purified guinea pig tTG was used for the generation of TG100. Although, the epitope of the TG100 antibody is not precisely known, it is thought to be located between amino acids 447-538 located within the calcium domain and first barrel (According to manufacturer). Both monoclonal antibodies 2G3H8 and 5G7G6 were generated in BALB/c mice, and both identify different epitopes. 2G3H8 recognises an epitope between amino acid 314 and 329 within the catalytic region whereas 5G7G6 recognises a region between 548-558 within the first beta barrel. All of these antibodies identify epitopes that are dependent on the conformational status of the enzyme, thus depending whether tTG is in its extended or closed conformation on the cell surface these antibodies may not be able to recognise its corresponding epitope. The inability of Cub7402 to recognise cell surface tTG by flow cytometry is perplexing since both TG100 and Cub7402 epitopes are mapped to the same region and are both generated from guinea pig. Although the exact epitope for TG100 in a given region of 90 amino acids has not yet been identified, a more precise mapping of the epitope may help clarify the observations made in this study.

Considering both monoclonal antibodies 2G3H8 and 5G7G6 bound non-
specifically to monocytes and did not detect cell surface tTG on monocyte derived DCs, employing both flow cytometry and confocal microscopy, it was concluded that they were not suitable for neither application. These results were in contrast to observations made by Bayardo et al (2012) who found both monoclonal antibodies 2G3H8 and 5G7G6 were capable of recognizing surface tTG on stimulated THP-1 cells, a monocytic leukaemia cell line. The difference in results obtained may be reflected in the different cell types employed. We used freshly isolated monocytes while Bayardo opted for a leukemic cell line, which may have altered tTG expression, considering tTG has been shown to be increased in several cancer cells (Herman, Mangala and Mehta, 2006; Mangala et al., 2006; Mehta et al., 2010). Another probable reason may be due to the difference in methodology employed in each study.

Mucosal Dendritic cell tTG expression

To further establish whether surface tTG on DCs may possess a functional role in coeliac disease we next determined if dendritic cells in the gut expressed tTG. This study initially performed by Raki et al, (2007) detected cell surface tTG on mucosal CD11c+ DCs in both healthy and coeliac duodenal biopsies when employing monoclonal antibody 6B9. However, since publishing this data, it is now known that 6B9 does not recognise tTG but rather CD44 (Stamnaes et al., 2008) and their original observations have yet to be confirmed employing additional antibodies. Therefore, in attempt to investigate whether CD11c+ positive cells in the small intestine express tissue transglutaminase, immunofluorescence studies were carried out on duodenal tissue sections. Since both monoclonal antibodies TG100 and Cub7402 detected tTG on the presence of monocyte derived DCs in-vitro both antibodies were used. Antibodies 2G3H8 and 5G7G6 were also tested but non-interpretable results were obtained.

In contrast to our in-vitro findings that showed monocyte derived DCs to express cell surface tTG, CD11c+ cells identified in the mucosa did not co-express tTG in neither normal nor coeliac biopsies. These contrasting results may be due to the fact that dendritic cells found in the lamina propria are of different origin and do not arise from peripheral blood monocytes (Bogunovic, 2010). However, previous reports have suggested that dendritic cells in the lamina propria do originate from monocytes (Varol, 2007) but this remains to be elucidated in humans.

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When analysing the images acquired from the CD11c and tTG co-localisation studies, we noted cells located most prominently beneath the basement membrane, which were strongly positive for tTG. These cells are likely to be macrophages, as they are found in abundance in the duodenal mucosa and are known to express high levels of tTG when generated *ex-vivo* from monocytes in the presence of M-CSF. (Hodrea et al, 2010). Since the co-expression of tTG by intestinal macrophages has never been determined we investigated, employing immunofluorescence studies, whether macrophages co-expressed tTG in duodenal sections. In these studies, we have shown for the first time that mucosal macrophages do express tissue transglutaminase. These CD68 positive tTG cells were most evident under the sub-epithelial in untreated coeliac samples. The relevance of tTG to macrophage function, especially in the coeliac lesion, has yet to be established. Macrophages are highly phagocytic cells that are constantly engulfing antigenic particles and dying cells. Of note, macrophages from the TG2-/- mouse demonstrated impaired ability to phagocytose apoptotic cells (Toth et al., 2009). Thus the expression of tTG by macrophages in the coeliac lesion may reflect their increased phagocytic activity due the accumulation of apoptotic cells as a result of the inflammatory lesion.

**Quantification of tTG expression in intestinal biopsies**

The majority of studies that have evaluated tTG expression in the coeliac lesion have opted for qualitative or semi quantitative analysis employing immunohistochemistry techniques as opposed to immunofluorescence. Here, we used an immunofluorescence technique, which allowed the generation of high-resolution images for the quantification of the fluorescent signal using specialised image analysis software. Although this approach requires much more time for samples to be analysed, it minimizes subjectivity allowing more accurate interpretation of results even in small groups of samples.

We found a significant increase in tTG mucosal expression in samples from untreated coeliac patients in comparison to healthy controls. In healthy individuals high levels of tTG expression were detected in the muscularis mucosa while only low levels were observed in the lamina propria and epithelium. In contrast, we observed an increase in tTG expression in the lamina propria of untreated coeliac patients and similar
findings were described by others (Esposito et al., 2003; Hansson et al., 2002). Individual tTG positive cells were identified more frequently in the lamina propria of patients with active coeliac disease, most notable beneath the basement membrane. The over-expression of extracellular tTG may be explained by the translocation of tTG from the intracellular to the extracellular environment by cells in the lamina propria in response to the inflammatory response initiated by the influx of gliadin peptides. We also detected epithelial over-expression for tTG in the majority (7/10) of our untreated samples while our healthy controls exhibited no tTG expression within the crypt and surface epithelium. It is likely that the increased expression of tTG in untreated patients is due to the significant amount of IFN-γ secreted by gliadin specific T cell and TNF-α from activated macrophages and/or dendritic cells in the lamina propria. These two cytokines in combination have been shown to significantly induce tTG expression in intestinal biopsies and several cell lines tested ex-vivo, thus further magnifying the adaptive immune response to gliadin within the coeliac lesion (Bayardo et al., 2012).

The observations made in this study, are at variance with several previous studies. Employing a semi-quantitative approach to analyse mucosal tTG expression Sakly et al (2005) found that coeliac patients exhibited less epithelial tTG expression than controls. Maiuri et al (2005) did not detect any tTG expression within the epithelial compartments in either treated or untreated coeliac mucosa employing immunofluorescence. Finally, using immunohistochemistry Villanacci et al (2008) and Mercan et al (2003) found no difference in total tTG expression or distribution between healthy controls and coeliac patients. These conflicting findings are probably a consequence of methodological differences such as sample preparation, staining technique and sample cohort. Although tTG expression by quantitative immunofluorescence methods could be used in the evaluation of coeliac disease, this is unlikely to replace the present diagnostic tests. Moreover, further investigations are needed to clarify the role of this altered expression and distribution of tTG in the pathogenesis of coeliac disease.

Potential role of smooth muscle alpha actin auto-antibodies

The mechanisms that result in total villous atrophy and reorganisation of the mucosal architecture characteristic of the coeliac lesion are still very much unknown. In this respect, the potential involvement of the cytoskeleton has been suggested by
several studies. It has been shown that gliadin can induce rapid actin rearrangement and polymerization in coeliac patient biopsy tissue (Reinke et al., 2010) and disrupts the micro villous structure with disorganisation of the actin network (Myrsky et al., 2008; Sander et al., 2005). Interestingly, the detection of IgA smooth muscle autoantibodies in the serum of coeliac patients may also target actin the major component of the cytoskeleton. Several groups (Achour et al., 2010; Granito et al., 2004; Pedreira et al., 2005; Porcelli et al., 2013) including ours (manuscript preparation) have demonstrated that the presence of these antibodies are more frequent in the serum of patients who present with total villous atrophy compared to patients with subtotal or mild villous atrophy. A possible reason for the appearance of these auto-antibodies could be the generation of neo-epitopes exposed in polymerized F actin filaments that are usually hidden in monomer G actin (Granito et al., 2004).

Actin has 6 isoforms, all of which are highly conserved, and participate in many cellular processes including muscle contraction, cell motility, cell division, cell shape, cell signalling and the formation of stress fibers (Perrin et al., 2010; Khaitlina et al., 2001; Schoenenberger et al., 2011). Four isoforms i.e. alpha smooth muscle actin, gamma-smooth muscle alpha actin, alpha-skeletal muscle actin, and alpha-cardiac actin are tissue restricted, whereas beta and gamma actin are expressed ubiquitously in all cells (Perrin et al., 2010). Myofibroblasts express cytoplasmic β-actin and γ-actin and α-smooth muscle alpha actin isoforms (Serini et al., 1996).

Smooth muscle alpha actin is highly expressed by myofibroblasts in the mucosal surfaces. This cell is fundamental in the maintaining the structural framework of the mucosal architecture (Lahar et al., 2011). We considered that a change in the expression of this antigen in the gut might be responsible for the generation of auto antibodies directed against actin. However, quantitative immunofluorescence analysis showed no significant difference in smooth muscle alpha actin expression in untreated coeliac and treated coeliac patients compared to healthy controls. These findings did not support the hypothesis that the raised IgA antibody response to smooth muscle antigen is induced by its increased mucosal expression. Interestingly, interferon gamma, the Th1 proinflammatory cytokine, which is over-expressed in the coeliac lesion (Lionetti & Catassi, 2011) has been reported to moderately inhibit the production of α-smooth
muscle alpha actin in myofibroblasts (Tanaka, 2003). However, this does not explain our findings in which no difference in smooth muscle alpha actin expression in the untreated coeliac lesion and healthy mucosa was detected. The only increase in smooth muscle alpha-actin expression was observed in the mucosa from treated coeliac patients. It is known that myofibroblasts transiently upregulate smooth muscle alpha actin expression during the healing process (Darby et al., 1990) thus, this increased expression may reflect the role of myofibroblasts in the healing of the mucosal lesion.

**Interaction between tissue transglutaminase/ smooth muscle alpha actin**

From our image analysis of tTG, we found the enzyme highly expressed around crypts in which cells displayed an elongated, stellate cell morphology similar to myofibroblasts. Since actin has been shown to be a good substrate of tissue transglutaminase (Akimov et al., 2000; Nemis et al., 1997), we investigated the relationship between tTG and smooth muscle alpha actin in the coeliac mucosa compared to healthy controls. When the expression of tTG was examined in the myofibroblast, identified as a smooth muscle alpha actin positive cells, we found the myofibroblast to express significantly higher levels of tTG in untreated samples compared to treated and healthy duodenal mucosa. The significance of this finding is not yet known. However, the increased co-expression of the two antigens may be of relevance to the presence of alpha actin auto antibodies in the serum of untreated patients. tTG may catalyse the crosslinking of actin generating novel immunogenic epitopes which are then exposed to the mucosal immune system, triggering the production of IgA auto-antibodies directed against actin filaments.

The over expression of tTG by the myofibroblast may also be a sign of cell apoptosis (Autuori et al., 1998) or cell stress (Lentile et al., 2007) in response to the inflammatory milieu characteristic of the coeliac lesion and indicating a loss of cell function. Myofibroblasts are essential for crypt development and preservation. Crypts harvest stem cells for epithelial differentiation and villi maintenance (Verbeke et al., 2002). Thus the impaired function of the myofibroblast could disrupt this intricate network contributing to loss of villi structure and villous atrophy commonly seen in untreated coeliac patients. It has been proposed and preliminary experiments have shown that tTG autoantibodies are important in the progression of the coeliac lesion by

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interacting and interfering with extracellular or cell membrane bound tTG (Halttunen & Mäki, 1999). In this proposed scenario IgA tTG autoantibodies bind to tTG overexpressed on the surface of myofibroblasts disrupting the mesenchymal-epithelial cross talk, inhibiting effective stem cell differentiation and epithelial cell renewal. This results in villous atrophy (Halttunen & Mäki, 1999). It has also been shown that IgA tTG antibodies can inhibit fibroblast-induced TGF-beta mediated differentiation while inducing the proliferation of crypt like epithelial cells (Halttunen & Mäki, 1999). This was demonstrated employing a three-dimensional fibroblast epithelial cell co-culture assay that mimicked the *in-vivo* crypt-villous axis. The inhibition of fibroblast differentiation was mediated via the blocking of tTG activity, which in turn prevented the generation of the active form of TGF-beta. The presence of anti-tTG IgA deposits beneath the basement membrane and along the crypts and mucosal vessels in patients with coeliac disease support this proposed mechanism (Kaukinen et al., 2005). The potential role of tTG antibodies is further supported by their ability to display partial and dose-dependent inhibitory effects on the transamidating activity of human tTG (Byrne et al., 2007).

### 6.5 Conclusion

Taken together, the data reveal that monocyte derived dendritic cells express surface tTG whereas mucosal myeloid dendritic cells exhibit no such expression. These conflicting results reinforce the idea that these cells are two separate entities with different functional capacities. Thus, we are unable to conclude whether surface tTG on monocyte derived dendritic cells plays a functional role in the pathogenesis of coeliac disease. Moreover, we found tTG overexpression in the lamina propria, intestinal epithelium and different mucosal cells are a characteristic of coeliac disease. However, further investigations are necessary to establish how far an altered expression and distribution of tTG contributes to CD pathogenesis.
Chapter 7 Final Discussion

The established celiac lesion exhibits a complex interplay of inflammatory changes, and morphological features including increased numbers of intraepithelial lymphocytes (IELs), decreased enterocyte height, villous atrophy, and crypt hyperplasia (Tack et al., 2010). The aim of this study was to examine the features of the coeliac lesion and the role of antigen presenting cells and the significance of tTG and CD163 in the coeliac lesion.

The role of the antigen-presenting cell in the coeliac lesion

Under homeostatic conditions mucosal dendritic cells and macrophages exist in a quiescent steady state maintaining tolerance to dietary antigen (du Pré & Samsom, 2011) (Figure 7.1 (1)). However, in an activated state during coeliac disease, possibly triggered by a co-infection (Tack et al., 2010) or other innate activation signals (Gianfrani et al., 2005; Tucková et al., 2002), antigen presenting cells activate gluten specific Th1 cells, initiating pathogenesis (Figure 7.1 (2)). Our findings, in agreement with previous studies, demonstrate that the myeloid CD11c+ dendritic cell is most likely to play a role in the activation and maintenance of gluten reactive T cells as demonstrated by the accumulation of DCs in the coeliac lesion predominantly located beneath the basement membrane (Figure 7.1 (4)). In support of this, Raki et al found that freshly isolated CD11c+ cells from the untreated coeliac lesion were the most efficient APC in presenting gliadin to gluten specific T cells (Ráki et al., 2006).

In contrast to the accumulation of DC, no change in the expression of the classical macrophage marker CD68 was found in the coeliac lesion compared to healthy duodenal mucosa. On investigating the frequency of antigen presenting cells in the peripheral blood of untreated coeliac patients we found a significant decrease in the myeloid DC subset while the monocytes subsets remained unchanged. This finding is of interest considering that myeloid DCs were elevated in the coeliac lesion, suggesting a migration of blood myeloid DCs to the mucosa in response to inflammatory mediators (Figure 7.1 (3)).

CD4+ T cells activated by gliadin peptides secrete potent amounts of Th1 cytokines, mainly IFN-gamma (I. Monteleone et al., 2004), which coordinates a series of
immunological events that result in mucosal remodeling and villous atrophy (Przemioslo et al., 1995). IFN-γ orchestrates the trafficking of specific immune cells to sites of inflammation through up-regulating expression of adhesion molecules and chemokines (Schroder et al., 2004). The increased expression of CCR6 and CD83 observed in this study on myeloid dendritic cells in the peripheral blood of untreated coeliac patients may be in response to inflammatory mediators, such as IFN-γ, derived from the coeliac lesion. CCR6 and its ligand CCL20 are responsible for the chemoattraction and migration of immature dendritic cells during mucosal immune responses (Ito et al., 2011). CCR6 positive dendritic cells may migrate to the coeliac lesion where they encounter gliadin which in turn promotes the down regulation of CCR6 and upregulation of CCR7. CCR7 is essential for DC homing to the lymph nodes where they subsequently prime gliadin reactive T cells (Hubo et al., 2013). Thus the increase in CCR6 expression on DC may explain our previous observation of a decreased frequency in peripheral blood myeloid dendritic cells along with the subsequent accumulation of CD11c positive cells in the coeliac lesion.

Additional flow cytometry and tissue staining experiments would help establish whether tissue dendritic cells originate from the peripheral blood. Considering a large proportion of monoclonal antibodies are not suited for paraffin embedded sections, fresh frozen tissue would have to be acquired for any additional in-situ staining experiments conducted in the future.

Tissue Transglutaminase expression on antigen presenting cells

Tissue transglutaminase, the main autoantigen of coeliac disease, plays a vital role in coeliac disease pathogenesis. tTG deamidates gliadin peptides enhancing their immunogenicity and recognition by the immune system (Klöck et al., 2012). However, very little is known about the location of this deamidation and the potential cells, if any, involved in this process. It has been proposed that dendritic cells in the duodenal mucosa may recognise and uptake gliadin peptides via cell surface tTG (Hodrea et al., 2010; M Ráki et al., 2007). This would subsequently promote their deamidation intracellularly within the lysosome and presentation to gliadin reactive T cells. Furthermore, the subsequent internalisation and transport of gliadin complexed with tTG to the endocytic compartment of the cell would promote deamidation of gliadin peptides.
peptides rather than transamidation due to its acidic environment. However, the results obtained in this study did not support this hypothesis as CD11c+ cells in the coeliac lesion and healthy mucosa did not co express tTG. This observation was in contrast to our in-vitro findings, which found surface tTG expressed on monocyte derived dendritic cells. The conflict in these results may be explained by the possibility that mucosal dendritic cells are not of monocyte origin, a phenomenon that has not yet been proven in humans (Andersson et al., 2012; Collin et al., 2011; Domínguez & Ardavin, 2010; Varol et al., 2009). Thus, at this juncture we are unable to state that monocyte derived dendritic cells play a functional role in the pathogenesis of coeliac disease.

Interestingly, we identified mucosal macrophages expressing tTG in the coeliac lesion predominantly located beneath the basement membrane, a phenotypic change most likely induced by IFN-gamma, a cytokine known to influence macrophage function (Figure 7.1 (8)) (Schroder et al., 2004). IFN-gamma alone and in combination with TNF-alpha has been shown to significantly induce tTG expression in intestinal biopsies cultured ex-vivo (Bayardo et al., 2012). The potential importance of tTG expression by mucosal macrophages in the coeliac lesion is not known. Cell surface tTG on mucosal macrophages may act as a receptor for gliadin peptides thus promoting the antigen presenting function of the macrophage, a process that is normally inactive in a steady state (Mosser & Edwards, 2008; Murray & Wynn, 2011). Therefore, the direct uptake of gliadin by surface tTG expressed on the macrophage may promote presentation of gliadin to gluten reactive T cells, helping to maintain the inflammatory lesion. It is not known why all mucosal macrophages in the coeliac lesion do not express tissue transglutaminase but we propose it may relate to the close proximity of the macrophage to IFN-gamma producing intraepithelial lymphocytes (Olaussen et al., 2002).

Further in-situ co-localisation studies employing antibodies for CD68 and gliadin, paralleled with in-vitro experiments aimed at investigating the role of surface tTG on the macrophage would help establish whether surface tTG plays a functional role in the pathogenesis of coeliac disease.

The characterisation of CD163 in the inflammatory lesion
An additional phenotypic change observed in the untreated coeliac mucosa was the loss

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of cell surface CD163 by the mucosal macrophage (Figure 7.1 (5)). We found a significant decrease in mucosal CD163 expression in the untreated coeliac lesion along with a decrease in the co-localisation of CD163 and CD68 surface area. CD163 has been previously used in the differentiation between the M1, classical activated macrophage and the M2, alternatively activated subset, which display pro-inflammatory and anti-inflammatory phenotypes, respectively (Buechler et al., 2000; J. A. Harris et al., 2012). The M1 macrophage subset is generated in response to microbial products or IFN-γ and lacks CD163 expression (Mosser & Edwards, 2008). In contrast, M2 macrophages produce low amounts of IL-12 and IL-23 and high levels of IL-10 and transforming growth factor beta (TGF-β) and expresses high levels of CD163 (Mosser & Edwards, 2008). Thus, our results reveal a skew in the macrophage phenotype in the coeliac lesion, from a M2 phenotype to the M1 phenotype. These two subsets are interchangeable depending on their microenvironment (Bain & Mowat, 2011), thus it is not surprising that the M1 subset is more prominent in the coeliac lesion. High levels of IFN-gamma are characteristic of the coeliac lesion (Nilsen et al., 1998), a cytokine known to support the generation of the classically activated M1 subset (Sica & Mantovani, 2012). This subset is maintained by the Th1 proinflammatory milieu and in return contributes to IFN-gamma and TNF-alpha production thus helping to maintain the inflammatory cascade within the coeliac lesion. Another potential role for M1 macrophages in the pathogenesis of coeliac disease, is their ability to recruit Th17 cells to the coeliac lesion, further exacerbating the immune response. M1 macrophages produce IL-6 and IL-23 cytokines (Mosser & Edwards, 2008), which can give rise to Th17 cells in the presence of TGF-beta. Th17 cells have been implicated in coeliac disease (Castellanos-Rubio et al., 2009; Lahdenperä et al., 2012) and are now known to play a pathogenic role in an array of autoimmune diseases (Jäger & Kuchroo, 2010; Kim et al., 2013).

The decrease in CD163 co-expression on CD68 positive macrophages in the coeliac lesion is probably a consequence of the increased rate of CD163 shedding from the mucosal macrophage cell surface. When shed from the cell surface CD163 exists in its soluble form (Krzysztof Kowal et al., 2011) and is found significantly increased in the serum of adults and children newly diagnosed with coeliac disease as exhibited in this study (Figure 7.1.6). Therefore, our results suggest that mucosal macrophages are the
main cell source that contributes to elevated soluble CD163 found in the serum of untreated coeliac patients. Furthermore, upon analysing CD163 expression of peripheral blood monocytes in untreated coeliac patients we found an inverse relationship between surface CD163 and soluble CD163. This indicates the loss of CD163 from the monocyte cell surface, which further contributes to elevated serum soluble CD163 found in untreated coeliac patients (Figure 7.1.7). The loss of CD163 from the surface of the monocyte may also represent monocyte activation in the peripheral blood.

The role of soluble CD163 in the coeliac lesion is not known. It may simply represent a biomarker of in-vivo macrophage activation (H J Møller et al., 2002) or may possess functional properties that are capable of modulating the immune response (Högger & Sorg, 2001). However, our limited functional experiments used were unable to show any immune modulating effects of recombinant CD163.

Future work will require further optimisation of our soluble CD163 functional assay employing purified soluble CD163 as opposed to recombinant CD163. Preliminary work has been carried out in the lab, with the aim of purifying soluble CD163. Briefly, monocytes were cultured in the presence of dexamethasone to upregulate surface CD163 expression, followed by a PMA stimulation to induce CD163 shedding from the cell surface. Thus depending on the concentration of soluble CD163 present in the supernatant, affinity chromatography could be utilised to purify the molecule. The successful isolation of soluble CD163 would allow us to further investigate the modulating effects of this molecule on the adaptive immune response.

**Increased mucosal tissue transglutaminase expression**

In addition to mucosal macrophages we found that myofibroblasts, identified by their expression of smooth muscle alpha actin, expressed significantly higher levels of tTG in untreated coeliac disease biopsies (Figure 7.1.9) when compared to treated and healthy duodenal mucosa. The significance of this finding is not yet known. However, the co-expression of these two antigens (tissue transglutaminase and smooth muscle alpha actin) may explain the finding of alpha actin autoantibodies in the serum of untreated coeliac patients (Achour et al., 2010; Granito et al., 2004; Pedreira et al., 2005). Considering that actin is a suitable substrate of tTG (Griffin et al., 2002; Nemes Jr., 1997),
tTG may catalyse the crosslinking of actin molecules generating novel immunogenic epitopes, which are subsequently exposed to the mucosal immune system. This could trigger the production of IgA autoantibodies directed against actin filaments.

Myofibroblasts are essential for crypt development and preservation (Hinz et al., 2007; Mifflin et al., 2011; Powell et al., 1999). Thus the altered behavior of myofibroblasts, reflected by tTG overexpression, could disrupt this intricate network and contribute to the remodelling of the mucosal architecture seen in untreated coeliac patients.

Coeliac disease is characterised by IgA tTG autoantibodies. However, whether antibodies to tTG are simply a biomarker of the disease or are of pathogenic importance has yet to be established. It has been proposed that antibodies to tTG may contribute to the pathogenesis of villous atrophy by interacting with cell surface tTG (C Esposito et al., 2002). One proposed mechanism that may be of relevance to the presence of the increased co-expression of tTG and smooth muscle alpha actin is the interaction of tTG IgA autoantibodies to cells with membrane bound tTG. In this proposed scenario, IgA tTG autoantibodies could bind to tTG overexpressed on the surface of myofibroblasts, disrupt the mesenchymal-epithelial cross talk and inhibit effective stem cell differentiation and epithelial cell renewal. This results in villous atrophy (Halttunen & Mäki, 1999). It has also been shown that IgA tTG antibodies can inhibit fibroblast-induced TGF-beta mediated differentiation while inducing the proliferation of crypt like epithelial cells (Halttunen & Mäki, 1999).

In conclusion, we have identified several unique changes in the antigen presenting cell population present in the coeliac lesion. These findings offer a reasonable mechanism in which promote the initiation and accumulation of activated gliadin specific T cells found in the coeliac lesion. Furthermore, these changes help sustain the Th1 inflammatory response characteristic of the untreated coeliac lesion, highlighting the importance of antigen presenting cells in regulating the adaptive immune response.
Peripheral Blood

Lumen

Normal Duodenal mucosa

Villous atrophy

1. Villi
2. Gliadin
3. Accumulation of CD11c+ myeloid dendritic cells in the coeliac lesion
4. Upregulation of tTG by mucosal macrophages
5. Shedding of CD163 from the surface of mucosal macrophages and release into the circulation
6. Production of tTG
7. Homing of myeloid dendritic cells via CCR6 to the coeliac lesion
8. Gliadin
9. tTG autoantibodies bind to surface tTG on the myofibroblast, interfering with the cells function

Duodenal Mucosa

- Gliadin
- tTG
- Soluble interferon gamma
- Deamidated gliadin
- CD68+CD163+ mucosal macrophages (M2)
- Mucosal CD11c+ Myeloid DC
- tTG expressing macrophages
- CD68+CD163+ myofibroblast
- CD163+ CD11c+ peripheral blood myeloid DC
- CD163+ CD11c monocyte
- IgA tTG antibodies
- tTG expressing macrophages
- Crypt

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Figure 7.1: (1) Under homeostatic conditions mucosal dendritic cells and macrophages exist in a quiescent steady state maintaining tolerance to dietary antigen. (2) Ingested wheat is only partially digested. When the gliadin peptides enter the lamina propria, tissue transglutaminase (tTG) deamidates glutamine residues. Negative charges in gluten peptides, as well as the presence of proline residues at specific positions, facilitate their binding into the peptide pocket of HLA-DQ2 (or -DQ8) expressed by antigen-presenting cells (APCs; most likely the CD11c + myeloid dendritic cell. Gluten presentation promotes the activation of a gliadin-specific T helper 1 CD4+ response in the intestinal lamina propria, which is dominated by Interferon IFN-gamma. Interferon gamma co-ordinates a diverse array of immunological events that participate in the induction of mucosal damage that eventual result in villous atrophy. (3) IFN-gamma along with other pro-inflammatory mediators may be responsible for the migration of peripheral blood myeloid DCs into the lamina propria where they are found accumulated beneath the basement membrane and lamina propria in the untreated coeliac lesion. The increase in the number of mucosal dendritic cells magnifies the inflammatory cascade by priming and activating additional gliadin specific T cells. This results in the sustained production of IFN gamma by TH 1 cells, which gives rise to the classically activated M1 cell. The classically activated M1 macrophage possess enhanced microbicidal capacity, secrete high levels of pro-inflammatory cytokines, reactive nitrogen and oxygen intermediates and help sustain a TH1 response. (5) The shift in phenotype from a M2 to a M1 phenotype is accompanied by the shedding of CD163 from the macrophage cell surface generating soluble CD163. (6) The increase in the production of soluble CD163 over spills into the peripheral blood where higher levels are found in the serum of untreated coeliac patients. (7) In the peripheral blood, monocyte activation is also evident from the shedding of CD163 from the monocyte cell surface further contributing to the elevated levels of soluble CD163. Tissue transglutaminase is found over expressed in the coeliac lesion and in different cell types. (8) Macrophages located beneath the basement membrane over express tTG in the untreated coeliac lesion. (9) Pericryptal myofibroblasts surrounding the crypts were also shown to over express tTG. It is likely that IFN-gamma is responsible for the over expression of tTG by these cells. The significance of the over expression of tTG by these cells in not known. One proposed scenario is the binding of IgA tTG autoantibodies to tTG overexpressed on the surface of myofibroblasts, disrupt the mesenchymal-epithelial cross talk and the inhibition of effective stem cell differentiation and epithelial cell renewal resulting in villous atrophy. Thus, even though anti-TG antibodies are not the principal cause of celiac disease, they may indirectly contribute to tissue damage.
Chapter 8  References


Arentz-Hansen, H., Körner, R., Molberg, O., Quarsten, H., Vader, W., Kooy, Y. M., ... McAdam, S. N. (2000). The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a


Chirumbolo, S. (2012). State-of-the-art review about basophil research in immunology and allergy: is the time right to treat these cells with the respect they deserve? Blood Transfusion = Trasfusione Del Sangue, 10(2), 148-64. doi:10.2450/2011.0020-11


Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nature Immunology, 12(2), 151–9. doi:10.1038/ni.1981


Matrix metalloproteinase pattern in celiac duodenal mucosa. Laboratory Investigation; a Journal of Technical Methods and Pathology, 85(3), 397–407. doi:10.1038/labinvest.3700225

Matrix metalloproteinase pattern in celiac duodenal mucosa. Laboratory Investigation; a Journal of Technical Methods and Pathology, 85(3), 397–407. doi:10.1038/labinvest.3700225

Reduced number and function of peripheral dendritic cells in coeliac disease. Clinical & Experimental Immunology, 487–496. doi:10.1111/j.1365-2249.2007.03431.x


Human dendritic cell deficiency: the missing ID? Nature Reviews. Immunology, 11(9), 575–83. doi:10.1038/nri3046

Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. Journal of Leukocyte Biology, 82(6), 1365-74. doi:10.1189/jlb.0307166

A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. The Journal of Experimental Medicine, 204(8), 1757-64. doi:10.1084/jem.20070590


Unregulated IL-23/IL-17 immune response in autoimmune diseases. Diabetes Research and Clinical Practice, 88(3), 222–6. doi:10.1016/j.diabres.2010.03.014


Number of pericytial fibroblasts correlates with density of distinct mast cell phenotypes in the crypt lamina propria of human duodenum: implications for the homeostasis of villous architecture. The Anatomical Record. Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology,


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Esposito, C., Paparo, F., Caputo, I., Rossi, M., Maglio, M., Shlattero, D., ... Troncone, R. (2002). Anti-tissue transglutaminase antibodies from coeliac patients inhibit transglutaminase activity both in vitro and in situ. *Gut, 51*(2), 177–181. doi:10.1136/gut.51.2.177


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Nature Genetics, 40(4), 395-402. doi:10.1038/ng.102


Jensen, P. E. (2007). Recent advances in antigen processing and presentation. Nature Immunology, 8(10), 1041-8. doi:10.1038/ni1516


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John, S., Thiebach, L., Frie, C., Mokkapati, S., Bochtel, M., Nischt, R., ... Smyth, N. (2012). Epidermal transglutaminase (TGase 3) is required for proper hair development, but not the formation of the epidermal barrier. PloS One, 7(4), e34252. doi:10.1371/journal.pone.0034252


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Telci, D., Collighan, R. J., Basaga, H., & Griffin, M. (2009). Increased TG2 expression can result in induction of transforming growth factor beta1, causing increased synthesis and deposition of matrix proteins, which can be regulated by nitric oxide. *The Journal of Biological Chemistry, 284*(43), 29547–58. doi:10.1074/jbc.M109.041806


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