THE ROLE OF HBD-2 AND HBD-3
IN HUMAN T CELL DEVELOPMENT

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Abstract

Human β-defensins (hBDs) are a family of cationic peptides able to directly kill a wide range of microorganisms including bacteria, fungi and viruses. In addition to their antimicrobial activities, defensins also contribute to the modulation of both the host innate and adaptive immunity. In this project, we demonstrate that the αCD3/28 co-stimulation of human CD4\(^+\) T cells in the presence of 10µg/ml hBD-2 or hBD-3 together causes an up-regulation in numbers of CD4\(^-\)CD69\(^+\)CD25\(^+\) and CD4\(^-\)CD69\(^-\)CD25\(^+\) T cell subsets, indicating that the treatment of hBD-2 and 3 enhances CD4\(^+\) T cell activation. Consistent with this finding, proliferation assay using CFSE suggests that hBD-2 and hBD-3 treatment in vitro induces the proliferation of CD4\(^+\) T cells following by 96hrs culture. Analysis of expression of the regulatory T cells (Tregs) specific marker, FoxP3, reveals a shift in the CD4\(^-\)CD127\(^-\)CD25\(^+\) Treg subset at 18hrs. However, at the later time point, we found that the percentage of FoxP3\(^+\)cells decreased in the CD4\(^+\)CD127\(^-\)CD25\(^+\) Treg population, whereas the presence of the FoxP3\(^+\)CTLA-4\(^+\) Treg subset increased. These data indicate that Treg suppressive function may be potentially defective following the co-incubation of purified T cells with either hBD-2 or hBD-3 for 42hrs in vitro due to the apparent loss of FoxP3 expression. We further characterise the role of hBD-2 and hBD-3 in driving human CD4\(^+\) T cells polarisation. Our in vitro data suggests that treatment with hBD-2 and hBD-3 can not only induces effector T cell (Teff) differentiation into RORγt\(^+\)T-bet\(^+\) (Th17/Th1) cells, but can also trigger the differentiation of Treg expressing RORγt and T-bet rather than the master controller of Treg function, FoxP3. This apparent plasticity of T cell phenotype allows them to convert from Treg to Th1/17-like effector T cell phenotype following 18hrs in culture. By 42hrs in culture, treatment with hBD-2 and hBD-3 induced both Teff cell and Treg cell differentiation towards the Th17-like phenotype. Compared with the
treatment with hBD-2, treatment with hBD-3 induced a more pronounced effect to increase levels of RORγt in CD4⁺ T cells. This elevated expression may, in turn, be responsible for the induction of higher IL-17A secretion. Consistent with this idea, it was found that treatment with hBD-3 but not hBD-2 was capable of inducing the higher level of secretion of IL-17A. Additionally, treatment with hBD-3 induced an increased expression of IL-6, which is capable of driving the differentiation of naïve T cells towards IL-17-producing Th17 cells. Functionally, using the Treg suppression assay, the data suggested that hBD-2 may dampen down Treg cell ability to induce suppression of Teff cell activity. Interestingly, co-culture with hBD-2 would also appear to increase Teff cell resistance to Treg immunoregulation in vitro. Further investigation using microarray gene analysis revealed chemokine C-C motif ligand 1 (CCL1) as potential genes responding to hBD-2 treatment. The blockade of CCL1 has been reported to inhibit Treg suppressive function. Thus, this study explored the function of these antimicrobial candidates in regulating CD4⁺ T cell plasticity which could result in hBD-2 and hBD-3 being able to regulate its own production, but also may regulate Treg and Teff cell development and function, thus strengthening the link between innate and adaptive immunity.
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Abbreviations

AMPs  antimicrobial peptides
APCs  antigen presenting cells
CCL20  CC chemokine ligand 20
CCR6  CC chemokine receptor 6
CD  Crohn’s Disease
CD3  cluster of differentiation 3
CFSE  carboxyfluorescein diacetate succinimidyl ester
CTLA-4  cytotoxic T-lymphocyte antigen 4
DCs  dendritic cells
EAE  Experimental autoimmune encephalomyelitis
ELISA  enzyme-linked immunosorbent assay
FACS  fluorescence-activated cell sorting
FoxP3  forkhead box transcription factor 3
hBDs  human β-defensins
IFN-γ  interferon γ
IL  interleukin
mBDs  murine β-defensins
PBMCs  peripheral blood mononuclear cells
RORγt  Retinoic acid (RA)–related orphan receptor γt
T-bet  T-box transcription factor
TCR  T cell receptor
Teffs  effector T cells
TGF-β  transforming growth factor beta
Th17  type 17 T helper cells
TLR  Toll-like receptor
Tregs  regulatory T cells
UC  ulcerative colitis
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Chapter 1: Introduction

1.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) play a crucial role in the innate immune system to protect the host from microbial infection (Wang, 2014). The majority of mammal AMPs have been identified in a variety of epithelial surfaces that directly interface with the environment such as skin, respiratory tract, intestine and reproductive tract (Mukherjee and Hooper, 2015). In human, the key AMP families are cathelicidins, such as LL-37, REG3 lectins and defensins (Kahlenberg and Kaplan, 2013, Mukherjee and Hooper, 2015).

Defensins form one family of AMPs which are a large group of 10 peptides in humans and 14 in mice (Muniz et al., 2012, Ouellette, 2005, Yamaguchi and Ouchi, 2012). These peptides are small proteins ranging in size from 2–4 kDa and they are all cationic peptides capable of directly responding to, and destroying, a wide range of microbial infections, such as bacterial, fungal and viral infections (Mukherjee and Hooper, 2015).

The mammalian defensins constructed a multi-gene family which consist of different types of orthologs which are identifiable by their cysteine spacing, intramolecular disulphide bonds and genomic organization (Semple and Dorin, 2012). The defensin family comprises three sub-families, α-defensins, β-defensins and θ-defensins (Jarczak et al., 2013).

Generally, β-defensins have 6-cysteine motif and the molecular structure is constructed by disulphide bonds between Cys I-V, Cys II-IV and Cys III-VI, forming the typical β-sheet structure of β-defensins (Wang, 2014, Semple and Dorin, 2012). In the human genome, computational analysis has shown that 11 β-defensin genes locate as a cluster
on chromosome 8p23.1 and the other β-defensin genes were found in clusters on chromosome 20p13, 20q11.1 and 6p12 (Schutte et al., 2002). The human β-defensin 2 gene (hBD-2, DEFB2) is located in the p22–23 region of chromosome 8 which is close to the hBD-1 gene (DEFB1). This indicates that hBD-1 and hBD-2 genes were created by duplication of a single parental gene (Bals et al., 1999). The hBD-3 gene (DEFB3) is located close to DEFB2 gene, which is only about 13 kb from the DEFB-2 gene (Lehrer and Ganz, 2002).

Most of β-defensin genes comprise of two exons. The exon 1 encodes the leucine-rich signal peptide, and the exon 2 encodes the mature peptide (Semple and Dorin, 2012). According to the sequence analysis of the murine β-defensin (mBD) gene cluster on chromosome 8, it was found that both of the DNA and protein sequence of the signal peptide encoded by exon 1 is highly conserved amongst mBD genes (Semple and Dorin, 2012). In contrast to the signal peptide, the mature peptide encoded by exon 2 is not as well conserved as the signal peptide, leading to the diversity of β-defensin sequences which has developed as a response to selective pressure by a range of pathogens (Semple and Dorin, 2012, Jarczak et al., 2013). The molecular diversity can also reflect the different antimicrobial activity and tissue distribution of β-defensins, which suggests that β-defensins can provide the host with a range of different responses to a variety of pathogens and also may modulate different immune functions (Jarczak et al., 2013).

In recent years, the biological functions of β-defensins have been not only restricted in their antimicrobial activities but also extended to immunomodulation and their involvement in other processes, such as cancer and wound healing. In this study, we will focus on the role of hBD-2 and hBD-3 in human CD4\(^+\) T cell polarization.
1.2 Multifunctional role of Human β-defensins in immune system

1.2.1 Antimicrobial activity of hBDs

hBD-2 was originally purified from psoriatic skin lesions using *Escherichia coli* affinity column (Harder et al., 1997). The antimicrobial activity of hBD-2 is mainly against Gram-negative bacteria, yeasts, and HIV but not against HSV (Winter and Wenghoefer, 2012, Ganz, 2003). Like hBD-1, the antimicrobial activity of hBD-2 is inhibited by high concentrations of NaCl (20mM to 150 mM) (Bals et al., 1998), although the concentration of both salt and the peptides can influence its antimicrobial activity (Jarczak et al., 2013). Unlike hBD-2, hBD-3 is effective in killing Gram-positive vancomycin-resistant *Enterococcus faecium* and *Staphylococcus aureus* as well as the yeast *Candida albicans* at physiological salt concentrations (Harder et al., 2001). These findings may indicate that the antimicrobial activity of defensins could be regulated by salt concentrations under different infectious or inflammatory conditions.
In contrast to hBD-1 which is constitutively expressed by epithelial cells, the expression of hBD-2 and hBD-3 is triggered during infections or following various inflammatory stimuli, such as activation of IL-1β (Ramasundara et al., 2009, Doss et al., 2010). The expression of the hBDs in epithelial cells is mediated by Nucleotide-binding Oligomerization Domain-containing Proteins (NODs) as a receptor recognizing intracellular bacterial that, in turn, activates Nuclear Factor (NF)-κB to trigger gene expression of β-defensins (Voss et al., 2006). Interestingly, it was found that up-regulation of hBD-2 expression was induced through a NOD1-dependent signalling pathway, while the expression of hBD-3 was enhanced by a mechanism that is mediated through a NOD-independent, EGFR-mediated pathway (Boughan et al., 2006).

The expression of hBD-2 was significantly increased by human colonic epithelial cell lines HT-29 and Caco-2 cells following exposure to pro-inflammatory levels of IL-1α and/or enteroinvasive E. coli (O29:NM), suggesting that hBD-2 plays an crucial role in the pathology of colitis and colitis-associated growth of microflora (Ho et al., 2013). Additionally, George et al. (2003) demonstrated that infection by Helicobacter pylori up-regulated hBD-2 and hBD-3 mRNA and protein expression in gastric cell lines both in vitro and in vivo, and hBD-2 as well as hBD-3 display strong antimicrobial activity against H. pylori. Further investigation by Paolillo et al. (2009) indicated that the mRNA and protein expression of hBD-2 were significantly enhanced in Caco-2 cells when exposed to Lactobacillus plantarum in a time and dose-dependent manner.

Additionally, the reduction of murine β-defensin 10 expression caused the impaired regulation of growth of several major components of the intestinal microbiota, such as Bacteroides fragilis, E. coli, E. faecalis, and C. albicans (Peyrin-Biroulet et al., 2010). Defb1 knockout mice were found to have increased level of Staphylococcus species in
their normally sterile urine (Morrison et al., 2002) and a defective ability to inhibit *Haemophilus influenza* growth in the airway (Moser et al., 2002). These findings demonstrate the importance of β-defensins in the innate defence against infections.

Collectively, hBDs function as antimicrobial agents and their expression is regulated by the signalling induced following exposure to both inflammatory and bacterial stimuli. However, the *in vitro* antimicrobial activity of β-defensins may not represent the full function *in vivo*, as the peptide concentrations required for *in vitro* antimicrobial activity are higher than those that are observed *in vivo* (Semple and Dorin, 2012). Despite the difference between *in vitro* culture condition and *in vivo* physiological conditions, it is suggested that β-defensins can not only inhibit bacteria growth by directly interacting with bacteria, but also modulate both innate and adaptive immunity (Semple and Dorin, 2012).

**1.2.2 hBDs regulate pro-inflammation in innate immune responses**

In addition to their antimicrobial activities against both Gram-positive and Gram-negative bacteria, defensins are also capable of mediating inflammatory innate immune reactions. These mediators are also known to possess chemoattractant activities able to recruit and activate other leukocyte populations into the inflamed area. (Oppenheim and Yang, 2005). Moreover, hBD-2 is capable of inducing recruitment of mast cells and neutrophils (Niyonsaba et al., 2002, Niyonsaba et al., 2004). In addition to hBD-2, mBD-14 (*DEFB14*), the mouse ortholog of hBD-3, was also shown to chemoattract both human and mouse CCR6-expressing cells (Taylor et al., 2008, Rohrl et al., 2008). hBD-3 was also demonstrated to suppress the apoptosis of neutrophils via binding to CCR6 at the neutrophil cell surface, increasing the expression of the anti-apoptotic protein Bcl-xL which, in turn, inhibits caspase-3 activity (Nagaoka et al., 2008).
However, hBD-3 and hBD-4 also were also shown to induce chemoattraction of macrophages which are not CCR6⁺, suggesting alternative receptors other than CCR6 may be involved in the interaction with β-defensins. Rohrl et al. (2010) demonstrated that it was the CCR2 receptor which mediates the migration of monocytes and macrophages in response to hBD-2 and hBD-3. Additionally to the interaction with CCR2 and CCR6, β-defensins also have been demonstrated to interact with Toll-like receptors (TLRs) on antigen presenting cells (APCs) (Dorin and Barratt, 2014). hBD-3 was also reported to induce the expression of the co-stimulatory molecules CD80, CD86, and CD40 on monocytes and myeloid dendritic cells (DCs), and interestingly, this up-regulation in expression is dependent upon TLR1 and TLR2 ligation (Funderburg et al., 2007), whilst mBD-2 enhances the activation of DCs in a TLR4-dependent manner (Biragyn et al., 2002). hBD-3 is also reported to increase the protein level of pro-inflammatory cytokines, including IL-1β, IL-6 and IL-8, in human monocytes via TLR1/2 (Funderburg et al., 2011). Alternatively, hBD-3 is capable of blocking CXCR4 activation by CXCL12 (Feng et al., 2013). In addition, hBD-2, hBD-3 and hBD-4 have been previously shown to induce pro-inflammatory mediators in keratinocytes including macrophage inflammatory protein-3α (MIP-3α), monocyte chemoattractant protein-1 (MCP-1), RANTES, IL-6, IL-10 and IP-10, through the G-protein and phospholipase C (G protein-PLC) signalling pathway (Niyonsaba et al., 2007).

As well as their pro-inflammatory role, β-defensins have also been reported to be suppressors of inflammation as well. Semple et al. (2010) demonstrated that hBD-3 and mBD-14 could inhibit TNF-α and IL-6 accumulation in human and mouse primary macrophages, respectively. They also showed that in the presence of hBD-3, the stimulation of macrophages by CD40/IFN-γ was significantly reduced in vivo (Semple
et al., 2010). Further investigation supported the finding that hBD-3 inhibits the transcription of pro-inflammatory genes in TLR4-stimulated macrophages (Semple et al., 2011). It is suggested that hBD-3 down-regulated the protein levels of pro-inflammatory cytokines, such as IL-6 and IL-12p40, and cell surface molecules, such as CD40 and CD86. In addition, hBD-3 reduces NF-κB signalling in cells transfected with MyD88 or TRIF, which are down-stream of TLR4 signalling, suggesting that the Myd88 and TRIF are the downstream targets of hBD-3 mediated suppression of the activation of macrophages (Semple et al., 2010).

Collectively, these findings suggest that β-defensins are able to regulate both pro- and anti-inflammatory immune responses by interacting with different receptors on several different types of immune cells.

1.2.3 hBDs regulate pro-inflammation in T-cell-mediated adaptive immune response

Recent studies have demonstrated that hBDs are also capable of regulating the adaptive immune response. Yang et al. (1999) demonstrated that hBD1–3 induced chemoattraction of CD4+ memory T cells and immature DCs through interaction with CCR6. Although hBD-2 has a lower affinity than CCL20 for the interaction with CCR6, both ligands can compete for binding to CCR6. Ghannam et al. (2011) demonstrated that hBD-2 was able to mediate the migration of the pro-inflammatory Th17 cell, in an in vitro model using induced inflammation in endothelial cells under flow conditions. Their data also showed that the Th17 derived cytokines, IL-17 and IL-22, could induce the secretion of hBD-2 by epidermal keratinocytes leading to the increase of inflammation.
As mentioned briefly above, in addition to the capability of hBDs to act as a chemoattractant to T cells, Boniotto et al. (2006) found that hBD-1, 2 and 3 are all capable of up-regulating the secretion of a variety of cytokines, such as IL-6, IL-8 and IL-10, and chemokines, such as MCP-1 by human peripheral blood mononuclear cells (PBMCs). These defensins also showed different abilities to induce the secretion of selected cytokines and chemokines. Following this study, Kanda et al. (2011) demonstrated that hBD-2 regulated cytokine mRNA expression and protein secretion by human CD3+ T cells \textit{in vitro}. Their data suggested that hBD-2 induced TNF-α, IFN-γ, IL-1β, IL-6, IL-10, and IL-22 production by these CD3+ T cells but inhibited IL-17 production. Additionally, the induction of cytokine production was found to be due to the enhanced expression of SOCS3 and the suppressed phosphorylation of STAT3 by hBD-2 treatment in αCD3/28-stimulated T cells (Kanda et al., 2011). These effects were suggested to be induced by hBD-2 via PTX-sensitive GPCR (Kanda et al., 2011). Additionally, investigation by Meisch et al. (2013) indicated that hBD-3 causes STAT1 tyrosine phosphorylation in human CD3’CD45RO+ T cells.

Further investigation suggested that hBD-2 induced IL-22 and oncostatin M in human CD3+ T cells (Kanda and Watanabe, 2012). The induction of T cell cytokines by hBD-2 treatment provides the evidence that treatment with hBD-2 potentially influences T cell differentiation following activation. Recent studies by Agatha Schwarz’s lab demonstrated that the treatment of T cells with murine β-defensin 14 (mBD-14) is capable of inducing a regulatory phenotype in CD4+ T cells. They used a murine model of contact hypersensitivity (CHS) induced by painting 2,4-dinitro-1-fluorobenzene (DNFB) on the skin of a mouse to test the suppressive function of mBD-14 on tissue inflammation. They found that the injection of mBD-14 before sensitization can protect the mice from the ear-swelling response compared to the non-injected control.
Additionally, the transfer of the splenocytes and lymph node cells obtained from mBD-14-treated mice can significantly suppress CHS response in the recipient mice (Navid et al., 2012). Finally, they showed that mBD-14 treatment in vitro can induce the expression of FoxP3, CTLA-4, CD62L and neuropilin on CD4⁺CD25⁻ T cells, which are considered as conventional T cells (Navid et al., 2012). These studies suggest that mBD-14 can induce regulatory phenotypes in vivo. More recently, a similar regulatory effect was demonstrated using an EAE mice model. The injection of mBD-14 into mice before immunization can significantly reduce the clinical score of experimental autoimmune encephalomyelitis (EAE), suggesting that mBD-14 treatment can induce Treg cell development to inhibit the inflammation in central nervous system (Bruhs et al., 2015). Interestingly, this regulatory effect induced by mBD-14 in CD4⁺CD25⁻ T cell is found to be IL-10-dependent because the T cells obtained from IL-10⁻/⁻ mice could not protect the recipient mice from ear-swelling (Navid et al., 2012).

Additionally, a recent study by Tomalka et al. (2015) demonstrated that the murine β-defensin, mBD-1, mediates the mucosal defense against C. albicans. They found that mBD-1 deficient mice exhibit impaired neutrophil infiltration which leads to the increased C. albicans infection. On the other hand, the deficiency of mBD-1 causes significant down-regulation of IL-1β, IL-6 and IL-17, suggesting defective Th17 lineage differentiation which is required in antifungal immune response against C. albicans infection.

In summary, these studies suggest a role for β-defensins in regulating T cell development and function. In respect to the functional diversity of human T cells in particular, it is of great interest to investigate the effect of hBD-2 and 3 in driving human CD4⁺ T helper cell differentiation.
Table 1.1 The antimicrobial activities and immunomodulatory function of hBD-2 and hBD-3.

<table>
<thead>
<tr>
<th>Expression sites</th>
<th>hBD-2</th>
<th>hBD-3</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium of tracheal, kidneys (also fetal kidneys), bladder, kernel, uterus,</td>
<td>Epithelium of tracheal, kidneys (also fetal kidneys), bladder, kernel, uterus, prostate, stomach, small intestine, liver, pancreas, thymus, marrow, leucocytes, cells forming keratin, and the skin;</td>
<td>Keratinocytes, tonsils, skin, esophagus, placenta, trachea, uterus, kidney, thymus, adenoid, pharynx, tongue, heart of adults, fetal thymus, skeletal muscle, gums, tongue, mucosa of cheeks and lips, dental follicle and pulp.</td>
<td>(Jarczak et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Expressed following microbial infection and inflammation.</td>
<td>Expressed following microbial infection or secretion of inflammatory cytokines, such as IL-1β.</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial activities</td>
<td>Antimicrobial activity against Gram-negative bacteria, yeasts, HIV and HPV;</td>
<td>Antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeasts, HIV and HPV;</td>
<td>(Jarczak et al., 2013, Wang, 2014)</td>
</tr>
<tr>
<td></td>
<td>Antimicrobial activity can be inhibited by NaCl</td>
<td>Antimicrobial activity is resistant to high NaCl concentration.</td>
<td></td>
</tr>
<tr>
<td>Immunomodulatory function</td>
<td>Chemotactic activity for monocytes, macrophages, neutrophils and immature dendritic cells;</td>
<td>Chemotactic activity for monocytes, macrophages, neutrophils and immature dendritic cells via CCR6, CCR2 or CXCR4;</td>
<td>(Jarczak et al., 2013, Semple et al., 2011, Semple et al., 2010, Meisch et al., 2013, Kanda et al., 2011)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Induces phosphorylation of JNK, ERK, and Akt in human CD3⁺ T cells;</td>
<td>Inhibits TLR4 downstream signaling, such as MyD88 and TRIF, in macrophages;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Suppresses phosphorylation of STAT3 and enhances expression of SOCS3 in αCD3/28-stimulated human CD3⁺ T cells;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enhances cytokine secretion and mRNA expression.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3 Research aims

Recent studies have suggested a role for defensins in Treg development (Navid et al., 2012), however the effect of hBD-2 and 3 on human CD4$^+$ T helper cell differentiation is not fully explored. Thus, the aim of this study is to investigate the role of hBD-2 and hBD-3 in driving human CD4$^+$ T cell activation, proliferation and differentiation.

1.4 Research objectives

- To investigate the effect of hBD-2 and hBD-3 on CD4$^+$ T cell activation and proliferation using human whole PBMCs;

- To investigate the effect of hBD-2 and hBD-3 on purified human CD4$^+$ T cell activation and proliferation;

- To characterize the polarization of purified human CD4$^+$ T cells in the presence or absence of hBD-2 and hBD-3 and investigate cytokine production in the cell culture;

- To assess the suppressive function of hBD-2 pre-treated Treg cells and the resistance of hBD-2 pre-treated Teff cells from Treg suppression.

- To investigate the transcriptome profile of human CD4$^+$ T cells after treatment with hBD-2 using microarray analysis.
Chapter 2: Methodology

2.1 Ethical approval

This study has been reviewed and approved by the NHS City Road and Hampstead Research Ethics Committee (Appendix 2.1) as well as the University Research Ethics Committee of University of East London (Appendix 2.2). Human venous blood samples were collected with written consent from healthy volunteers according to the NREC approvals (Ref: 13/LO/0296).

2.2 Antibodies and reagents

Synthesized peptides of hBD-2 and hBD-3 were purchased from Severn Biotech (UK). Anti-human CD3 (Clone: OKT3) and anti-human CD28 (Clone: CD28.6) antibodies were purchased from eBioscience. The following eBioscience mAbs were used in flow cytometry: FITC-conjugated anti-human CD69 (Clone: FN50); PE-conjugated anti-human CD25 (Clone: BC96); PE-conjugated anti-human CD127 (Clone: eBioRDR5); PE-Cyanine7-conjugated anti-human CD4 (Clone: RPA-T4); PE-Cyanine7-conjugated anti-human T-bet (Clone: 4B10); allophycocyanin (APC)-conjugated anti-human FoxP3 (Clone: 236A/E7); PE-conjugated anti-human RORγt (Clone: AFKJS-9). 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience) was used to trace cell proliferation.

2.3 Isolation and activation of human PBMCs

Initially, 50ml of peripheral blood was taken from healthy donors using heparinized vacutainer tubes. PBMCs were isolated by density gradient centrifugation using the Histopaque 1077 (Sigma, USA). Blood samples were diluted 1:1 (v/v) with AIM-V
medium (Gibco, UK). The diluted blood were carefully layered onto 10ml of Histopaque 1077 in a sterile conical centrifuge tube and then centrifuged at 400×g without break for 30mins. After centrifugation, the interface containing the whole PBMCs was carefully taken and moved into a new centrifuge tube. Subsequently, the cells were washed by medium twice to remove the excess Histopaque. After washing, the isolated PBMCs were seeded at 2.0×10^6 cells/ml in 96-well plates and stimulated with soluble anti-human CD3 (0.005μg/ml) and anti-human CD28 (0.005μg/ml) antibodies (eBioscience) in the presence or absence of 1, 3 and 10μg/ml hBD-2 in 200μl AIM-V medium. Cell cultures were incubated at 37℃, 5% CO₂. After 18hrs and 42hrs, PBMCs were assayed for cell surface marker expression and a range of cytoplasmic markers including CD4, CD69 and CD25 using flow cytometry.

2.4 Purification of human CD4⁺ T Cells

CD4⁺ T cell were isolated using EasySepTM human CD4⁺ T cell enrichment kit (StemCell, UK) according to the manufacturer’s instruction. Human PBMCs were suspended in a sterile polystyrene tube with recommended buffer (PBS with 2% FCS). Cell suspensions were incubated with antibody cocktail (CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A) for 10mins. Then, magnetic beads were added into cell suspension and incubated for 5mins. Finally, the tube was inserted into magnet. After 5mins incubation, the fraction of purified CD4⁺ T cells was poured into a new sterile tube. These isolated CD4⁺ T cells were stimulated using plate bound anti-human CD3 antibodies (0.1μg/ml) and soluble anti-human CD28 antibodies (2μg/ml), according to the published methodology (Kanda, 2011, Kanda and Watanabe, 2012).
2.5 **Enzyme linked immunoabsorbent assay (ELISA)**

For assay of IL-2, IL-6, IL-10 and IL-17A secretion, supernatants of cell culture were collected and the cytokine concentrations were determined by ELISA Ready-Set-Go kits (eBioscience, UK) according to the manufacturer’s instructions. Firstly, 96-well plates were coated with capture antibodies overnight at 4°C. Then, the wells were washed using ELISA wash buffer (1× PBS, 0.05% Tween-20) to remove unbound antibody and blocked using 1× ELISA/ELISPOT Diluent from the kit for 1hr. After washing, 100µl of diluted supernatant samples along with a serial dilution of recombinant cytokine protein were added and the plate was incubated at 4°C overnight in order to get the maximal sensitivity. After incubation, the samples were aspirated and the plate was washed using ELISA wash buffer. Detection antibody against each individual cytokine was added and incubated at room temperature for 1hr. Then, the plate was washed again and the diluted Avidin-HRP was added and incubated for 30mins. After washing, the substrate was added and the colour was allowed to develop for 15mins at room temperature. After 15mins incubation, the stop solution was added to the plate (2N H$_2$SO$_4$) to stop the reaction. Finally, The plates were read using the Thermo Multiskan plate reader (Thermo) at 450nm. The actual values for analysis were obtained by subtracting the values obtained at 570nm.

2.6 **Cell Proliferation assay**

Isolated PBMCs were washed twice with PBS to remove fetal calf serum (FCS) in the culture. Then, cells were suspended in PBS at 2.0×10$^6$ cells/ml in PBS. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was added (5mM) to a final concentration of 5µM, vortexed gently and the suspension allowed sitting for 15mins in 37°C incubator. To stop further labelling of the cells, an equal volume of 5% FCS/PBS
was added to the cell suspension. The cells were centrifuged for 5 min, 400×g. The supernatant was discarded and the cell pellet resuspended to obtain a single cell suspension. Cells were washed twice by suspending in 10 ml of 2% FCS/PBS. Cells were centrifuged for 5 min, 400×g. Finally, the labelled cells were resuspended in AIM-V at 2.0×10^6 cells/ml. Where described, IL-2 (0.02 μg/ml) was added to promote T cell proliferation.

2.7 Flow cytometry

Cells were prepared and incubated with fluorescence-conjugated antibodies for cell surface markers, such as CD4, CD69 and CD25. Briefly, cells were washed and suspended in 50 μl of FACS buffer (PBS containing 2% FCS). Then, 50 μl of antibody cocktail was added into each cell suspension. After gently flicking, the cell suspension was incubated on ice for 30 mins. Then, cells were washed using FACS buffer and were then analysed using an Accuri C6 Flow Cytometer (BD Bioscience, UK). For the staining of intracellular transcriptional factors, FoxP3, T-bet and RORγt, intracellular staining was performed using FoxP3/Transcription Factor Staining Buffer Set (eBioscience, UK). After labelling with surface antibodies as described earlier, cells were initially fixed and permeabilized using Fixation/Permeabilization solution and stained with selected antibodies in 1× Permeabilization Buffer. After staining, cells were washed and resuspended in FACS buffer ready for analysis using the Accuri C6.

2.8 RNA extraction

RNA samples were isolated and purified using PureLink RNA Mini Kit (12183018A, Life Technologies). Cultured human CD4+ T cells were collected and pelleted in RNase free tubes. After removing the supernatant, lysis buffer containing 1% β-mercaptoethanol was added in an appropriate amount according to the cell count. The
cell lysate was processed in order to extract RNA according to the manufacturer’s instruction in which a step of DNase on column digestion was also applied using PureLink DNase set (Life technologies, UK). The quality and quantity of RNA samples was determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, US). Samples with an absorbance of 260/280nm ratio between 1.8 and 2.1 were considered as accepted quality to be used in downstream experiments. The isolated RNA samples were stored at -80°C. cDNA was synthesised using the iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). RNA samples were extracted as described earlier. 300-400ng RNA was added in the reaction to make up a total volume of 20μl. The other components, such as Oligo(dt) primers, reverse transcriptase, are provided by the kit. The cDNA synthesis was performed using the Bio-rad T100TM Thermal Cycler (Bio-Rad, Inc., US). The reaction program was set as it is suggested in the manufacturer’s reaction protocol. cDNA samples were stored at -20°C.

2.9 Micro-array assay

An ethanol precipitation step was carried out in order to further purify RNA samples. RNA samples were applied to an Affymetrix HuGene Exon Expression Microarray Chip. Poly-A controls were used to monitor the entire target labelling process. Poly-A controls were dap, lys, phe, thr. Hybriziation controls were bioB, bioC, bioDn, creX. The signal intensities of the hybridization control probes are presented in Appendix 2.3. As it is shown in Appendix 2.3, the profiles of all samples are similar, indicating good hybridization and washing of the arrays. CEL files were produced and then analysed using Agilent GeneSpring GX 13 software. The data was normalized using the GeneSpring GX 13 software with the Robust Multi Array (RMA) algorithm and. Statistical analysis was performed using t test. p values less than 0.05 were considered
to be significant. In addition, fold changes of gene expression of more than 1.5 were considered biologically relevant. Principal Component Analysis (PCA) was carried out as described in Furmanski et al. (2013). Briefly, CEL files were analysed for PCA using the CRAN package (Bioconductor, U.S). Genes identified from GeneSpring and PCA was verified using qRT-PCR.

2.10 Quantitative RT-PCR

Relative gene expression levels of selected target genes were measured using quantitative real-time PCR (qPCR). RNA extraction and cDNA synthesis were performed using the same method as described earlier. qPCR were performed using the iTaq UniverSYBR Green RT-PCR kit (Bio-rad, UK) following the manufacturer’s protocol. The house-keeping gene, human hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*), was used as internal standard for the quantification of template as well as the normalisation of target gene expression. Amplification of *Hprt1* was quantified using a dilution series of neat cDNA, 1:10, 1:100, 1:1000 and 1:10000 prepared from stimulated control CD4\(^+\) T cells RNA and the relative standard curve for the gene of interest was generated by a 1:10 serial dilution of RNA prepared from cultured CD4\(^+\) T cells in the presence or absence of hBD-2 (10µg/ml) or hBD-3 (10µg/ml). The efficiency of the qRT-PCR was determined for each gene with the slope of a linear regression model (Pfaffl, 2001). The efficiencies (E) were calculated according to the equation: E= \((10^{(-1/slope-1)} \times 100)\).

The primers used for the reaction are listed below in Table 2.1. All primers used were purchased from Quantitech (Qiagen, UK). The primer sequences are protected by Qiagen. Each reaction contained approximately 100ng of cDNA, 300nM of forward and reverse primers, 10µl of SYBR green super-mix (containing, iTaq DNA polymerase,
dNTPs, MgCl₂). The reaction was made up to 20μl using nuclease free water (Qiagen Inc., US) in a 96 well plate (Thermo Scientific, U.K). Data analysis was performed according to using standard curve method (Bolha et al., 2012). The calculation of relative expression is shown in Appendix 2.4. Gene expression levels were first normalized to the expression levels of the Hprt1 and then calculated relative to the normalized gene expression in the stimulated control (Maine et al., 2014).

qRT-PCR reaction was performed under the recommended conditions for each primer used according to the manufacturer’s protocol: initial denaturation at 95°C, for 30s followed by 40 cycles of denaturation at 95°C for 30s and annealing at 60°C for 30s. In each experiment, an additional programme for the detection of melting curve was performed in order to check the melting temperatures of the products to assess primer-dimer formation and confirm the product size was as expected (Appendix 2.3).

**Table 2.1** Primers used in RT-PCR experiment

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl1</td>
<td>QT00203154</td>
</tr>
<tr>
<td>Ctla4</td>
<td>QT01670550</td>
</tr>
<tr>
<td>Il23r</td>
<td>QT00032914</td>
</tr>
<tr>
<td>Il21</td>
<td>QT00038612</td>
</tr>
<tr>
<td>Hprt1</td>
<td>QT00059066</td>
</tr>
</tbody>
</table>

### 2.11 Treg/Teff sorting and Treg suppression assay

Purified human CD4⁺ T cells were stained with anti-CD4-Pe-Cy7, anti-CD25-APC and anti-CD127-PE antibodies before FACS sorting. Then, CD4⁺CD25⁻CD127⁻ Treg cells and CD4⁺CD25-CD127⁺ Teff cells were sorted using BD FACS Ari II. After sorting, target Teff cells were stained with CFSE using the method described previously. Then,
CFSE labelled target Teff cells and Tregs were seeded in separate wells at 2.0×10⁶ cells/ml and incubated in the presence or absence of hBD-2 for 4hrs. After hBD-2 treatment, Teff and Treg cells were collected and washed with fresh medium twice and target Teffs were cultured alone at 5.0×10⁴ cells/well or co-cultured with Tregs at a Teff/Treg ratio 1:1 in 96-well plate. Teffs were stimulated using αCD3/28 antibody coated beads (Dynabeads, Life Technologies) at a bead/cell ratio 1:10. Proliferation of Teffs was assessed by flow cytometry following 4 days in culture.

### 2.12 FACS gating strategy

PBMCs were stained and analysed using flow cytometry (Accuri C6, BD). To exclude dead cells, a plot of forward scatter (FSC-A) vs. side scatter (SSC-A) was set at the beginning of analysis. Lower granularity on SSC-A and FSC-A indicates dead cells. In PBMCs, CD4 was used as the first marker and it is plotted against the SSC gated as shown in R1. Cells were then analysed for the expression of T cell activation markers, CD69 and CD25, within the CD4⁺ T cell gate. In the case of purified CD4⁺ T cells, T cell activation was analysed also using CD69 and CD25 similar to the PBMCs. In addition to activation, the Treg population was gated as CD4⁺CD25⁺CD127⁻ subset, whereas the activated effector T cell (Teff) population was gated as CD4⁺CD25⁺CD127⁺ subset. The expression of transcription factors, such as FoxP3, T-bet and RORγt was analysed using the gates for Treg cells or activated Teff cells.

### 2.13 Statistical analysis

Statistical analysis was performed using the Student t Test for statistical testing between two groups, or One-way ANOVA for three groups. Column graphs were generated by GraphPad Prism 5. Dot-plots and peak-plots were generated by Accuri CFlow software (BD Bioscience, UK). Differences between the means and the stimulated control value,
with $p$ values of 0.05 or less, were considered statistically significant. Unless otherwise stated, the figures in the results show one representative experiment of at least three independent experiments.
Chapter 3: Effects of hBD-2 on whole PBMCs population

3.1 Introduction

β-defensins were initially described as peptides with only direct antimicrobial activity and therefore were regarded as an essential innate immune component protecting the host from infection (Jenssen et al., 2006, Harder et al., 2007). Biragyn et al. (2002) were considered as the first to report that defensins may play a role as a mediator in both the innate and the adaptive immune response. Their studies demonstrated that mBD-2 up-regulates co-stimulatory molecules on immature DCs by interacting with TLR-4, thereby inducing DC maturation. Additionally, hBD-3 as well as LL-37 was shown to exhibit chemotactic activity for monocytes, immature DCs, neutrophils and T cells (Doss et al., 2010).

It has also been shown that the closely related, α-defensins cannot only regulate the maturation and differentiation of leukocytes but also act as chemotactic factors. In particular, the α-defensins, HNP1–3, have been reported to control the maturation of human monocyte-derived DCs and regulate the maturation of these cells as judged by CD83, CD86 and HLA-DR expression in a dose dependent manner (Rodriguez-Garcia et al., 2009).

In addition to HNP1, hBD-1 was also suggested to be chemotactic for monocyte-derived DCs by inducing the activation of these cells and production of pro-inflammatory mediators in these cells (Presicce et al., 2009). Moreover, Boniotto et al. (2006) revealed that human PBMCs treated with hBD-2 can up-regulate secretion of a number of cytokines, such as IL-6, IL-8 and IL-10 by these cells in vitro. These studies
reveal the fact that defensins may play a crucial role in regulating both the innate and adaptive immune responses.

Dysregulated expression of the defensins may also lead to the development of pathologies. The over-expression of hBD-2 in keratinocytes is considered to participant in the induction of autoimmune pathology, such as chronic psoriasis by inducing the infiltration of IL-17-producing T cells into psoriatic skin (Lowes et al., 2014). Kanda et al. (2011) detected elevated levels of hBD-2 in serum from patients suffering from psoriasis which correlated in a positive fashion with levels of TNF-α, IFN-γ, IL-1β, IL-6, IL-10 and IL-22, but were inversely correlated with IL-17 levels. Consistent with this finding, they found that mRNA levels of TNF-α, IFN-γ, IL-1β, IL-6, IL-10 and IL-22 were significantly higher in hBD-2 treated CD3+ T cells in contrast to the untreated control, suggesting that the defensins were working to up-regulate the pro-inflammation which may lead to autoimmunity.

β-defensins have also been shown to regulate the development of Tregs. The mouse homolog of hBD-2 and hBD-3, Defb14, has been indicated to be capable of switching CD4+ effector T cells into Treg cells with an accompanying increase in secretion of the immunosuppressive cytokine, IL-10 (Navid et al., 2012). Moreover, mBD-14 has been shown to be able to direct CD4+CD25- T cells into a regulatory phenotype by up-regulating the expression of FoxP3 and CTLA-4 in these cells (Navid et al., 2012).

In this chapter, we investigate the immune-modulatory effects of hBD-2 on human peripheral blood derived T cells in the culture of whole PBMCs. Synthetic hBD-2 peptides were used at a range of different doses and were added to the cultures of PBMCs together with soluble αCD3/28. Any effect of hBD-2 on T cell activation, proliferation and apoptosis of the CD4+ T cells was analysed using flow cytometry. We
also investigated the development of $\text{CD}4^+\text{CD}25^{\text{high}}$ Treg cells by analysing the expression of the marker for Tregs, FoxP3.

### 3.2 Results

#### 3.2.1 Treatment with hBD-2 up-regulates the number of percentage of FoxP3$^+$ cells in CD4$^+\text{CD}25^{\text{high}}$ T cells at 18hrs, but down-regulates FoxP3$^+$ cells at 42hrs.

It has been suggested in several studies that the commensal microbiota in intestine plays an important role in facilitating the immune system, including the generation of an appropriate environment for the development of Tregs (Brenchley and Douek, 2012, Muniz et al., 2012, Ostaff et al., 2013). Antimicrobial peptides (AMPs) are an essential component of this environment. In this chapter, we have selected to study hBD-2 in order to assess what impact this AMP might have on the adaptive immune response and in particular the emergence of Tregs.

Tregs represent a distinct phenotype of T cells that express CD4, $\alpha$ chain of IL-2R (CD25), and FoxP3 (Josefowicz et al., 2012). FoxP3 has been demonstrated as a transcriptional factor specifically expressed in CD4$^+\text{CD}25^{\text{high}}$ Tregs (Hori et al., 2003). It has been demonstrated that FoxP3 functions as a key regulatory gene for the development of Tregs, as retroviral gene transfer of FoxP3 converts naive T cells into a Treg phenotype similar to that of naturally occurring Tregs (Navid et al., 2012, Bilate and Lafaillle, 2012).

Additionally, CD69 is a type II C-lectin membrane receptor barely expressed on resting lymphocytes but then rapidly induced on effector T cells upon cell activation which makes it a good marker of choice to measure early activation of T cells (Gonzalez-Amaro et al., 2013). Recent studies have suggested that the cytoplasmic tail of CD69
can interact with the transcriptional factor STAT5, which is an essential mediator of IL-2 signalling in T cells. IL-2 enhances Treg function via increased expression of FoxP3, whilst inhibits Th17 differentiation and IL-17 production through the inhibition of STAT3 signalling (Gonzalez-Amaro et al., 2013).

In order to investigate the effect of hBD-2 treatment on the development of Tregs within a PBMC mixed cell culture, expression of CD4 and CD25 was used to identify putative Tregs and then intracellular expression of FoxP3 was analysed in this gated population. Upon incubation of PBMCs with 10μg/ml hBD-2 at 18hrs, a 50% increase in levels of FoxP3 fluorescence intensity was observed in CD4⁺CD25⁺ T cells (Figure 3.1A, upper left). In addition, the percentage of CD4⁺CD69⁺CD25⁺ T cells significantly increased upon co-incubation with 10μg/ml hBD-2 from 35% in the stimulated control to 39.9% in the presence of hBD-2. As it is shown in Figure 3.1C, there is an increase of the percentage of CD69⁺CD25⁺ T cells (Q2-UR) on incubation of PBMCs treated with hBD-2 at 18hrs (from 35% to 39.9%). Several recent reports suggest that CD69 may exert a regulatory function in different activated T cell subsets. Considered as a recent early activation marker, the expression of CD69 has also been found on FoxP3⁺ Tregs and may act as a marker for recently activated Treg cells which are able to recognize locally presented antigens in different tissues (Lieberman et al., 2012). Our data indicates that hBD-2 may increase the presence of CD69⁺CD25⁺ T cells at 18hrs and this observation may correlate with either the appearance of a greater number of CD25⁺CD69⁺ activated T cells or these may represent an increase in the presence of newly activated Tregs that express both CD69 and CD25. These data are consistent with previously published findings which reveal that mBD-14, an ortholog of hBD-2, is capable of inducing the differentiation of Treg cells (Navid et al., 2012).
However, interestingly, we found that the percentage of FoxP3\(^+\) cells in CD4\(^+\) CD25\(^{high}\) T cells was significantly reduced following 42hrs treatment with 10\(\mu\)g/ml of hBD-2 (Figure 3.1A, lower left; 3.1B), although there was no significant change in the numbers of either CD69\(^+\)CD25\(^+\) or CD69\(^-\)CD25\(^+\) T cells at this time point (Figure 3.1C). Previous studies suggested the essential role of FoxP3 in CD4\(^+\)CD25\(^+\) Treg cells (Hill et al., 2007, Sugimoto et al., 2006, Gavin et al., 2007). Additionally, ablation of a conditional FoxP3 allele in mature Treg cells was found to result in a loss of characteristic gene expression and suppressor function as well as the acquisition of effector T cell function (Williams and Rudensky, 2007). Thus, the heritable maintenance of a developmentally established Treg cell transcriptional and functional program requires continuous expression of FoxP3 (Josefowicz et al., 2012). Following 42hrs treatment with 10\(\mu\)g/ml of hBD-2, the reduced expression of FoxP3 could indicate a loss of immunosuppressive function of these CD4\(^+\)CD25\(^{high}\) Tregs.
Figure 3.1 hBD-2 treatment increased the percentage of FoxP3\(^+\) cells in CD4\(^+\)CD25\(^{\text{high}}\) T cell population at 18hrs, but down-regulates the percentage of FoxP3\(^+\) cells in the same subsets at 42hrs. A. PBMCs were activated with soluble anti-human CD3
(0.005μg/ml) and anti-human CD28 (0.005μg/ml) and co-incubated in the presence or absence of 1, 3 and 10μg/ml of hBD-2. After 18hrs and 42hrs incubation, cells were stained and analysed by flow cytometry. Data were collected from 3 independent experiments (n=3). Statistical analysis was performed using Student t test. *p<0.05 versus stimulated control (Stim Co); ** p<0.01 versus stimulated control (Stim Co).

B. Histograms showing expression of FoxP3 in CD4+CD25high T cells. C. Dot-plots of expression of CD25 and CD69 on CD4+ T cells.

3.2.2 hBD-2 treatment in the culture of PBMCs induces proliferation of both CD4+ and CD8+ T cells.

As discussed previously, we demonstrated that percentage of FoxP3+ cells in CD4+CD25high T cells is significantly reduced following 40hrs incubation with 10μg/ml of hBD-2 in these same CD4+CD25high T cell population. It has been suggested that the decrease of FoxP3 expression in CD4+CD25high Tregs may lead to their impaired suppressive function. Thus, we would hypothesize that there could be a loss of immunosuppressive activity induced by hBD-2 treatment in these cultures following 42hrs incubation. In order to assess the in vitro immunosuppressive activity of Treg cells after treatment with hBD-2, PBMCs were stained with CFSE prior to stimulation with αCD3/28 antibodies in order to assess any regulation of proliferation of CD4+ T cells following treatment with hBD-2. CFSE is able to bind to intracellular proteins by reacting with lysine side-chains and other available amine groups. When cells proliferate, CFSE labelled proteins are separated equally into the daughter cells, which therefore obtain half of their fluorescence from their parent cells (Hawkins et al., 2007). As a result, each generation of proliferation is marked by a halving intensity of CFSE fluorescence which can be characterised as several finger-like fluorescent peaks displayed in flow cytometry.
Data presented in Figure 3.2 and 3.3 suggested that the proliferation of CD4$^+$ T cell population as well as CD8$^+$ T cell population was increased at 72hrs and 96hrs after treatment with 10μg/ml hBD-2 (Figure 3.2A and 3.3A). The lower fluorescence intensity of CFSE in the treated cells indicates the higher level of proliferation that cells were undergoing. With the treatment of hBD-2, the increasing percentage of T cells expressing the lower fluorescence intensity can be identified (Figure 3.2B and 3.3B). At 96hrs there was a shift in percentage of cells that had divided from 53.8% in cells stimulated with αCD3/28 alone to 69.6% cells in cell cycle when treated with hBD-2. A similar, but less increased finding was observed at 72hrs (Figure 3.2B). Additionally, for CD8$^+$ T cells, there was an increase in percentage of cells that had proliferated from 66.5% to 73.5% at 72hrs and an increase from 69% to 81.9% at 96hrs (Figure 3.3B). These data suggests that hBD-2 treatment can induce T cell proliferation at 72hrs and 96hrs.

As discussed previously, the expression of FoxP3 in CD4$^+$CD25$^{\text{high}}$ Treg cells is significantly down-regulated following 42hrs incubation with 10ug/ml of hBD-2 in these same CD4$^+$CD25$^{\text{high}}$ T cells, which may imply defective immunosuppression of CD4$^+$CD25$^{\text{high}}$ Tregs. Tregs are a distinct lineage among newly arising CD4$^+$ thymocytes in expressing CD25. Additionally, it has been previously reported that CD4$^+$FoxP3$^+$ Tregs suppress virus-specific CD8$^+$ T cells proliferation and production of cytotoxic molecules. The depletion of Treg cells induced CD43$^+$ effector CD8$^+$ T cells to enhanced the levels of the cytotoxic molecules, such as granzyme A and B (Zelinskyy et al., 2013). We hypothesise that down-regulation of FoxP3 in Tregs would result in an increase of CD8$^+$ T cell proliferation due to impaired Treg suppressive function.
In summary, *in vitro* proliferation assay using CFSE stained PBMCs revealed that hBD-2 treatment enhances proliferation of both CD4\(^+\) and CD8\(^+\) T cells following 72hrs and 96hrs culture. These data suggests that any negative control of proliferation provided by the FoxP3\(^+\) Tregs of these CD4\(^+\) T cells has been partially lost (Figure 3.2 and 3.3).

**Figure 3.2** hBD-2 treatment induces CD4\(^+\) T cells proliferation *in vitro*. A. PBMCs were stained with CFSE (5µM) prior to the stimulation with soluble anti-human CD3 (0.005µg/ml) and anti-human CD28 (0.005µg/ml) and co-incubated in the presence or absence of 1, 3 and 10µg/ml hBD-2. Recombinant human IL-2 (0.02µg/ml) was added
to promote T cell proliferation. After 72hrs and 96hrs incubation, cells were stained and analyzed by flow cytometry. Data were collected from 3 independent experiments (n=3). Statistical analysis was performed using Student t test. *p < 0.05 versus stimulated control (Stim Co). B. Dot-plots and peak-plots of CFSE fluorescence intensity in CD4+ T cells.

**Figure 3.3** hBD-2 treatment induces CD8+ T cell proliferation *in vitro*. A. PBMCs were stained with CFSE (5μM) prior to the stimulation with soluble anti-human CD3
(0.005μg/ml) and anti-human CD28 (0.005μg/ml) and co-incubated in the presence or absence of 1, 3 and 10μg/ml hBD-2. Recombinant human IL-2 (0.02μg/ml) was added to promote T cell proliferation. After 72hrs and 96hrs incubation, cells were stained and analyzed by flow cytometry. Data were collected from 3 independent experiments (n=3). Statistical analysis was performed using Student t test. *p < 0.05 versus stimulated control (Stim Co). B. Dot-plots and peak-plots of CFSE fluorescence intensity in CD8+ T cells.

3.2.3 Co-culture with hBD-2 reduces induction of T cell apoptosis at 18hrs but enhances levels of apoptosis at 42hrs.

In order to analyse the level of apoptosis induced in CD4+ T cells by treatment with hBD-2, PBMCs were stained with Annexin-V to characterise any apoptotic cells following αCD3/28 co-stimulation in the presence and absence of hBD-2. Annexin-V is a phospholipid-binding protein that is capable of binding with phosphatidylserine (PS). In normal cells, PS is located in the inner leaflet of the cytoplasm membrane. However, upon initiation of apoptosis, PS loses its asymmetric distribution across the cell membrane and is trans-located to the cell surface. This then allows Annexin V, labelled with fluorescence, to bind to PS on the cell surface of the membrane indicating apoptosis occurring in cells.

Results presented in Figure 3.4 indicated that, at 18hrs, there was a small but significant decrease in Annexin-V expression following treatment with hBD-2 which suggested that the apoptosis of CD4+ T cells was partially suppressed in the presence of 10μg/ml hBD-2 (Figure 3.4, left hand panel). However, at 42hrs, there was a significant increase observed in Annexin-V staining which suggested that the apoptosis of CD4+ T cells was increased in the presence of 10μg/ml hBD-2 relative to the control (Figure 3.4, right
hand panel). These data suggest that hBD-2 may play a role in inhibiting CD4\(^+\) T cells apoptosis at 18hrs and induce CD4\(^+\) T cells apoptosis at 42hrs. According to a recently published paper, hBD-3 was found to be capable of suppressing neutrophil apoptosis by up-regulating in expression of Bcl-x(L) and suppressing the activation of caspase-3 (Nagaoka et al., 2010). However no role has been documented to date for a pro-apoptotic effect of hBD-2 on immune cells making these findings particularly interesting.

**Figure 3.4** Annexin-V staining on CD4\(^+\) T cells at 18hrs and 42hrs. PBMCs were stimulated with soluble anti-human CD3 (0.005\(\mu\)g/ml) and anti-human CD28 (0.005\(\mu\)g/ml) and treated with 1, 3 and 10\(\mu\)g/ml hBD-2. After staining by cell surface markers, cell suspension was washed by Annexin-V binding buffer. FITC-conjugated Annexin-V was added in cell suspension with a recommended concentration by the manufacturer’s instructions. Data were collected from 3 independent experiments (n=3). Statistical analysis was performed using Student t test. *\(p<0.05\)
3.2.4 *hBD-2 and hBD-3 induce the appearance of a CD69^+CD25^-CCR6^+ subset at 18hrs, but at 42hrs, hBD-2 and hBD-3 induced the appearance of a CD69^+CD25^-CCR6^+ subset and a CD69^-CD25^-CCR6^+ subset.*

CCR6 is a CC chemokine receptor which is an important receptor that is involved in regulating several many aspects of mucosal immunity (Ito *et al.*, 2011). It has been reported that not only the chemokine CCL20, but also the non-chemokine peptides hBD-2 and hBD-3 can function as ligands for human CCR6 (Yang, 1999, Schutyser *et al.*, 2003). In order to investigate whether expression of CCR6 was required for hBD-2 induced CD4^+ T cell activation, this CCR6^+ population was looked at specifically.

Firstly, we analysed the percentage of CCR6^+CD4^+ T cells in the PBMCs culture. We found that there was no significant change in the numbers of percentage of CD4^+CCR6^- T cell population following 18hrs culture or 42hrs culture (Figure 3.5A). Then, CD4^+CCR6^- T cells were gated and analysed for expression of the activation markers CD69 and CD25. As shown in Figure 3.5B, gates were set up to select CD4^+CCR6^- (Gate: R7) and CD4^+CCR6^+ (Gate: R6) cells. When compared with the CD4^+CCR6^- subset, CD4^+CCR6^+ cells aggregated collected in the CD25^- section quartiles at both 18hrs and 42hrs. At 18hrs, 53.2% of these cells were CD69^+CD25^- phenotype, increased from 22.0% in CCR6^- subset. There were also 29.0% of the CCR6^+ cells were CD69^-CD25^- cells and this had in CCR6^+ cells increased from 11.7% when compared to CCR6^- cells. However, for in respect of the CD69^-CD25^- subset, the percentage decreased from 19.3% to 5.8% in CCR6^- cells. The statistical analysis revealed that there was a significant increase of CD69^-CD25^+ and CD69^-CD25^- cells in the CD4^-CCR6^+ T cells, and significant decrease of CD69^-CD25^- phenotypes in CD4^-CCR6^+ T cells (Figure 5C). Interestingly, these data suggested that CCR6^+ T cells may respond more quickly to αCD3/28 activation as there are more cells expressing the
phenotype that is characteristic of the later stages of activation which is CD69⁺CD25⁺ and CD69⁻CD25⁺.

**Figure 3.5** CCR6 staining on CD4⁺ T cells in human PBMCs culture. A. the percentage of CD4⁺CCR6⁺ T cells in the PBMCs culture. B. dot plots showing the gate strategy to distinct CCR6⁻ and CCR6⁺ subsets in the samples and analysing the expression of CD69 and CD25 in these subsets. C. statistical analysis comparing the percentage of different CD4⁺ T cells phenotypes regarding to the activation markers between CCR6⁻ and
CCR6\(^{+}\) subsets. Data were collected from 3 independent experiments (n=3). Statistical analysis was performed using Student \( t \) test. *\( p<0.05 \)

Having established the use of gates to analyse the activation status of CCR6\(^{+}\) and CCR6\(^{-}\)CD4\(^{+}\) T cells, we went on to analyse the effect of co-culture of these cells with hBD-2 and hBD-3. Next, we found that the population of CCR6\(^{-}\)CD69\(^{+}\)CD25\(^{-}\) were significantly increased by 50\% in the presence of hBD-3 and to a lesser extent of hBD2 at 18hrs (Figure 3.6), whereas no such effect was observed in the case of CCR6\(^{-}\)CD69\(^{+}\)CD25\(^{-}\) subset cells did not show any significance. These data suggested that hBD-2 or hBD-3 induced more CCR6\(^{+}\) unstimulated T cells express CD69 at their earlier stage of activation. This induction may lead to the up-regulation of CCR6\(^{+}\) T cell activation. Moreover, as the percentage increase of CCR6\(^{+}\)CD69\(^{+}\)CD25\(^{-}\) was more dramatic increased greater in the presence of hBD-3 than when compared to hBD-2, it would appear that this CD4\(^{+}\)CCR6\(^{+}\) T cell population response more quickly at the early stage of activation, possibly reflecting a difference of affinity in binding to CCR6 between hBD-2 and hBD-3.

**Figure 3.6** hBD-2 and hBD-3 induced increase of CD69\(^{+}\)CD25\(^{-}\)CCR6\(^{+}\) T cell subset at 18hrs. Data were collected from 3 independent experiments (n=3). *\( p<0.05 \)
At 42hrs this CCR6 dependent effect was lost, we observed a very small (1.1 fold) but significant increase of both the CCR6\(^+\)CD69\(^+\)CD25\(^+\) subset and the CCR6\(^-\)CD69\(^-\)CD25\(^+\) in the presence of hBD-2. However, this very small increase mediated by the presence of hBD-2 may have no physiological meaning as it is so small (Figure 3.7 left). However, in the co-incubation with hBD-3, there was a very small but significant increase of the same subset only in CCR6\(^-\) but not in CCR6\(^+\) CD4\(^+\) T cells (Figure 3.7 right).

Finally, there was a significant increase observed in the presence of both hBD2 and hBD3 at 42hrs in CCR6\(^-\)CD69\(^-\)CD25\(^+\) T cells. This effect was not observed on the corresponding CCR6\(^+\) T cell population. The presence of CCR6\(^-\) cells, but not CCR6\(^+\) cells, in the CD69\(^-\)CD25\(^+\) subset increased when treated with hBD-2 or hBD-3 at 42hrs (Figure 3.8). Taken together, these data suggest that at 42hrs it is not CCR6\(^+\) cells but CCR6\(^-\) cells that have responded to hBD-2 and hBD-3 or, alternatively, that this is an affect that has occurred as a consequence of an earlier event in activation. These data suggest that there may be alternative receptors or pathways to CCR6 that may be ligated by hBD2 and hBD3 in order to induce CD4\(^+\) T cell activation by hBD-2 and hBD-3.
Figure 3.7 hBD-2 induced both CCR6^- and CCR6^+CD69^-CD25^- cell subset at 42hrs, whereas hBD-3 induced increase of CCR6^- but not CCR6^+ T cells in CD69^-CD25^- cell subset at 42hrs. Data were collected from 3 independent experiments (n=3). *p<0.05

Figure 3.8. hBD-2 and hBD-3 induced increase of CCR6^- but not CCR6^+ T cells in CD69^-CD25^- cell subset at 42hrs. Data were collected from 3 independent experiments (n=3). *p<0.05, **p<0.01
3.3 Discussion

As it has been reported that hBD-2 may act as a modulator of T cell cytokine production (Kanda et al., 2011), and also is able to suppress the immune response by inducing the differentiation of Treg phenotype (Navid et al., 2012), it was our original aim to further investigate the immunomodulatory effects of hBD-2 on human peripheral T cells. In this chapter, we suggest that human PBMCs treated with hBD-2 causes an up-regulation in numbers of CD4+CD25high T cells at 18hrs post-activation stimulus and these numbers remain elevated following 40hrs in culture. Characterisation of FoxP3 staining also reveals an enhanced expression of this transcription factor in the CD4+CD25high T cell subset at 18hrs.

However, we found that the percentage of FoxP3+ T cells is significantly decreased following 42hrs treatment with hBD-2 in these same CD4+CD25high T cell population in vitro. It has been demonstrated that FoxP3 plays a key transcriptional factor to the development and function of Treg cells (Josefowicz et al., 2012, Bilate and Lafaille, 2012). These results could indicate a loss of function of Treg cells upon co-culture with hBD-2. Indeed, it has been previously shown that an experimentally induced reduction in the expression of FoxP3 resulted in impaired suppressive function of Treg cells (Wan and Flavell, 2007). Additionally, it is of note that it has been shown that in developing Treg cells, CD28 co-stimulation induces FoxP3 expression, which most likely occurs through the activation of NF-κB signalling pathway and binding of c-Rel to non-coding sequence elements in the Foxp3 gene (Tai et al., 2013).

However, as we had observed that there was an apparent up-regulation in FoxP3+ Tregs by 18hrs, but an apparent loss of these cells as they appear to lose FoxP3 expression at 42hrs. Therefore, we hypothesised that this finding might result in a loss of Treg
suppressive activity and that this effect would promote the proliferation of effector T cells in our cell cultures. Consistent with this idea we found that, using analysis of CFSE stained PBMCs, the proliferation of CD4$^+$ T cells in vitro is enhanced by hBD-2 treatment after both 72hrs and 96hrs. This result suggests the negative control of proliferation of these CD4$^+$ effector T cells by Tregs has been partially lost.

Several previous studies have indicated hBD-2 has direct effects on T cells. Boniotto et al. analysed the effects of both hBD-2 and hBD-3 on the induction of secretion of cytokines and chemokines by PBMCs using a protein-array, in order to describe a role for human β-defensins in the induction of pro-inflammatory response in these cells (Boniotto et al., 2006). Their investigation suggest the expression of IL-6, IL-8 and IL-10 is enhanced by co-culture of with hBD-2 and human PBMCs. Further studies by Kanda et al. (2011) revealed the effects of hBD-2 on the purified human CD3$^+$ T cell population. They illustrated that the effects of hBD-2 on T cell were to enhance secretion of TNF-α, IFN-γ, IL-1β, IL-6, IL-10 and IL-22. These data suggest that hBD-2 is able to manifest feedback to T cells in order to sustain maintain or halt their own cytokine production. hBD-2 was also shown to induce an increase in secretion of the immunoregulatory cytokine, IL-10, which can itself induce the development of Treg cells. On the other hand, hBD-2 also induced the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1β, IL-6 and IL-22 expression by human CD3$^+$ T cells (Kanda et al., 2011). TNF-α, IL-1β and IL-6 can themselves also induce the production of IL-12 and IL-12 inhibits Treg cell conversion and expansion. It has also been shown that IL-6 together with TGF-β abrogates Treg generation and drives naive T cells to develop into Th17 cells (Kimura and Kishimoto, 2010). Th17 cells may then produce IL-17, IL-21 and IL-22 which are able to induce inflammation in tissues (Korn et al., 2009). Collectively, these data suggest that hBD-2 may indirectly and directly
affect the development and function of both Treg and Th17 cells through the regulation of cytokine. Thus this complex regulation of the immune response by the defensins involves the secretion of a wide range of cytokines with apparently opposing effects and as such a homeostatic control of the immune response may be maintained.

The role of CCR6 in mediating these reported hBD2 and hBD3 effects remains controversial (Lee et al., 2015). Some researchers have reported that CCR6 functions as a receptor for hBDs (Yang, 1999, Schutyser et al., 2003), whilst other report that its expression is not necessary to mediate hBD-2 and hBD-3 effects (Soruri et al., 2007). Others suggest that it is a question of affinity. It is suggested that hBD-2 was less effective than CCL20 in interacting with CCR6 (Yang, 1999). However, although the affinity of hBD-2 and for CCR6 is lower than CCL20, hBD-2 was shown to be capable of inducing the arrest of human Th17 cells on arrest to inflamed epithelial cells in vitro via CCR6 (Ghannam et al., 2011). Interestingly, in contrast to CCL20, co-incubation with hBD-2 did not lead to Ca\(^{2+}\) mobilization in CCR6-expressing Th17 cells (Ghannam et al., 2011). Because Ca\(^{2+}\) mobilization plays an essential role in T cell activation (Shi et al., 2013, Joseph et al., 2014), these findings suggest that there may be other receptors capable of responding to hBD-2 during the process of T cell activation. Kanda et al. (2011) demonstrated that hBD-2 may also regulate the activation and cytokine production in CD3\(^+\) T cells via PTX-sensitive G-protein coupled receptors, rather than CCR2, CCR6, or TLR4. In our study, we demonstrated that hBD-2 and hBD-3 may influence the events of early activation in a CCR6 dependent fashion but then influence the later stages of activation in a CCR6 independent fashion. These data may suggest the possibility that alternative receptors could be ligated by hBDs in order to induce CD4\(^+\) T cell activation in vitro, and further study is required in order to identify which receptors are involved in this process.
3.4 Conclusion

In this chapter, our results reveal that hBD-2 may play both a positive and negative role in the development of Treg cells by regulating the expression of FoxP3. Initially, the positive regulatory effect is found by an up-regulation in numbers of CD4⁺CD25⁺ Tregs that also express elevated levels of FoxP3. The later negative regulatory effect is identified by a loss of FoxP3⁺ cells in these CD4⁺CD25⁺ Treg population, leading to a limited ability of these Treg cells to slow down the proliferation of CD4⁺ effector T cells in the presence of hBD-2. In addition, we observed an increase of early activated CD69⁺CD25⁻CCR6⁺ CD4⁺ T cells population but not CD69⁺CD25⁺CCR6⁻ CD4⁺ T cell in the presence of hBD-2 or hBD-3 at 18hrs. This suggested that hBD-2 or hBD-3 may act via CCR6 thereby inducing the appearance of more CCR6-expressing unstimulated T cells which also express CD69 at their earlier stage of activation. However, by 42hrs, only the later activated T cells, CD69⁺CD25⁻CCR6⁻ and CD69⁺CD25⁺CCR6⁻ subsets appeared to respond to hBD-2 and hBD-3, whereas CCR6-expressing subsets did not show any significant changes. This data may suggest that there are alternative receptors or pathways to induce CD4⁺ T cell activation by hBD-2 and hBD-3 at the later stages in activation.

These data suggest a role for hBD-2 in regulating the adaptive immune response, however, as these investigations were carried out on mixed PBMCs we could not rule out the possibility that another cell type other than the CD4⁺ T cell was responsible for mediating the observed effects of hBD-2 and hBD-3. In Chapter 4, we will focus on analysing purified human CD4⁺ T cells in order to investigate whether these findings are intrinsic to CD4⁺ T cells.
CD4\(^+\) T cells

4.1 Introduction

CD4\(^+\) T cells and CD8\(^+\) T cells comprise the two major lineages of peripheral T lymphocytes. Both CD4 and CD8 are co-receptors expressed on peripheral T cells that play essential roles in T cell development. Distinct from MHC I - restricted CD8 cytotoxic T cells, CD4 T cells are MHC II - restricted T helper cells. CD8\(^+\) T cells function as effector cells capable of inducing cell death of virus infected cells or cancer cells, whereas CD4\(^+\) T cells can differentiate to become one of several different T helper cell phenotypes which play different and important roles in the activation of other immune cells and may therefore regulate the immune response (Kleinewietfeld and Hafler, 2013, Korn et al., 2009, Vahedi et al., 2013). However, additional to their ability to act as helper cells, they can also become “troublemakers” leading to the development of autoimmune diseases and allergies (Hirahara et al., 2013).

Upon recognition of antigen, CD4\(^+\) naïve T cells may be polarised to become Th1, Th2, Th17, Tfh and regulatory T cell (Treg) With the exception of Tregs, all other T helper subsets play a role in combating infection. In the case of CD4\(^+\) Tregs, these cells are described mainly as being a distinct lineage of cells which form an essential component of the immune response and are responsible for maintaining the homeostasis of the immune system (Josefowicz et al., 2012, Kleinewietfeld and Hafler, 2013, Bilate and Lafaille, 2012). Treg cells function as inhibitors of activation and proliferation of other effector T cells including CD4\(^+\) T effectors, CD8\(^+\) T cells, NK cells, B cells and APCs (Kleinewietfeld and Hafler, 2013, Josefowicz et al., 2012). Tregs can be characterized not only by expression of CD4 on their cell surface but also by their high expression of
CD25 (also known as IL-2Rα) and by the expression of the transcriptional factor FoxP3 (Kleinewietfeld and Hafler, 2013). FoxP3 has been considered as ‘a lineage-specification factor’ for Treg cell differentiation and function (Ramsdell and Ziegler, 2014). Also, the maintenance of suppressive function by Treg cells requires continuous expression of FoxP3 (Williams and Rudensky, 2007). In humans, Treg cells were found to also have low expression of IL-7R (CD127) (Liu et al., 2006, Hartigan-O'Connor et al., 2007).

CD4+ T cell activation and proliferation is regulated by Treg cells. The mechanism of Treg-mediated suppressive function is mainly dependent on cell-to-cell contact mechanisms, such as CTLA-4-dependent blockade of co-stimulation, the release of suppressive cytokines (e.g. TGF-β, IL-10 or IL-35), or the production of suppressive metabolites (e.g. adenosine) (Kleinewietfeld and Hafler, 2013, Tai et al., 2012, Shevach, 2009). FoxP3 plays a key role in Treg suppressive function (Rowe et al., 2012, Josefowicz et al., 2012). Binding of FoxP3 cannot only induce CTLA-4 expression and the production of cytokines which mediate immune-suppression (Hossain et al., 2013), but also can interact with RORγt protein to inhibit Th17 differentiation (Chen et al., 2011).

More recently, a novel mechanism was reported whereby auto-Treg cells release CD73-expressing exosomes to suppress CD4+CD25+ T-cell proliferation following TCR activation (Smyth et al., 2013). Tregs can mediate their suppressive function via transfer miRNA-containing exosomes to Th1 cells in order to inhibit proliferation and cytokine production by effector T cells (Okoye et al., 2014).
In Chapter 3, we reported that co-culture of mixed PBMCs with hBD-2 induced human CD4\(^+\) T cell activation and proliferation \textit{in vitro}. In addition, we found that hBD-2 treatment may trigger both a positive and negative effect in the development of human Tregs by regulating the expression of FoxP3 at different time points. Following 18hrs co-culture, hBD-2 induced an up-regulation in the expression of FoxP3 in CD4\(^+\)CD25\(^{\text{high}}\) Tregs. By 42hrs, however, hBD-2 treatment caused a down-regulation of FoxP3 expression in these CD4\(^+\)CD25\(^{\text{high}}\) Tregs which may cause these Tregs to lose their ability to control the proliferation of CD4\(^+\) effector T cells, thus leading to the up-regulated levels of proliferation. It is not clear whether the observations described in Chapter 3 are due to a CD4\(^+\) T cell intrinsic effect or to the presence of another cell contained within the PBMC population. Therefore, we will focus on purified human CD4\(^+\) T cells in this chapter and investigate whether the effect of hBD treatment on CD4\(^+\) T cells is, in fact, intrinsic to CD4\(^+\) T cells themselves.

4.2 Results

4.2.1 hBD-2 treatment in purified CD4\(^+\) T cells induces an increase in the percentage of FoxP3\(^+\) cells in human CD4\(^+\)CD25\(^{\text{high}}\) T cells by 18hrs, but suppresses the Foxp3\(^+\) cells by 42hrs.

In order to prepare a culture of purified T helper cells, human CD4\(^+\) T cells were isolated using magnetic bead enrichment kit and then stimulated in culture using plate bound anti-CD3 (0.1\(\mu\)g/ml) and soluble anti-CD28 (2\(\mu\)g/ml) antibodies. Cell cultures were observed using an inverted microscope (Figure 4.1 and Figure 4.2). Cell clusters indicate the degree of activation as T cell activation is accompanied by the up-regulation of expression of adhesion molecules on the T cell surface which allows T cells to stick together forming clumps. Compared to the stimulated control, the diameter of T cell clusters co-incubated with hBD-2 and 3 increased by 18hrs and 42hrs. This
data suggests that there could be a greater number of CD4\(^+\) T cells activated in the presence of hBD-2 and hBD-3 when compared to the stimulated only control.

Following this observation using the microscope, CD4\(^+\) T cells were isolated from the cultures and stained with fluorescence conjugated antibodies and analysed using flow cytometry. Following co-incubation of CD4\(^+\) T cells with hBD-2 and an activation stimulus for 18hrs, there was an increased induction of CD69\(^+\)CD25\(^+\) T cells from 11.8 to 20.7\% in the presence of 10ug/ml hBD-2 (Figure 4.3 A, upper panel). This finding is consistent with the data obtained using whole PBMCs (Chapter 3, Figure 3.1). Although the percentage of CD69\(^-\)CD25\(^+\) subset remains at 10.0\% in the experiment presented in Figure 4.3A, statistical analysis showed that there was a small but significant increase by 1.2 fold of the CD69\(^-\)CD25\(^+\) population (Figure 4.3 B). In addition to this effect observed with hBD-2, co-culture with hBD-3 increased the presence of CD69\(^+\)CD25\(^+\) subset from 11.8\% to 22.3\%, and induced an increase in the presence of CD69\(^-\)CD25\(^+\) subset from 10.0\% to 12.6\%. Statistical analysis showed that co-culture with hBD-3 significantly induced the percentage of the CD69\(^-\)CD25\(^+\) subset at 18hrs by a fold change which is greater than the effect observed with hBD-2.

Moreover, following co-incubation of CD4\(^+\) T cells with hBD-2 for 42hrs, the percentage representation of the CD69\(^+\)CD25\(^+\) T cell subset significantly increased from 10.3\% to 14.05\%, and the percentage representation of the CD69\(^-\)CD25\(^+\) T cell subset significantly increased from 21.2\% to 27.5\% (Figure 4.3 A, lower panel). Interestingly, we found that co-incubation of T cells with hBD-3 had a more dramatic effect on T cell differentiation than co-culture with hBD-2. In the case of hBD-3, there was an increase in the presence of CD69\(^+\)CD25\(^+\) from 10.3\% to 26.7\% and an increase of CD69\(^-\)CD25\(^+\) subset from 21.2\% to 33.4\% (Figure 4.3 A, lower panel). It seems that after the phase of
early activation, the expression of CD69 was down-regulated while the expression of CD25 was up-regulated. CD25⁺CD69⁺ T cells developed into CD25⁺CD69⁺ T cells and then into the CD25⁺CD69⁻ phenotype. Taken together, these results suggested an up-regulation in CD4⁺ T cell activation by both hBD-2 and hBD-3.

**Figure 4.1** Morphology of purified CD4⁺ T cell culture with or without the treatment of hBD-2 and 3 at 18hrs. Purified CD4⁺ T cells were stimulated using plate bound anti-human CD3 (0.1μg/ml) and soluble anti-human CD28 (2μg/ml) in the presence or absence of hBD-2 (10μg/ml) or hBD-3 (10μg/ml). The unstimulated control sample was purified CD4⁺ T cells cultured without antibody stimulation. After 18hrs incubation, plates were taken out of incubator and placed under microscope to observe and photograph the cell layout and clustering.
Figure 4.2 Morphology of purified CD4\(^+\) T cell culture with or without the treatment of hBD-2 and 3 at 42hrs. Purified CD4\(^+\) T cells were stimulated using plate bound anti-human CD3 (0.1\(\mu\)g/ml) and soluble anti-human CD28 (2\(\mu\)g/ml) in the presence or absence of hBD-2 (10\(\mu\)g/ml) or hBD-3 (10\(\mu\)g/ml). The unstimulated control sample was purified CD4\(^+\) T cells cultured without antibody stimulation. After 42hrs incubation, plates were taken out of incubator and placed under microscope to observe and photograph the cell layout and clustering.
Figure 4.3 hBD-2 and 3 induce CD69 and CD25 expression on human CD4$^+$ T cells.  

A. Dot-plots for CD69 and CD25 expression on human CD4$^+$ T cells from one experiment;  

B. Statistical analysis of CD69$^+$CD25$^+$ and CD69$^-$CD25$^+$ subsets in the presence of hBD-2 and 3 at 18hrs and 42hrs. Data were collected from 5 independent experiments (n=5). *p<0.05
4.2.2 Proliferation of CD4$^+$ T cells was increased following treatment with hBD-2 and 3 for 72hrs and 96hrs.

In order to investigate the effect of co-culture of T cells with hBD-2 and hBD-3 on T cell proliferation, purified human CD4$^+$ T cells were stained with CFSE prior to stimulation with αCD3/28 antibodies. Data presented in Figure 4.4 suggested that the proliferation of CD4$^+$ T cells was increased from 38.4% in the stimulated control to 48.7% in the presence of hBD-2 and the percentage increased to 52.8% in the presence of hBD-3. These data suggest that co-incubation of T cells with an activation stimulus as well as hBD-2 or hBD-3 induces significantly increased T cell proliferation at 96hrs (Figure 4.5A). This finding could be considered to be consistent with the observed increase in activation of T cells in the presence of hBD-2 and hBD-3. As proliferation of T cells is mediated, in part, by interleukin-2 (IL-2), the IL-2 production in the supernatant at 18 and 42hrs was detected using ELISA. The data shown in Figure 4.5B demonstrates that co-culture with hBD-2 and hBD-3 induces a significant up-regulation of IL-2 production by CD4$^+$ T cells by 18hrs. Although the overall level of IL-2 went down by 42hrs, there is still a trend towards an increase in IL-2 production in the presence of hBD-3 (Figure 4.5). It is possible that at this time point IL-2 may have been absorbed out of the system by the increased presence of the IL-2Ra (CD25) expressed on the surface of the activated T cells thereby accounting for the drop in IL-2 levels observed in the cultures. These data suggest that co-culture with hBD-2 or hBD-3 induces CD4$^+$ T cells to produce more IL-2 which in turn is able to promote CD4$^+$ T cell proliferation in vitro.

This data is seemingly inconsistent when considered alongside the finding that the presence of CD69$^+$CD25$^+$ putative Tregs is increased in these cultures. These findings could imply loss of immunosuppressive function of the Tregs following treatment with
hBD-2 or hBD-3 as expression of FoxP3 is a requirement for the suppressive function of Treg cells.

Furthermore, CD25 is a subunit of the high-affinity IL-2 receptor (IL-2R). It is therefore possible that as Tregs are described to mop up excess IL-2 via the binding of CD25 to IL-2 in the local environment and as IL-2 is crucial for Treg cell expansion and maintenance, decreased level of IL-2 in the local environment may lead to a reduction in T cell proliferation (Boyman and Sprent, 2012, Zhang et al., 2013). Interestingly, high level of IL-2Rα expression is required for Treg cells homeostasis and this function is considered as FoxP3-dependent (Josefowicz et al., 2012). Dysregulated Tregs may no longer be capable of their suppressive function thus allowing a higher level of proliferation of the Teffs cells (Tai et al., 2013).

Figure 4.4 hBD-2 and hBD-3 treatment induces the proliferation of purified CD4+ T cells in vitro. Purified CD4+ T cells were stained with CFSE (5μM) prior to activation. CFSE labelled CD4+ T cells were activated with plate bound anti-human CD3 (0.1μg/ml) and soluble anti-human CD28 (2μg/ml) and treated with 10μg/ml hBD-2 or hBD-3. After 96hrs incubation, cells were stained and analysed by flow cytometry.
Figure 4.5 hBD-2 and hBD-3 treatment significantly enhanced the proliferation of CD4⁺ T cells following 96hrs culture and increased the protein level of IL-2 in the supernatant of CD4⁺ T cell culture in vitro. A. the statistical analysis of the fold change of proliferated CD4⁺T cells; B. IL-2 concentration was detected in the supernatants using ELISA. The factor of treatment was analysed using One-way ANOVA and the factor of time was analysed using Two-way ANOVA. Data were collected from 3 independent experiments (n=3). * p<0.05; ** p<0.01

4.2.3 FoxP3 and CTLA-4 expression of CD4⁺ T cells co-cultured with hBD-2 and 3.

In order to investigate the expression of FoxP3 in CD127⁻CD25⁺ Treg cells, we used intracellular staining techniques on cell isolated from the purified CD4⁺ T cell cultures. Interestingly, we demonstrated that the percentage of FoxP3⁺ cells within the CD127⁻CD25⁺ Treg population significantly increased from 28.0% to 34.6% in the presence of hBD-2. Also, we observed that following co-culture with hBD-3, the percentage of FoxP3⁺ cells within the CD127⁻CD25⁺ Treg population significantly increased even more dramatically from 28.0% to 44.1% (Figure 4.6 and 4.8). These findings suggested that treatment with hBD-2 and hBD-3 can induce more CD4⁺ cells to differentiate into CD127⁻CD25⁺FoxP3⁺ Treg phenotype when CD4⁺ T cells are activated by αCD3/28 in vitro. We also assessed CTLA4 expression in the CD127⁻CD25⁺ Treg subset. However,
we could not find any cell surface expression of CTLA4 on the Treg subsets suggesting that if present, the expression of CTLA4 was below the levels of detection in our assay (Figure 4.7). We then analysed intracellular CTLA4 expression and found that treatment with hBD-2 significantly induced the appearance of a population of FoxP3⁺CTLA4⁺ subset within the CD127⁻CD25⁺ population of Treg cells. In comparison, treatment with hBD-3 induced a significant increase in the FoxP3⁺CTLA4⁻ subset (Figure 4.7 and 4.8). These results indicated that the treatment with hBD-2 may promote both FoxP3 and intracellular CTLA4 expression in CD127⁻CD25⁺ Tregs by 18hrs, whereas hBD-3 treatment only induced FoxP3 expression in CD127⁻CD25⁺ Tregs by 18hrs. These findings are consistent with our observation using PBMCs (Chapter 3) as well as the previously published findings which reveal mBD-14, a homologue of hBD-2, is capable of inducing the development of Tregs in CD4⁺CD25⁻ T cells in mice (Navid et al., 2012). However, by 42hrs, we found the presence of the FoxP3⁺ population of Tregs was significantly decreased after the treatment with hBD-2 or hBD-3, even though the overall percentage of FoxP3⁺ cells was raised in all samples from 18 to 42 hrs in culture (Figure 4.6 B and 4.9). These data suggest that CD127⁻CD25⁺ Treg cells may lose their expression of FoxP3 following the treatment with hBD-2 or hBD-3 for 42hrs and this may lead to the defective function of these CD127⁻CD25⁺ Treg cells. We then went on to investigate the expression of CTLA4 in CD127⁻CD25⁺ Treg cells. Surprisingly, we found that there was a significant up-regulation of FoxP3⁺CTLA4⁺ cells in the presence of hBD-2 or hBD-3, although there was a significant decrease in the presence of FoxP3⁺CTLA4⁻ cells with the co-culture of hBD-2 or hBD-3 (Figure 4.9).
Figure 4.6 Percentages of FoxP3⁺ subsets in Treg and Teff cells at 18hrs. Purified human CD4⁺ T cells were stimulated using αCD3/28 antibodies and co-cultured with hBD-2 or 3 for 18hrs. Treg population was characterized as CD127⁻CD25⁺ subset. Teff population which has been activated was characterized as CD127⁺CD25⁺ subset. Gating on the Treg subset and Teff subset, the percentages of FoxP3⁺ cells were measured respectively. A. Cells that were cultured for 18hrs; B. Cells that were cultured for 42hrs. Data were collected from 5 independent experiments (n=5).
Figure 4.7 Intracellular staining for FoxP3 and CTLA-4 expression in CD127⁻CD25⁺ Treg cells at 18hrs and 42hrs. Purified human CD4⁺ T cells were stimulated using αCD3/28 antibodies and co-cultured with hBD-2 or 3 (10µg/ml). Cells were gated on CD4⁺CD127⁻CD25⁺ Treg subset. The left panel shows the cell surface staining of CTLA-4 and the rest of the dot-plots show the staining for intracellular CTLA-4. Data were collected from 3 independent experiments (n=3).
Figure 4.8 Statistical analysis of FoxP3 and CTLA-4 expression at 18hrs. FoxP3\(^+\)CTLA4\(^+\) and FoxP3\(^+\)CTLA4\(^-\) T cell subsets were gated on CD127\(^-\)CD25\(^+\) T cells. Fold changes were calculated relative to the percentage of Stim Co. Data were collected from 3 independent experiments (n=3). \(*p<0.05\)
Figure 4.9 Statistical analysis of FoxP3 and CTLA-4 expression at 42hrs. FoxP3<sup>+</sup>CTLA4<sup>+</sup> and FoxP3<sup>+</sup>CTLA4<sup>-</sup> T cell subsets were gated on CD127<sup>-</sup>CD25<sup>+</sup> T cells. Fold changes were calculated relative to the percentage of Stim Co. Data were collected from 3 independent experiments (n=3). *p<0.05

4.3 Discussion

Recent studies have shown that hBD-2 is capable of inducing an increase in the phosphorylation of JNK, ERK, and Akt, following activation with αCD3/28 suggesting that treatment of T cells with hBD-2 may enhance the degree of activation of CD3<sup>+</sup> T cells in vitro (Kanda et al., 2011). In addition to the effect observed with hBD-2, treatment with hBD-3 was also reported to stimulate STAT1 tyrosine phosphorylation in CD3<sup>+</sup>CD45RO<sup>+</sup> T cells but was unable to induce MAPK activation indicating,
possibly, that hBD-2 and hBD-3 exert their effects differently on the induction of T cell activation (Meisch et al., 2013). Thus the distinct roles of hBD-2 and 3 in human CD4⁺ T cell activation remains to be characterised. In Chapter 3, we demonstrated that treatment with hBD-2 and 3 up-regulate the numbers of CD4⁺CD69⁺CD25⁺ T cells following 18hrs in cultures of human PBMCs. To investigate whether this effect is a CD4⁺ T cell intrinsic effect, we assessed the role of hBD-2 and 3 in purified human CD4⁺ T cell activation and proliferation.

The data presented in this chapter demonstrated that following co-stimulation of T cells with αCD3/28 and hBD-2 or hBD-3 significantly up-regulated the presence of CD69⁺CD25⁺ T cells at both 18 and 42hrs in purified CD4⁺ T cell culture. We additionally demonstrated that treatment with hBD-2 significantly increased the presence of the CD69-CD25⁺ T cells subset at both time points, whereas treatment with hBD-3 only induced a significant increase in the presence of CD69⁺CD25⁺ T cell by 18hrs post-activation stimulus. These data may reflect the different potencies between hBD-2 and 3 and indicate that hBD-2 may push CD4⁺ T cell activation more quickly through the CD69⁺CD25⁺ stage, or hBD-3 may inhibit activation of CD4⁺ T cells in this double positive phase. These data suggest that the presence of both hBD-2 and 3 may augment human CD4⁺ T cell activation following αCD3/28 in vitro. Consistent with this finding, we also showed that CD4⁺ T cell proliferation also enhanced in the presence of hBD-2 or 3 by 96hrs. These results reveal the capability of hBD-2 and 3 in possibly accelerating and enhancing human CD4⁺ T cell activation and proliferation.

Additionally, our data indicate that co-incubation with hBD-2 or hBD-3 in vitro induces an increase in the presence of FoxP3⁺ cells within the CD127⁻CD25⁺ Treg population of cells following 18hrs in culture. This data is consistent with the finding that, the closely
related homologue, murine defensin mBD-14, also induces FoxP3 expression in mouse CD4⁺CD25⁺ Treg cells (Navid et al., 2012). FoxP3 has been reported to be a key transcription factor in Treg development and suppressive function (Josefowicz et al., 2012). Transduction of FoxP3 up-regulates expression of CD25 on the T cell surface as well as intracellular CTLA4 in the cytoplasm, and both markers are associated with Treg phenotype and function, and also triggers the suppression of IL-2 secretion (Hori et al., 2003, Yamaguchi et al., 2011). Our intracellular staining analysis showed that following 18hrs treatment with hBD-2 there was a significant increase in the percentage of FoxP3⁺CTLA4⁺ cells within the CD127⁻CD25⁺ Treg population. These results suggest that treatment with hBD-2 and 3 may induce the regulatory phenotype in human CD4⁺ T cells.

However, surprisingly, following 42hrs in culture, we observed a significant decrease in the percentage of FoxP3⁺ cells in the presence of hBD-2 or hBD-3. This finding suggests that by this time point, treatment with hBD-2 and 3 may cause down-regulation of FoxP3 expression in Treg cells in vitro, and this in turn may lead to defective Treg suppressive function of these cells in controlling effector T cell activation and expansion.

Interestingly, when we investigated particular subsets of cells, we observed an increase in the number of FoxP3⁺CTLA4⁺ cells following 42hrs culture in the presence of hBD-2 or hBD-3, suggesting that the expression of intracellular CTLA4 had been unregulated in these CD127⁻CD25⁺FoxP3⁺ Tregs following treatment with hBD-2 and hBD-3. However, as CTLA4 protein expression on Treg cell surface was below levels of detection, the significance of this finding is hard to evaluate. According to Tai et al. (2012), CTLA4 proteins expressed on Tregs are rapidly cycling between the cell surface
and interior, which lead to the instability of cell-surface CTLA4 protein which may alter the ability of interacting with co-stimulatory ligands on APCs which leads to down-regulation of immune response. Navid et al. (2012) reported that in vitro co-incubation of T cells with mBD-14 could induce an up-regulation of expression of surface CTLA4 in mouse Treg cells. However, it is still unclear whether this up-regulation in expression can alter Treg function.

It is also possible that the up-regulation of intracellular CTLA4 expression induced by treatment with hBD-2 or hBD-3 may result from the presence of anti-CD28 in the cultures. As described previously, CTLA4-independent Treg suppression is mediated by CD25 and deprivation of IL-2. However, this mechanism only occurs under conditions using αCD28 for co-stimulation instead of APCs (Pandiyan et al., 2007), which circumvents CTLA4-mediated co-stimulatory blockade (Tai et al., 2012). In this study, we were using αCD28 as co-stimulation in the CD4⁺ T cell culture, which means that Treg suppression may occur independently of expression of CTLA4. Hence, Treg suppressive function may still be affected by the down-regulation of FoxP3 expression even though CTLA4 expression is apparently increased inside the cells. In this case, an in vitro suppressor function assay of Treg cells is required to be performed in order to investigate the ability of Tregs to induce suppression of effector T cells following treatment with hBD-2 and hBD-3 (Chapter 6).

Classically, thymic derived Tregs (tTregs) have been considered to be a distinct lineage from effector T cells. In the lineage perspective model, tTregs are regarded as self-reactive T cells which escaped from negative selection in the thymus, and this T cell phenotype is selected to control self-reactive naïve T cells in the periphery (Josefowicz et al., 2012, Ono and Tanaka, 2016). However, a recently paper by Ono and Tanaka
(2016) suggested that the induction of Tregs may occur as a natural consequence of T cell activation and may provide a mechanism whereby the immune response may be negatively regulated in terms of T cell activation, thus providing a ‘feedback control mechanism’. In this model, some of the activated T cells generate FoxP3+ memory-like T cells (or T effector cells) which act as positive regulators to induce immune response, while some of the activated T cells are induced to express FoxP3 (Tregs) which then allows them to compete the binding sites on APCs with FoxP3– Teff in order to delay T cell activation (Ono and Tanaka, 2016). According to this hypothesis, self-antigens or previously recognized antigens can induce a positive immune response by Teffs and a negative regulation by Tregs. This model suggested that upon TCR activation, both FoxP3– Teff response and FoxP3+ Treg regulation can be triggered together, and enhanced TCR signaling can induce both FoxP3– Teff response and FoxP3+ Treg regulation.

In summary, FoxP3 expression was regarded to be a consequence of T cell activation and provides a negative feedback control on human T cell activation (Ono and Tanaka, 2016). Interestingly, FoxP3 was reported to have the capacity to bind to various transcription factors in order to induce the activation in conventional T cells and to regulate their functions (Ono and Tanaka, 2016, Josefowicz et al., 2012). It has also been suggested that IL-2 secreted by activated T cells may induce and prolong FoxP3 expression in Tregs, while the expressed FoxP3 inhibits IL-2 production by repressing NFκB and NFAT transactivation reporters (Ono and Tanaka, 2016, Hench and Su, 2011, Smith and Popmihajlov, 2008). In our study, we found that treatment with hBD-2 and hBD-3 increased FoxP3 expression in Treg cells following 18hrs in culture together with an activation stimulus, suggesting an enhanced differentiation of Tregs following delivery of an activation stimulus together with hBD-2 or hBD-3. This induction of
FoxP3 expression may occur as the result of the ability of treatment with hBD-2 and 3 to induce enhanced IL-2 production by 18hrs. By 42hrs, treatment with hBD-2 or hBD-3 induced a decrease of FoxP3\(^+\) Treg cells which may suggest a weakened control of T cell activation and subsequently leading to the enhanced positive feedback via effector T cells.

Taken together, when considering which hypothesis of Treg differentiation best fits the data presented in this chapter, it would seem that the point of view of a feedback control perspective (Ono and Tanaka, 2016) appears to fit best. Treatment with hBD-2 and hBD-3 may induce FoxP3 expression, following, or in parallel with, an enhancement in T cell activation and therefore induce both Teff response and Treg response simultaneously.

Considering the well documented plasticity of T effector cells in the periphery in response to the local environment in which the T cell is found, the down-regulation of FoxP3 expression following co-culture with hBD-2 or hBD-3 for 42hrs implies a loss of the Treg phenotype and its associated functions. It might also suggest the differentiation towards an altered T cell immune response that might arise due to the influence of the local environment. For example, in the case where an infection has triggered an inflammatory immune response resulting in inflammation and an up-regulation of expression of the hBDs.
Figure 4.10 A proposed schematic model summarising the key findings in Chapter 4. A. hBD-2 or hBD-3 treatment enhances human CD4$^+$ T cell activation. B. hBD-2 or hBD-3 treatment enhances human CD4$^+$ T cell proliferation. C. hBD-2 or hBD-3 treatment up-regulates the induction of CD127$^-$CD25$^+$FoxP3$^+$ Treg population at 18hrs, however, down-regulates the percentage of CD127$^-$CD25$^+$FoxP3$^+$ Treg population following 42hrs treatment.
4.4 Conclusion

In this chapter, we discussed the effect of treatment of CD4 T cells with hBD-2 and 3 on activation and proliferation in vitro. Our data suggest that treatment of CD4$^+$ T cells with hBD-2 or hBD-3 leads to an increase in the presence of CD4$^+$CD69$^+$CD25$^+$ and CD4$^+$CD69$^+$CD25$^+$ T cell subsets following 18hrs and 42hrs in culture. We also observed an enhanced rate of CD4$^+$ T cell proliferation following 96hrs in the presence of hBD-2 or hBD-3 and this was accompanied by an increased IL-2 production in the supernatant of the treated CD4$^+$ T cell cultures. We also showed using intracellular staining that the presence of FoxP3$^+$CD4$^+$CD127$^-$CD25$^+$ Treg cell was increased following treatment with hBD-2 and hBD-3 for 18hrs but the presence of this FoxP3$^+$CD4$^+$CD127$^-$CD25$^+$ cellular subset was reduced by 42hrs in culture. This data is consistent with the finding reported in Chapter 3 using human PBMCs, suggesting that this is a CD4$^+$ T cell intrinsic effect. Additional to our observations regarding FoxP3 expression, we also investigated intracellular CTLA4 expression in CD4$^+$CD127$^-$CD25$^+$ Tregs. Although there was an increase in percentage representation of FoxP3$^+$CTLA4$^+$ cells within the CD4$^+$CD127$^-$CD25$^+$ population of Treg cells, further investigation is still required in order to assess stability and plasticity of the Treg phenotype following treatment with hBD-2 and 3. Thus, in the next chapter, we will investigate the role of hBD-2 and 3 in driving human CD4$^+$ T cell plasticity.
Chapter 5: Regulation of human CD4$^+$ T cell plasticity following treatment with hBD-2 and hBD-3

5.1 Introduction

Differentiation of CD4$^+$ T helper cells into distinct functional phenotypes is crucial for immune homeostasis. These subsets are defined as separate lineages based on the expression of selective signature cytokines and master transcriptional factors (Zhou et al., 2009, Lazarevic et al., 2013). Upon antigen presentation, CD4$^+$ naïve T cell can polarize to become one of a variety of different effector phenotypes, including Th1, Th2, Th17, follicular T helper cells (Tfh) and Treg cells. The direction of polarisation is determined and regulated by the cytokines in the microenvironment and the strength of TCR activation signal (Zhou et al., 2009).

Recent studies have demonstrated that hBDs can play a role in the regulation of T cell cytokine production. It is reported that hBD-2 can enhance mRNA level and secretion of IFN-γ, TNF-α, IL-1β, IL-6, IL-10 and IL-22, and reduce those of IL-17 in αCD3/28-stimulated CD3$^+$ T cells (Kanda et al., 2011). Interestingly, these are all cytokines that display either pro or anti-inflammatory effect and as such, it would be expected that they be secreted at an inflamed site.

Additionally, hBD-3 has been reported to induce IL-2 and IL-10 production, but not IFN-γ (Meisch et al., 2013). All three cytokines are involved in regulating T cell function and differentiation. These studies also demonstrated that hBD-2 and hBD-3 are capable of regulating STAT3 and STAT1 phosphorylation, respectively, two important regulators of helper T cell differentiation and functions (Kanda et al., 2011, Meisch et
Several cytokines, in particular, have been implicated in driving T cell plasticity. For example, IL-6 together with TGFβ has been shown to be involved in Th17 differentiation (Korn et al., 2009). IL-10 has been shown to induce Treg differentiation (Bilate and Lafaille, 2012), while IFN-γ can induce Th1 differentiation (Cope et al., 2011). Hence, we hypothesized that hBD-2 and hBD-3 may be capable of regulating the differentiation of these T cell subsets by modulating the expression of these cytokines.

Both human and murine β-defensins have also been shown to play a role in T cell mediated immune-regulation. Navid et al. (2012) demonstrated that mBD-14 was involved in the induction of differentiation of mouse Treg cells. In their research, the treatment of CD4+ T cells with mBD-14 in mouse can induce expression of the molecular signature of Tregs, such as FoxP3, CTLA4 and neuropilin expression. In addition, the injection of mBD-14 into C57BL/6 mice was shown to suppress the induction of contact hypersensitivity by inducing the differentiation of Ag-specific Tregs in an IL-10 dependent fashion (Navid et al., 2012).

Taken together, these studies suggest that β-defensins have the capacity play a role in the regulation of T cell polarisation and plasticity. However, little is known about the molecules involved in driving this process. In this chapter, we show that treatment of human CD4+ T cells with hBD-2 and hBD-3 is capable of regulating expression of the master transcriptional factors in Th1, Th17 and Treg cells.
5.2 Results

5.2.1 Intracellular staining of RORγt, T-bet and FoxP3 in human CD4⁺ T cells culture at 18hrs

In Chapter 4, we demonstrated that treatment of purified CD4⁺ T cells with hBD-2 and hBD-3 can induce T effector cell activation and proliferation whilst also apparently inducing Treg cells expansion. Based on this apparently conflicting data, we hypothesized that treatment of T cells with hBD2 and 3 might induce differentiation of Treg cells but, that subsequently, these cells lose their ability to suppress the differentiation of Teff cells. To determine the identity of the T cells following treatment, intracellular staining was undertaken, analysing the expression of T-bet, RORγt and FoxP3, as the hallmark transcriptional factors of Th1, Th17 and Tregs respectively (Josefowicz et al., 2012, Korn et al., 2009, Lazarevic et al., 2013). As shown in Figure 5.1, when focussing on the CD69⁺CD25⁺ T cell subset, there was a significant increase in the presence of RORγt⁺T-bet⁺ T cells but not RORγt⁺T-bet⁻ or RORγt⁻T-bet⁺ subsets, following treatment with hBD-2 and hBD-3 for 18hrs in culture, suggesting that there is an induction of differentiation of a T cell subset which displays characteristics of both Th1 and Th17 cell (Figure 5.2A). The expansion of the RORγt⁺T-bet⁺ T cell subset represents an expansion of an intermediate phenotype (Th1/17) in between Th1 and Th17 (Geginat et al., 2013). It has been suggested that, a double positive Th1/Th17 cell is capable of producing pro-inflammatory cytokines, including IFN-γ and IL-17 (Kleinewietfeld and Hafler, 2013). Also, according to the study by Lee et al. (2012), T-bet can play an essential part in pathogenic Th17 cells induction. This suggests that the co-incubation with hBD-2 or hBD-3 may induce a more pro-inflammatory phenotype, Th1/17, by 18hrs and the induction of T-bet⁺ may cause the pathogenicity of Th17 cells. This finding is consistent with a role for the defensins in inducing autoimmune
inflammatory conditions such as IBD and colitis (Ramasundara et al., 2009, Ho et al., 2013).

Additionally, we found that when analysing the CD69⁺CD25⁺ cells (Figure 5.1), which we expected to contain more Treg cells, we, once again, observed a significant increase of RORγt⁺T-bet⁺ dual expressing cells when co-incubated with hBD-2 or hBD-3 (p=0.11) (Figure 5.2A). There was also a significant increase or close to significant increase in this CD69⁺CD25⁺ subset towards increased expression of RORγt⁺T-bet⁺ putative Th1 T cells in the presence of hBD-2 and hBD-3 (p=0.07) (Figure 5.2A). Further characterization of RORγt and T-bet expression also performed in CD25⁺CD127⁻ Treg cells revealed that there was a significant increase of RORγt⁺FoxP3⁺ phenotype in these CD25⁺CD127⁻ Treg subset when co-cultured with hBD-2 or hBD-3. This suggested co-incubation with hBD-2 or hBD-3 induces an increase in the number of FoxP3⁺ cells within the CD25⁺CD127⁻ population of Tregs but additionally, some of these FoxP3⁺ cells also appeared to express T-bet (Figure 5.2B). This is consistent with the finding in Chapter 4, in which we reported a significant increase of the percentage of FoxP3 staining in Tregs by 18hrs. In addition, we also found a trend towards a decrease of in the presence of RORγt⁺FoxP3⁻ subset when treated with hBD-2, and a significant decrease of the RORγt⁺FoxP3⁻ subset when treated with hBD-3, whereas the percentage of T-bet⁺FoxP3⁺ cells were significantly increased when treated with hBD-2 or hBD-3. This suggests that, by 18hrs in culture, CD25⁺CD127⁻ Treg may differentiate into an intermediate phenotype (Th1/Treg, T-bet⁺FoxP3⁺ cells, Figure 5.2B) that has characteristics of both Th1 and Treg cell following treatment with hBD-2 or hBD-3. These so-called Th1-like Treg cells observed with treatment with hBD-2 and hBD-3 has been reported to secret IFN-γ (Dominguez-Villar et al., 2011), which are
also associated with several autoimmune disease in humans, such as Type I diabetes (T1D) (Kleinewietfeld and Hafler, 2013).

**Figure 5.1** Gating strategy for intracellular staining of RORγt and T-bet staining in CD69+CD25+ T effector cells or CD69−CD25+ Treg cells following 18hrs culture. Purified human CD4+ T cells were stimulated using αCD3/28 antibodies and treated with hBD-2 (10µg/ml) or hBD-3 (10µg/ml) for 18hrs. Gating was done on the CD69+CD25+ subset or CD69−CD25+ subset, within these two gates, the percentages of RORγt′T-bet′, RORγt′T-bet+RORγtT-bet+ cells were measured respectively.
Figure 5.2 Intracellular staining of RORγt, T-bet and Foxp3 of human CD4+ T cells at 18hrs with the treatment of hBD-2 or hBD-3. A. Fold change analysis performed according to the percentage of different subsets collected from at least 5 independent experiments (n=5). B. Fold change analysis performed according to the percentage of different subsets gating on Treg population from 5 independent experiments (n=5). The
significance of the differences observed in fold changes was assessed by One-way ANOVA, *p<0.05.

5.2.2 Intracellular staining of RORγt, T-bet and FoxP3 in human CD4+ T cells culture at 42hrs.

In addition to the data observed at 18hrs, we investigated the effect of treatment co-incubation with hBD-2 and hBD-3 for 42hrs on T cell function (Figure 5.3). In contrast to the 18hrs data, there was an increased population of RORγt+T-bet- effector T cells in the presence of hBD-2 (p=0.08) or hBD-3 (p=0.06) (Figure 5.4A) possibly suggesting the differentiation of the Th1/Th17 cell observed at 18hrs towards the Th17 phenotype at this later time point. Also, when analysing the CD69+CD25+ Treg cell population, there was a similar significant increase of RORγt+T-bet- subset in the presence of hBD-3 (Figure 5.4A). This suggests that, following 42hrs in co-culture, Treg cells may be induced to differentiate to become a RORγt+ Treg phenotype which is capable of further differentiation into an IL-17 producing effector T cells (Mercer et al., 2014). Furthermore, when analysing the effect of hBD-3 treatment on CD25+CD127- Treg cells, we found a significant increase of RORγt+Foxp3- and T-bet+Foxp3- subsets and significant decrease of RORγt+Foxp3+ and T-bet+Foxp3- subsets (Figure 5.4B) suggesting a loss of FoxP3 expression in these treated T cells. This data suggests that hBD-3 may induce differentiation away from Treg phenotype towards Th17 and Th1 phenotype. However, when analysing the presence of Tregs form following hBD-2 treatment, we only found a significant induction of T-bet+Foxp3- subset, and a trend of increase in RORγt+Foxp3- (Figure 5.4 B). Interestingly, a closely significant difference between hBD-2 and hBD-3 in the induction of RORγt+Foxp3- subset may reveal the difference between hBD-2 and hBD-3 that hBD-3 is more capable of promoting Th17 differentiation or maintaining the stability of Th17 phenotype. It is consistent with the
data that we obtained using our IL-17A ELISA which data showed that treatment with hBD-3, but not hBD-2 significantly induced IL-17A production by CD4\(^+\) T cells in the culture (Figure 5.5). Because IL-6 plays a key role in driving Th17 differentiation as well as IL-17A production, it became important to investigate whether the expression of this cytokine is regulated by either treatment with hBD-2 or hBD-3. We then performed an IL-6 ELISA and interestingly, we found that hBD-3 but not hBD-2 induced a significant increase of IL-6 secretion, this data is consistent with the finding that IL-17A secretion is also increased following treatment with hBD3 but not hBD2 suggesting that treatment with hBD-3 specifically induces the differentiation of a Th17 like phenotype, in part, by up regulating expression of IL-6 (Figure 5.5). However, whilst hBD-2 also drives the differentiation of a Th17 like phenotype this may not be a pathogenic Th17 phenotype as there is no increase in IL-17A secretion observed. Additionally, we found that both hBD-2 and hBD-3 treatment significantly increased the level of IL-10 in the supernatant of CD4\(^+\) T cell culture, suggesting that hBD-2 and hBD-3 are capable of inducing the anti-inflammatory factor that can negatively regulate immune response.

In addition, we found that both hBD-2 and hBD-3 are capable of inducing IL-10 production in purified CD4\(^+\) T cells. It is consistent with the finding that hBD-2 induced IL-10 production in human CD3\(^+\) T cells (Kanda et al., 2011), however, it was not found that the level of IL-10 was significantly changed by the treatment of hBD-3 (Kanda and Watanabe, 2012). Because CD3\(^+\) T cells consist of CD4\(^+\) T cells, CD8\(^+\) T cells, NKT cells and HLA DR\(^+\) T cells (Orru et al., 2013), in future studies, we should further characterize the effect of hBD-2 and hBD-3 on the other T cell subsets.
Figure 5.3 Gating strategy for intracellular staining of RORγt and T-bet in CD69+CD25+ T effector cells or CD69−CD25+ Treg cells following 42hrs culture. Purified human CD4+ T cells were stimulated using αCD3/28 antibodies and treated with hBD-2 (10µg/ml) or hBD-3 (10µg/ml) for 42hrs. Gating on the CD69+CD25+ subset or CD69−CD25+ subset, the percentages of RORγt+T-bet−, RORγt+T-bet+, RORγt−T-bet−, and RORγt−T-bet+ cells were measured respectively.
Figure 5.4 Intracellular staining of RORγt, T-bet and Foxp3 of human CD4+ T cells at 42hrs with the treatment of hBD-2 or hBD-3. A. Fold change analysis performed according to the percentage of different subsets collected from at least 5 independent experiments (n=5). B. Fold change analysis performed according to the percentage of
different subsets gating on Treg population from 5 independent experiments (n=5). The significance of the differences observed in fold changes was assessed by One-way ANOVA, *p<0.05, **p<0.01.

Figure 5.5 Cytokine ELISA for IL-6, IL-10 and IL-17A production in the supernatants of T cell culture. One-way ANOVA was performed for significant analysis with Turkey as post-test. Data were collected from 5 independent experiments (n=5). *p<0.05, **p<0.01.

5.2.3 Phenotyping of CD4^+CD25^+ T cells in the presence or absence of hBD-2 and 3

To understand the effect on plasticity of treatment of activated CD4^+ T cell co-cultured with hBD-2 or hBD-3, CD4^+CD25^+ T cells were gated on, and analysed following for expression of T-bet, RORγt and FoxP3 following intracellular staining. In Figure 5.6, phenotypes in of the stimulated control were set to 1 fold (blue line) and the fold changes of each phenotype following treatment with in the presence of hBD-2 (red line) and hBD-3 (green line) are displayed in the radar charts. The upper left radar chart shows that, following 18hrs co-culture with hBD-2 or 3, activated CD4^+ T cells tends appear to differentiate towards a Th1-like phenotype regarding in respect to the expression of the transcriptional factor, so that the distribution of the hexagon extends to the Th1 side and not to the, whereas Th17 proportion is hold backside.
However, interestingly, following 42hrs co-culture with hBD-2 (Figure 5.4, upper right), the differentiation of CD4⁺CD25⁺ T cell moves from a Th1-like phenotype to a Th17-like phenotype and following treatment with hBD3 the CD4 T cells take on a more TH17/Treg type phenotype. It has been reported in the literature that IL-6 is able to induce Th1 and Treg cells to differentiate to become Th17 cells (Muranski and Restifo, 2013). Compared to hBD-2, hBD-3 induced CD4⁺CD25⁺ T cell differentiation towards Th17/Treg phenotype and this was accompanied by an increase in IL-6 and IL-17 secretion.

**Figure 5.6** Phenotypes of human CD4⁺ T cell in the presence or absence of hBD-2 and hBD-3. The radar charts show the fold change of different T cell subsets characterized in activated CD4⁺CD25⁺ T cells. Data were collected from 5 independent experiments (n=5).
5.3 Discussion

Previously in Chapter 4, we demonstrated that co-culture of human purified CD4⁺ T cells with hBD-2 together with αCD3/28 causes an up-regulation in the numbers of CD69⁺CD25⁺ Teff and CD69⁺CD25⁺ Treg cells at 18hrs and these numbers remain increasing following 42hrs in culture. However, we also showed that co-culture with hBD-2 and hBD-3 resulted in a significant increase in the percentage of proliferated CD4⁺ T cells suggesting a positive effect on the rate of proliferation observed in T cells which data is seemingly at odds with the previous finding that treatment with hBD-2 appears to induce the differentiation of Treg cells.

Recent studies have demonstrated that there is an “unexpected” plasticity between Treg and Teff cells (Kleinewietfeld and Hafler, 2013). Treg cells are capable of converting into Th1-like cells or Th17-like cells in which RORγt or T-bet is expressed respectively. When this occurs the effects are reported to be pro-inflammatory (Zhou et al., 2009, Nakayamada et al., 2012, Soler and McCormick, 2011). Additionally, this plasticity can also be determined by the strength and duration of T cell stimulation signals (Miskov-Zivanov et al., 2013). It is still unclear whether hBDs play a role in these processes. In Kanda’s research (2011), it was demonstrated that treatment of T cells with hBD-2 could induce secretion of IFN-γ, IL-1β and IL-6, but suppress IL-17 production suggesting a role for hBD-2 in T cell polarisation. IFN-γ is a signature cytokine of Th1 cells and IL-1β and IL-6 can induce Th17 differentiation but with no increase observed in this study in IL-17 secretion it might suggest that differentiation towards Th1 phenotype is a dominant of treatment of T cells with hBD-2. In addition, any role for hBD-2 in inducing the plasticity of Tregs towards another phenotype with a consequent loss of suppressive function needs to be investigated.
Based on these findings, we undertook intracellular staining analysis for the characterisation of the master transcriptional factor of Th1, Th17 and Treg cells. The data presented here suggest that treatment with hBD-2 and hBD-3 induced effector T cells to differentiate into RORγt^T-bet^ Th1/17 phenotype following 18hrs in culture. RORγt^T-bet^ effector T cells are reported to appear in response to viral infection (Hegazy et al., 2010), but this phenotype is also demonstrated to drive chronic inflammation in autoimmune disease (Geginat et al., 2013). This suggests that hBD-2 and hBD-3 not only have an anti-microbial effect, but also have the capacity to induce effector T cells to differentiate towards a more pro-inflammatory phenotype with pathogenic capability such as Th17 cells.

In addition, when considering the data obtained at following 18hrs in culture, we observed that treatment the co-culture with hBD-2 or hBD-3 may induce Treg cells exhibiting to lose in FoxP3 expression and a gain in T-bet expression. This finding suggests that hBD-2 and hBD-3 can not only induce Teff cells to differentiate into Th1/17 pro-inflammatory phenotype, but also trigger Treg plasticity by converting into a Th1-like phenotype, which may cause the loss of their immune-regulatory function and secretion of IFN-γ. This finding is consistent with a role for the defensins in inducing autoimmune disease, such as psoriasis and IBD (de Jongh et al., 2005, Ho et al., 2013).

Despite the fact that T-bet is the master control gene, expression of which drives Th1 cell differentiation and function, it is also reported to play an essential role in Th17 differentiation. It has been revealed that T-bet expression can induce endogenous TGF-β3 expression in Th17 cells and modulate other factors to drive Th17 conversion into a pathogenic phenotype. This makes T-bet expression part of the molecular signature of
pathogenic Th17 cells (Lee et al., 2012). Accordingly, in autoimmune disease, Treg cells may lose FoxP3 expression, but increase RORγt expression and produce IL-17 (Mercer et al., 2014, Joller and Kuchroo, 2014). It is unclear in the literature whether hBD-2 or hBD-3 can induce Treg cells to differentiate into Th17 phenotypes. We found that treatment with hBD-2 or hBD-3 enhanced T-bet expression in both Teff and Tregs by 18hrs. We then observed a significant increase of RORγt expression in both Teff and Tregs by 42hrs in culture in the presence of hBD-2 and hBD-3. This expression pattern is consistent with a Th17-like phenotype in both effector T cells and Tregs. This suggests that with further treatment with hBD-2 or hBD-3, CD4+ T cells may be converted via a Th1-like intermediary cell into a Th17-like T cell. However, surprisingly, we observed that only co-culture with hBD-3 induced RORγt expression in Treg cells but hBD-2 did not. Consistent with this phenomenon, hBD-3 did, but hBD-2 did not, induce IL-17A production following 42hrs in culture. As IL-6 is the key factor to drive Th17 differentiation, IL-6 secretion in the cultures was measured and the data suggested that co-culture with hBD-3 induced an increase in IL-6 production which would in turn, lead to increased IL-17 secretion. Taken together, these data demonstrate that treatment with hBD-2 and hBD-3 may modify the effector T cells immune response and also drive Treg cells to lose Foxp3 expression thus also influencing the homeostasis of the immune response. The consequences of this latter finding may lead to defective Treg suppression and convert Tregs into Th1-like or Th17-like phenotype which could potentially drive further inflammation.
Figure 5.7 A proposed schematic model summarising the key findings in Chapter 5. A and B. RORγt and T-bet expression in CD69$^+$CD25$^+$ T cells or CD69$^-$CD25$^+$ T cells. C.
The plasticity of Treg cells characterised by RORγt and T-bet expression. n.s. denotes no significant difference.

5.4 Conclusion

In summary, we report a role of hBD-2 and hBD-3 in human CD4+ T cells activation, differentiation and proliferation. Our *in vitro* study demonstrated that treatment with hBD-2 and hBD-3 could not only induce Teff cells to differentiate into RORγt+T-bet+ cells, they could also trigger Treg cells to express RORγt and T-bet which may induce Tregs plasticity converting from Treg to Th1/17-like phenotype by 18hrs post activation stimulus. By 42hrs, treatment with hBD-2 and hBD-3 induced both effector and Treg cells to differentiate towards a Th17-like phenotype and away from the Th1-like phenotype. Compared with hBD-2, treatment with hBD-3 induced the appearance of Treg cells expressing more RORγt which in turn produced IL-17A. Consistent with this, it was treatment with hBD-3 but not hBD-2 that induced the production of IL-17A as well as IL-6, which is a key inducer of Th17 polarization in T cells.
Chapter 6: Function assay of human CD4⁺CD25⁺CD127⁻ Treg cells in the presence of hBD-2 in vitro

6.1 Introduction

Recent studies have provided strong evidence that CD4⁺CD25⁺CD127⁻FoxP3⁺ Treg cells appear to be critical in regulating immune response to self-antigens and are able to suppress the proliferation of co-cultured effector T cells (Teffs) (Hartigan-O'Connor et al., 2007, Liu et al., 2006).

As FoxP3⁺ Treg cells play an important role in suppressing the activation and proliferation of Teffs, impaired Treg regulation may result in the failure of immune suppression and contribute to the development of autoimmunity (Costantino et al., 2008). Accordingly, possible causes of impaired Treg-mediated suppression leading to autoimmunity include: 1) defective Treg function; 2) inadequate numbers of Treg cells and 3) resistance of Teff cells to the suppressive function of Tregs (Buckner, 2010). Either just one, or a combination of these events, may lead to defective Treg-mediated suppression.

In Chapter 5, we demonstrated that the treatment of CD4⁺ T cells with hBD-2 induced human Treg cells to differentiate into T-bet⁺ Th1-like phenotype following 18hrs in culture. In addition, following 42hrs co-culture with hBD-2, Treg cells were induced to differentiate into RORγt⁺ Th17-like phenotype. Based on these results, we hypothesized that the treatment of CD4⁺ T cells with hBD-2 may cause defective Treg function which leads to the loss of the suppression on Teff cell activation and proliferation. Thus, in this chapter, we used an in vitro suppression assay to test this possibility.
### 6.2 Results

6.2.1 Treatment of human CD4$^+$ T cells with hBD-2 may not only enhance Teff cell resistance to suppression but also result in a defective ability of Treg cell to mediate suppression.

To investigate the capability of hBD-2 treated Tregs to mediate suppressive action on the proliferation of Teff cell, a Treg suppression assay was developed as shown in Figure 6.1. According to previously published studies documenting the phenotypic characterization of Treg subsets, potent suppressor Tregs are identified as the CD4$^+$CD25$^+$CD127$^-$ subset contained within human peripheral blood. These are distinct from Teff cells which are CD4$^+$CD25$^-$CD127$^+$ (Figure 6.1A) (Liu et al., 2006). Following FACS sorting of these two subsets of cells, Teff (CFSE labelled) and Treg cells were incubated in the presence and absence of hBD-2 for 4hrs. Then, Treg cells were cultured together with Teff cells at Teff/Treg ratio of 1:1 and stimulated with αCD3/28 Dynabeads for 4ds. The various combinations of treated and untreated Tregs and Teff cells used are shown as Figure 6.1B. The effect of hBD-2 on Treg-induced suppression, or Teff resistance to suppression, was compared in each case with the data obtained using non-treated (N) populations of cells, Teff(N)+Treg(N) combination. As shown in Figure 7A, typically, the proliferation of Teff(N) alone is 61.8%. When Teff(N) are co-cultured with Treg(N), the proliferation of Teff is typically decreased from 61.8% to 45.8%. This suggests that the proliferation of Teff cells was suppressed by Treg cells as expected and that the assay is working. Interestingly, Teff(T) cell proliferation increased from 61.8% to 66.9%. This suggests that hBD-2 treatment of this sorted population alone can induce Teff proliferation, which is consistent with previous results shown in Chapter 4. In addition, the percentage of proliferation of Teff in the case of the Teff(T)+Treg(N) combination increased from 45.8% to 64.4%, compared with Teff(N)+Treg(N) (Figure 6.2). This finding suggests that co-incubation of Teff
cells with hBD-2 may enhance the resistance of these Teff cells to Treg mediated suppression. Moreover, when considering the Teff(N)+Treg(T) combination, the proliferation of Teffs increased from 45.8% to 64.6%. This suggests that treatment of Treg cells with hBD-2 may cause these Treg cells to lose their ability to suppress Teff proliferation.

In order to carry out a more detailed analysis of the effects of treatment of Tregs and Teff cells with hBD2, statistical analysis was carried out on the percentage of cells that had undergone, 2,3,4 and 5 cycles of T cell proliferation. The data shown suggests that there is a significant increase of proliferation observed in Teff cells that had undergone 2-5 cycles following their treatment with hBD2 and in the presence of untreated Tregs; Teff(T)+Treg(N). We also found a significant increase in the number of Teff cells that had undergone 2 and 3 rounds of proliferation in the presence of treated Treg cells: Teff(N)+Treg(T). In respect the cells that had undergone 4 or 5 cycles there is a trend towards increasing proliferation but the differences are not significant. Surprisingly, we did not observe an additive effect towards increased proliferation when both treated Teff and Treg cells were cultured together: Teff(T)+Treg(T). This may be due to the possibility that there is an increase in cell death induced by hBD-2 when both treated cells are co-cultured. These data demonstrated that treatment with hBD-2 may cause Treg cells to partially lose their ability to mediate Teff cell suppression as well as cause Teff cells to be resistant to Treg-mediated inhibition. This is consistent with our finding that hBD-2 induces human CD4+ T cell proliferation.
Table 6.1 Codes for different population of Teff and Treg cells with or without treatment of hBD-2.

<table>
<thead>
<tr>
<th>Codes</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Teff(N)</td>
<td>Effector T cells which were NOT treated with hBD-2</td>
</tr>
<tr>
<td>Teff(T)</td>
<td>Effector T cells which were treated with hBD-2</td>
</tr>
<tr>
<td>Treg(N)</td>
<td>Regulatory T cells which were NOT treated with hBD-2</td>
</tr>
<tr>
<td>Treg(T)</td>
<td>Regulatory T cells which were treated with hBD-2</td>
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Figure 6.1 Cell sorting strategy and diagram of the experiment design of the experiment analysing the effects of hBD-2 treatment on Treg suppressive function. A. FACS
sorting of Teff cells and Treg cells. Gates were set according to the results obtained using single stained controls. Interphase cells which may contain CD127\(^+\)CD25\(^+\) cells were excluded in order to obtain a pure population of Teff cells and Treg cells. **B.** Diagram of the experimental design of Treg suppression assay.

**Figure 6.2** The suppression of proliferation of CFSE labelled Teff by Tregs. Histograms showing proliferation of CFSE labelled Teff cells. Teff and Treg cells were co-cultured and analysed for the ability of the Tregs to suppress the proliferation of the CFSE labelled Teff cells. Data was collected after 4ds in culture. Teff cells were gated on as the CFSE\(^+\) population. The percentage of proliferating cells is shown (reduced CFSE fluorescence indicates proliferation).
Figure 6.3 Statistical analysis of fold changes in total number of Teff cells in proliferation or those cells having undergone 2, 3, 4 or 5 cycles only. Bar graphs representing the fold changes of percentage of undergone cycles in Teff proliferation. Data were collected from 7 independent experiments (n=7). Statistical significance: *p<0.05.

6.3 Discussion

In Chapter 5, we demonstrated that, using intracellular staining for the master transcriptional factor of Th1, Th17 and Treg cells, hBD-2 treatment in CD4$^+$ T cells culture induced the differentiation of T cells expressing more than one master transcriptional factor. The data presented in Chapter 5 suggest that hBD-2 induced Treg cells to differentiate into T-bet$^+$FoxP3$^+$ Th1/Treg phenotype following 18hrs in culture. This finding suggests that hBD-2 treatment may induce T cell plasticity by inducing Treg cells to differentiate towards a subset which is potentially an IFN-γ producing Th1-like phenotype while the suppressive activity of the developing Treg population is
suppressed (Kleinewietfeld and Hafler, 2013). By the later time point of 42hrs treatment of CD4+ T cells with hBD-2 may also induce Teff cells to differentiate towards a more pro-inflammatory phenotype with possible pathogenic capability such as Th17 cells. These possibilities are consistent with a role for the defensins in inducing autoimmune disease, such as IBD and colitis.

A recent study by Navid et al. (2012) indicated that a mouse defensin mBD-14 can induce FoxP3, CTLA-4 and Nrp-1 expression in mouse Tregs. However, the induction of FoxP3, CTLA-4 and Nrp-1 expression is only thought to correlate with the induction of Treg phenotype but does not necessarily equate to an enhancement of Treg function. Hence, we developed a Treg suppression assay using hBD-2 treated or non-treated Tregs and Teff to investigate any effect of treatment of these cells with hBD-2 on both Treg suppressive function and the resistance of Teff against Treg inhibition. It was our original aim to further characterise whether hBD-2 could cause Treg cells to be incapable of properly inhibiting Teff proliferation, or alternatively, to induce resistance of Teff to Treg cell mediated inhibition thus explaining our increase in proliferation of effector T cells in the presence of hBD2. In our study, the data demonstrated that, with Teff:Treg ratio of 1:1, hBD-2 treated Tregs were incapable of mediating proper suppressive function compare to non-treated Tregs. Additionally, hBD-2 treated Teffs were observed to be partially resistant to the suppression function of Tregs. Together, these data suggest that treatment with hBD-2 may indeed dampen down Treg suppressive ability whilst simultaneously increasing Teff resistance to Treg immunoregulation in vitro. Interestingly, Kanda et al. (2011) suggested that treatment with hBD-2 is able to induce the phosphorylation of JNK, ERK, and Akt in human T cells. It is suggested that PI3K-Akt signalling activated by CD28 can inhibit FoxP3 expression, which leads to the loss of stability of Treg differentiation and function (Luo
and Li, 2013). These findings raise the possibility that treatment with hBD-2 may influence the CD28 co-stimulation pathway to regulate FoxP3 expression.

In Chapter 5, we reported our finding that the treatment of CD4+ T cells with hBD-2 increased IL-10 production following 42hrs in culture. This phenomenon suggests an induction of Treg function. However, according to the review by Shevach (2009), it is pointed out that in vitro Treg suppression may not be mediated only by IL-10 but may require the presence of additional factors. This may explain the conflict between increased IL-10 production and decreased Treg mediated suppression. It is reported that IL-35 and galectin-1 may be the soluble suppressor cytokines which mediate Treg suppression in vitro (Shevach, 2009). IL-35 is a member of the IL-12 heterodimeric cytokine family and consists of two subunit encoded by two different genes, Ebi3 (express a subunit of IL-27) and Il12a (express a subunit of IL-12). It has been shown that expression of both Ebi3 and Il12a mRNA is up-regulated in Treg cells that perform normal suppressive function. Interestingly, Treg cells isolated from both Ebi3−/− and Il12a−/− knockout mice exhibit repressed inhibitory activity in vitro when Teff cells are stimulated with αCD3/28 coated beads (Shevach, 2009). Additional to IL-35, galectin-1 is another candidate that plays a role in Treg mediated in vitro suppression. Galectin-1 is a member of a highly conserved family of β-galactoside binding proteins, which is involved in Treg-Teff or Treg-DC interactions (Garin et al., 2007). Galectin-1 protein is expressed preferentially in Treg cells and its expression is up-regulated on T cell receptor (TCR) activation. Blockade of galectin-1 protein markedly down-regulated the inhibitory functions of both human and mouse Treg cells. Also, Treg cells from galectin-1-deficient mice exhibited diminished Treg cell activity (Shevach, 2009). Thus, these findings suggest alternative factors which may alter Treg function when using αCD3/28 coated beads as stimulus of T cell activation in vitro. In future studies, further
investigation should focus on the role of these candidates, such as IL-35 and galectin-1, in mediating defective Treg function in the presence or absence of hBD-2. APCs can also be used to replace αCD3/28 coated beads as the stimulus of T cell activation to validate the effect of hBD-2 on Treg function in terms of Treg–APC interaction.

More recently, Bruhs et al. (2015) demonstrated that hBD-3 induced human CD4⁺CD25⁻ T cells to express the Treg specific markers, FoxP3, CTLA4 and glycoprotein A repetition predominant (GARP), following 24hrs pre-incubation in vitro before activation. Their suppression assay using these hBD-3-pretreated CD4⁺CD25⁻ T cells suggested an increase in percentage suppression of proliferating responder cells compared to the non-treated CD4⁺CD25⁻ T cells (Bruhs et al., 2015). These findings suggest that the role of hBD-3 in Treg cell differentiation and function is to promote Treg function in the periphery. These results are clearly at odds with the results reported in this chapter and so the reasons for this should be explored. The differences may be caused by two reasons: Firstly, in the case of the study reported here, Teff cells and Treg cells were pre-treated with hBD-2 for 4hrs before activation in our function assay, while in the study of Bruhs et al., cells were pre-treated for 24hrs prior to setting up the suppression assay. Future studies should focus on effect of hBDs pre-treatment on CD4⁺CD25⁻ T cells for different periods of time. Secondly, the antibody-coupled beads used in Bruhs et al. study were preloaded with anti-CD3, anti-CD28 and anti-CD2, whereas in our assay, we used the beads only coupled with anti-CD3 and anti-CD28. CD2 signalling has been suggested to induce FoxP3 expression and enhance CD4⁺CD25 high Treg cell function (De Jager et al., 2009) as well as being able to down-regulate the expression of Bim, a pro-apoptotic factor in Treg cells, thereby increasing the survival of Treg cells (Kashiwakura et al., 2013). In our study, we did not apply
anti-CD2 stimulation and this may have decreased the stability of the Treg population in our cultures.

Figure 6.4 A proposed schematic model summarising the key findings in Chapter 6. A. the balanced Treg suppression can control the proliferation of Teff cells so as to inhibit pro-inflammation. B. hBD-2 pre-treatment can cause the impaired Treg function which may lead to pro-inflammation. C. hBD-2 pre-treatment can also cause enhanced resistance of Teff cells to Treg suppression.
6.4 Conclusion

In this chapter, our data suggest that the treatment of human CD4$^+$ T cells with hBD-2 may cause repressed Treg ability to inhibit Teff proliferation. At the same time, we found that hBD-2 treatment also caused an increase in Teff cell resistance to Treg mediated immunosuppression in vitro thereby presenting evidence that the apparent loss of immunoregulatory function observed in our previous chapters in the presence of hBD2 may result from two different hBD-2 mediated effects occurring simultaneously.
Chapter 7: Microarray analysis of the transcriptome of human CD4\(^+\) T cells in the presence or absence of hBD-2

### 7.1 Introduction

In the previous chapters, we demonstrated that treatment of human CD4\(^+\) T cells with hBD-2 induced an up-regulation of the Treg subset following 18hrs in culture *in vitro*, but drove these expanded Treg cells to express ROR\(\gamma\)t by 42hrs in culture and to differentiate into a Th17-like phenotype. The results of our suppression assays suggested that treatment with hBD-2 caused Treg to be defective in their ability to mediate suppression of Teff leading to enhanced Teff cell proliferation *in vitro*. In addition, hBD-2 treated Teff cells were found to exhibit resistance to Treg suppression *in vitro*. These data suggest that treatment with hBD-2 may play a role in regulating human CD4\(^+\) T cell functions. However, the gene expression profile of human CD4\(^+\) T cells following hBD-2 treatment is yet to be characterised. In this chapter, using microarray analysis, we demonstrated the differences between the gene transcriptome profiles of purified human CD4\(^+\) T cells activated by \(\alpha\)CD3/28 antibodies in the presence or absence of hBD-2. We also validated the microarray data using RT-PCR of several candidate genes which were found to contribute to the difference in gene expression profile between hBD-2 treated and non-treated samples.

### 7.2 Results

#### 7.2.1 hBD-2 regulates human CD4\(^+\) T cell gene expression at 18hrs and 42hrs.

To investigate the transcriptional profile associated with CD4\(^+\) T cell activation in the presence or absence of hBD-2, we analysed the mRNA profile of *in vitro* stimulated human CD4\(^+\) T cells using whole exon expression microarrays. We performed Principal
Component Analysis (PCA) to compare the gene transcriptome profiles of purified CD4+ T cells activated by αCD3/28 antibodies in the presence of absence of 10μg/ml hBD-2 at 18hrs and 42hrs post activation stimulus. Figure 7.1 shows the PCA result of samples including stimulated control (Stim Co.) and hBD-2 treated stimulated samples (hBD-2 treated). First of all, in Figure 7.1 A2-6, samples are clearly separated into two groups using Principal component (PC) 1 which is the largest difference observed between transcriptomes following 18hrs and 42hrs cultures. This reflects the difference in the gene expression profiles between 18hrs (circle) and 42hrs (triangle) cultures, suggesting that the phenotypes of the cells following 18hrs in culture are quite distinct from the phenotypes of the cells following 42hrs in culture. However, in the case of PC2, we did not find any significant difference of clustering of samples between the two different time points. Interestingly, in the case of PC3 (Figure 7.1 C1-2, C3-6), although the samples randomly distribute in the plots, the 18hrs hBD-2 treated samples (red circles) are clearly separated from 18hrs Stim Co. samples (black circles). Whilst not being significant, this reflects a trend towards differences between the gene expression of Stim Co. and hBD-2 treated samples. In addition, samples co-incubated with hBD-2 for 42hrs (red triangles) grouped and separated from 42hrs Stim Co. samples (black triangles) in PC6 (Figure 7.1, F1-5), suggesting differences in gene expression between Stim Co. and hBD-2 treated samples following 42hrs culturing. However, neither of these components can significantly distinguish samples according to different treatment and time points, as there are overlaps between groups (Figure 7.1, C6 and F3). Therefore, 18hrs samples and 42hrs samples need to be analysed separately to identify differentially expressed candidate genes in hBD-2 treated and non-treated cells at different time points.
Figure 7.1 PCA analysis of all stimulated samples. Human CD4$^+$ T cells were isolated and activated using αCD3/28 antibodies in the presence or absence of hBD-2 (10µg/ml). After 18hrs and 42hrs culturing, RNA samples were purified and gene expression analysed using Affymatrix microarray. A-F and 1-6 represent the 2 dimensions of the PCA results and help to identify the individual graphs in the PCA results. The solid grey ovals are used to highlight the samples clustered in Comp3 and the dash grey ovals are used to highlight the samples clustered in Comp6.

To identify the genes that are differently expressed in hBD-2 treated and non-treated cells, samples from 18hrs and 42hrs were analysed using PCA separately. Figure 7.2 shows the PCA results using 18hrs Stim Co. and hBD-2 treated samples. It is found that the hBD-2 treated samples were distinguished from Stim Co. samples in PC2 which exhibits 42% variability, rather than in PC1 or PC3. This reflects the fact that the transcriptome of hBD-2 treated samples is distinct from the transcriptome of Stim Co.
samples and the candidate genes in PC2 contribute to this variation. Then, we analysed the samples cultured for 42hrs using PCA and the result showed that the hBD-2 treated samples separated from Stim Co. in PC3 which has 35% variability, but not in PC1 or PC2 (Figure 7.3). This suggests that the genes pulled out in PC3 contribute to the different expression profile in hBD-2 treated cells from Stim Co. cells.

**Figure 7.2** PCA results of stimulated samples at 18hrs. The black circles represent the two samples of stimulated control, and the red circles represent the two samples of hBD-2 treated samples. Samples are clearly separated by their treatment conditions in the Comp 2, suggesting that the genes in this component contribute to the difference of transcriptome profiles between hBD-2 treated and non-treated samples. A, B, C and 1, 2, 3 represent the 2 dimensions of the PCA results and help to identify the individual graphs in the PCA results.
Figure 7.3 PCA results of stimulated samples at 42hrs. The black circles represent the two samples of stimulated control, and the red circles represent the two samples of hBD-2 treated samples. Samples are clearly separated by their treatment conditions in the Comp 3, suggesting that the genes in this component contribute to the difference of transcriptome profiles between hBD-2 treated and non-treated samples. A, B, C and 1, 2, 3 represent the 2 dimensions of the PCA results and help to identify the individual graphs in the PCA results.

In order to further characterise gene expression profiles in the hBD-2 treated samples compared to the non-treated control samples, we used GeneSpring GX 13.1 software to normalize the microarray data and perform statistical analysis. The results were summarized as a gene list of 24 candidates that were transcriptionally regulated by hBD-2 treatment (Figure 7.4, Appendix 7.1). These genes were found to be differently
expressed by fold change greater than 1.5, which is considered as biologically relevant. The functions of these genes include RNA binding in mitochondria (Mt-td, Mt-tm), DNA replication (Hist1h2aa, Cdrt1512), cell apoptosis (Ddx46), NFκB signalling (Tnip3), chemoattraction (Ccl1), non-coding RNA (Rasa2-it1) and pseudogenes (Rnu4, Rnu6. Amongst these genes, we found that chemokine C-C motif ligand 1 (Ccl1) was down-regulated in hBD-2 treated CD4$^+$ T cells. As discussed in Chapter 6, we demonstrated that the co-incubation of CD4 T cells with hBD-2 induces defected Treg suppressive function. Thus, we hypothesized that genes involved in Treg function may be differently expressed in hBD-2 treated samples at 18hrs or 42hrs. When we looked at particular genes, we found that both Ccl1 and Ctla4 contributed to the variation observed between treated and untreated samples at 18hrs post activation stimulus in PC2 (Figure 7.5 A). This indicates that both these genes were differently expressed between hBD-2 treated and untreated CD4$^+$ T cells. Further verification of these results was performed using qPCR which shows that expression of both Ccl1 and Ctla4 was down-regulated following 18hrs in culture of hBD-2 treated samples relative to the untreated control (Figure 7.5 C and D). It has been reported the neutralization of CCL1 can inhibit Treg suppressive function but not compromise Teff function (Hoelzinger et al., 2010). Additionally, Treg expression of CTLA-4 was necessary for immune suppression and prevention of in vivo autoimmunity (Tai et al., 2012, Bilate and Lafaille, 2012, Josefowicz et al., 2012). Therefore, these data suggest that the in vitro co-culturing with hBD-2 may induce down-regulation of both Ccl1 and Ctla4 gene expression in human CD4$^+$ T cells which leads to defective ability to meditate Treg suppressive function.

In addition, we reported in previous chapters that hBD-2 treatment of CD4 T cells induced RORγt expression at 42hrs, indicating that hBD-2 may promote polarisation
towards a Th17-like CD4\(^+\) T cell phenotype. In our ELISA data, it suggested that hBD-2 treatment of CD4 T cells hardly induced both IL-6 and IL-17 production upon \(\alpha\)CD3/28 stimulation. This indicates that there might be other pathways to enhance and maintain ROR\(\gamma\)t expression in the presence of hBD-2. Accordingly, IL-21 is able to induce ROR\(\gamma\)t expression in the absence of IL-6 (Muranski and Restifo, 2013, Korn et al., 2009). Therefore, we looked into our microarray data and found that \textit{Il21} gene expression contributed to the variation of hBD-2 treated samples and the control samples (Figure 7.5 A). Further validation using qPCR shows that hBD-2 induced \textit{Il21} gene expression following 18hrs in culture (Figure 7.5 E). These data indicate that even though hBD-2 did not induce IL-6 production, it could up-regulate IL-21 expression which in turn might increase the level of ROR\(\gamma\)t expression. However, because \textit{Il23r} mRNA expression was not significantly changed (Figuer 7.5 F), this suggests that hBD-2 may not induce a stable Th17 cell polarization and subsequent differentiation of pathogenic Th17 cells, as the stability and expansion of pathogenic Th17 requires IL-23R expression (Muranski and Restifo, 2013, Lee et al., 2012). This is consistent with our finding in Chapter 5 that the co-incubation with hBD-2 did not induce IL-17 production \textit{in vitro}.
**Figure 7.4** Heatmap of 24 genes whose expression changed more than 1.5 fold following 18hrs hBD-2 treatment. Statistical analysis was performed using Student $t$ test and $p$ value less than 0.01 was considered as significant. High scores (reds) correspond to up-regulated, and low scores (blues) correspond down-regulated genes in hBD-2 treated CD4$^+$ T cells (Stim + hBD-2) when compared with the non-treated samples (Stim Co.).
**Figure 7.5** PCA analysis of genes regulated by hBD-2 treatment. **A** and **B.** PCA shows the genes that contribute to the components which make the samples distinct between Stim Co. and hBD-2 treated samples. **C-F.** qPCR validation of genes isolated from PCA analysis. Data were collected from 5 independent experiments (n=5).

**7.2.2 Preliminary results of qPCR indicate that certain genes are differently expressed in hBD-3 treated human CD4$^+$ T cells relative to the untreated control.**

To investigate whether there is similar regulation of gene expression in human CD4$^+$ T cells treated with hBD-3, one set of RNA samples were purified from CD4$^+$ T cells cultured together with or without hBD-3. The preliminary data obtained shows that treatment with hBD-3 may induce a decrease of *Ccl1* expression (Figure 7.5 A). This effect is similar to that observed following treatment of CD4$^+$ T cells with hBD-2.
(Figure 7.4 B). However, interestingly, we found a trend of increase of Ctlα4 gene expression in the presence of hBD-3, which is the reverse effect to that observed following treatment with hBD-2. This is consistent with our flow cytometry data indicating an up-regulation of intracellular CTLA-4 expression in CD4⁺CD127⁻CD25⁺ subset following co-culture with hBD-3. These data suggest that treatment with hBD2 and hBD3 may induce different effects on CD4⁺ T cells. This may be due either to the cell being able to distinguish which hBD it is responding to thereby inducing a different set of signals in the target cell, or alternately that treatment with hBD3 delivers a more potent signal than treatment with hBD2. Moreover, the qPCR results show that there was up-regulation of Il21 expression following 18hrs and 42hrs in culture (Figure 7.5 C). This finding indicates that treatment with hBD-3 may up-regulate IL-21 expression which in turn may induce Th17 differentiation. This is similar to our finding in hBD-2 treated cells (Figure 7.4 C). However, interestingly, unlike hBD-2, co-incubation with hBD-3 also up-regulated Il23r expression following 18hrs in culture, and this trend increased following 42hrs in culture (Figure 7.5 D). These data suggest that treatment with hBD-3 may induce the expression of IL-23R which leads to a number of downstream events, including stabilization of Th17 polarization, pathogenic Th17 differentiation and IL-17 cytokine production. These possibilities are consistent with the ELISA data presented in Chapter 5 in which treatment with hBD-3, but not hBD-2, induced up-regulation of IL-17 secretion by CD4⁺ T cells following 42hrs in culture.
Figure 7.6 Preliminary results of qPCR showing genes differently expressed in hBD-3 treated human CD4\(^+\) T cells (n=1).

7.3 Discussion

In previous chapters, we reported the effect of hBD-2 and hBD-3 treatment on human CD4\(^+\) T cell activation and differentiation. Our data presented in this thesis demonstrated that treatment of CD4\(^+\) T cells with hBD-2 and hBD-3 induced CD4\(^+\) T cell activation by up-regulating the expression of CD69 and CD25 at 18hrs and 42hrs following an activation stimulus. We also reported that co-culturing of CD4\(^+\) T cells with hBD-2 or hBD-3 induced Teff cells to differentiate towards a Th1/17-like phenotype by 18hrs post-activation stimulus and towards a Th17-like phenotype at 42hrs post-activation stimulus. In addition, our Treg suppression assay showed that hBD-2 treatment lead not only to Tregs with defective function but also Teff cell exhibiting resistance to Treg mediated suppression. However, the regulation of target
gene expression of CD4+ T cells following treatment with hBDs is still not well understood.

In this chapter, we use the microarray study to investigate the transcriptome profile of αCD3/28 stimulated human CD4+ T cells in the presence or absence of hBD-2. Our PCA analysis revealed that variation in certain components reflected the different gene expression in hBD-2 treated CD4+ T cells relative to the untreated control. This is the first time that the CD4+ T cell transcriptome profile was shown to be differently expressed following treatment with hBD-2.

The PCA data and qPCR data presented here demonstrated that treatment with hBD-2 induced down-regulation of Ccl1 and Ctla4 expression but up-regulated Il21 expression in vitro. In addition to hBD-2, our preliminary data shows that treatment with hBD-3 may have a similar effect as hBD-2 in the down-regulation of Ccl1 expression but interestingly, causes an up-regulation of expression of Ctla4, Il21 and Il23r. It has been reported that hBD-2 and hBD-3 play a role in the pathophysiology of colitis and colitis-associated microflora (Ho et al., 2013). Our data presented here has important consequences for understanding how the expression of hBD-2 and 3 is involved in the regulation of gene expression in CD4+ T cells which in turn may drive the CD4+ T cell intrinsic pro-inflammatory response. Our findings suggest that hBD-2 may down-regulate Ccl1 gene expression at 18hrs post activation stimulus. CCL1 has been reported to play a role in mediating Treg suppressive function and anti-CCL1 treatment has been developed as a tumour immunotherapy by which the suppressive function of tumour-associated Treg cells can be inhibited in order to enhance the Teff cell immune response against cancer cells (Hoelzinger et al., 2010). The down-regulation of CCL1 gene expression by hBD-2 may be the cause of the observed defective Treg function.
Additionally, we reported that treatment with hBD-2 may also down-regulate CTLA-4 mRNA expression *in vitro*. CTLA-4 interacts with co-stimulatory molecules CD80/CD86 on APCs to down-regulate or prevent T cell co-stimulation signalling pathway (Shevach, 2009). This mechanism is involved in Treg–mediated suppression, in addition to its important cell intrinsic role in limiting responses of activated T cells and the role of CTLA-4 in maintenance of homeostasis can be significantly modified by the genetic background (Josefowicz *et al*., 2012). Thus, the down-regulation of CTLA-4 expression in the presence of hBD-2 may reflect the impaired ability of Treg cells interaction with APCs, leading to defective Treg suppressive function.

In addition, it is the first time that an up-regulation of *Il21* expression in the presence of hBD-2 has been reported. IL-21 has been reported to be involved in driving Th17 lineage development (Korn *et al*., 2009). At the early stage of Th17 development, IL-21 is secreted by early Th17 cells and thus performs a ‘self-amplification autocrine loop’ via the IL-21 receptor. Its level of expression is also increased following IL-1 signalling (Muranski and Restifo, 2013). Our data suggested that hBD-2 treatment caused an increase of *Il21* mRNA expression. This may reveal the fact that treatment with hBD-2 could potentially encourage the early Th17 development. However, Th17 polarization requires IL-23/IL-23R signalling to maintain the stability of Th17 lineage which is important for the matured Th17 cells (Korn *et al*., 2009). Interestingly, we did not find significant change of *Il23r* expression level in hBD-2 treated CD4\(^+\) T cells compared the non-treated control, but the preliminary data on hBD-3 treated cells showed an up-regulation of *Il23r* expression level. These findings may suggest that Th17 polarization induced in the presence of hBD-2 may not be as stable as that induced following treatment with hBD-3, leading to the immature Th17 phenotype. This is consistent with our IL-17A ELISA data indicating that hBD-3, but not hBD-2,
induced IL-17A production in CD4+ T cells. We also found that hBD-3 treatment could enhance \textit{Il23r} expression. \textit{Il23r} is reported to play an essential part in the development of pathogenic Th17 cells (Lee \textit{et al.}, 2012). This suggests that compared to hBD-2, hBD-3 alone may be capable of inducing the pathogenic phenotype of Th17 cells.

In addition, we also found that the expression of some other genes were differently regulated in hBD-2 treated human CD4+ T cells which have been confirmed to be associated with RNA binding in mitochondria (\textit{Mt-td, Mt-tm}), DNA replication (\textit{Hist1h2aa, Cdrt15l2}), cell apoptosis (\textit{Ddx46}), NFκB signalling (\textit{Tnip3}), non-coding RNA (\textit{Rasa2-it1}) and pseudogenes (\textit{Rnu4, Rnu6}). Interestingly, Tnip3 was found to be up-regulated by hBD-2 treatment. TNIP3 is reported to be a negative regulator of NF-κB signalling, which is also involved in the signalling events downstream of TNF-α and in particular in the regulation of NF-κB signalling (Nair \textit{et al.}, 2009, Vereecke \textit{et al.}, 2009). Recent studies reported that TNIP3 was found to be up-regulated in Crohn’s Disease biopsies (Noble \textit{et al.}, 2010) and psoriasitic skin lesions (Johnston \textit{et al.}, 2013). This suggests that hBD-2 up-regulated TNIP3 expression which may in turn suppress NF-κB signalling leading to the blockade of IL-17A cytokine production. Moreover, our findings suggest the involvement of epigenetic regulation, such as regulation of the \textit{His1h2aa} gene which encodes a replication-dependent histone which is a member of the histone H2A family. The role of this gene in human T cell function is still not well understood. It is reported that the down-regulation of His1h2aa is correlated with the development of necrotizing enterocolitis in neonatal mice, suggesting the association with pro-inflammatory conditions (Jung \textit{et al.}, 2015). Further validation of these targets need to be done in future studies.
**Figure 7.7** A proposed schematic model summarising the key findings in Chapter 7. A. CCL1 contributes to the maintenance of Treg function. Down-regulation of CCL1 by hBD-2 or hBD-3 may lead to the impaired Treg suppression. B. the difference between hBD-2 and hBD-3 treatment *in vitro*. hBD-2 induces CD4⁺ T cells to produce IL-21 and differentiate to become RORγt⁺ T cells. However, the lack of IL-6 induction limits the production of IL-17A. In contrast, hBD-3 induces both IL-6 and IL-21 which can, in turn, promote IL-17A production, leading to pro-inflammation. Preliminary data suggests an up-regulation of *IL23r* gene expression following the treatment of hBD-3, this finding will require further characterisation in future studies.

### 7.4 Conclusion

In this chapter, we demonstrated that hBD-2 treatment of CD4⁺ T cells regulated gene expression following αCD3/28 treatment for 18 and 42hrs *in vitro*. Our microarray data revealed chemokine *Ccl1* as a potential target gene product responding to hBD-2 treatment. The blockade of CCL1 has been reported to inhibit Treg suppressive function.
Further investigation of gene expressions using qPCR revealed that hBD-2 treatment also down-regulated *Cila4* expression and up-regulate *Il2l* expression, suggesting a role for hBD-2 in regulating CD4⁺ T cell plasticity and Treg function, which is consistent with the results of our polarization and function studies presented in previous chapters. In addition, our preliminary qPCR data showed the different patterns of expression in a number of key target genes in hBD-3 treated CD4⁺ T cells compared with hBD-2 treated cells.
Chapter 8: General Discussion and Future Directions

8.1 General discussion

We initially demonstrated in Chapter 3 that co-culture of human PBMCs with hBD-2 together with anti-CD3/28 caused an up-regulation in numbers of CD69\(^+\)CD25\(^+\) T cells by 18 hrs and these numbers remain increased following 40 hrs in culture. Characterisation of FoxP3 expression also revealed an up-regulation of this transcription factor in the CD4\(^+\)CD25\(^{high}\) T cell subset. These data are consistent with published findings which reveal that mBD-14, an ortholog of hBD-2, is capable of inducing CD4\(^+\)CD25\(^-\) T cells differentiate into CD4\(^+\)CD25\(^+\) Tregs in mice (Navid et al., 2012). We also observed that expression of FoxP3 is significantly reduced following 42 hrs in culture following treatment with 10 \(\mu\)g/ml of hBD-2 in these same CD4\(^+\)CD25\(^{high}\) T cells. It has been suggested that the diminished FoxP3 expression in CD4\(^+\)CD25\(^{high}\) Tregs may diminish their suppressive ability. Thus, we analysed T cell proliferation using CFSE stained PBMCs and revealed that hBD-2 treatment enhances the proliferation of CD4\(^+\) T cells and CD8\(^+\) T cells \textit{in vitro} following 72 hrs and 96 hrs, suggesting that there may be a defect in the ability of treated Tregs to suppress Teff proliferation. However, this did not rule out the possibility that treatment with hBD-2 may induce the resistance of Teffs to regulation by Tregs. In addition, we observed an increase of the CD69\(^-\)CD25\(^-\)CCR6\(^+\) T cell population but not CD69\(^+\)CD25\(^-\)CCR6\(^-\) T cell population in the presence of hBD-2 or hBD-3 at 18 hrs. This suggested that co-culture with hBD-2 or hBD-3 either enhanced CCR6 expression in early activated CD69\(^+\)CD25\(^-\) T cells or that the CCR6\(^-\) cells were specifically responding to treatment with hBD2. Interestingly, this effect was not maintained at 42 hrs. By 42 hrs, only CD69\(^-\)CD25\(^-\)CCR6\(^-\) and CD69\(^+\)CD25\(^-\)CCR6\(^-\) subsets appeared to respond to hBD-2 and hBD-3 suggesting that there may have been some degree of apoptosis induced in
the CCR6\(^+\) cells. Alternatively, as both of the responding subsets observed at 42hrs are CCR6\(^-\) cells, it may suggest that there are alternative receptors or pathways by which hBD-2 and hBD-3 may induce CD4\(^+\) T cell activation by hBD-2 and hBD-3 that are independent of expression of CCR6.

In the following chapter, Chapter 4, we moved away from the use of mixed cells and focussed on purified CD4\(^+\) T cells in order to investigate whether or not the observed effects were intrinsic to CD4\(^+\) T cells or required the presence of another cell type. We demonstrated that co-culture of purified T cells with hBD-2 and hBD-3 together with an activation stimulus mediated by αCD3/28 continued to induce an increase in the presence of CD69\(^-\)CD25\(^+\) and CD69\(^+\)CD25\(^-\) subsets at both 18hrs and 42hrs post activation stimulus. Additionally, we observed that by 96hrs post stimulus there was an increase in CD4\(^+\) T cell proliferation in the presence of hBD-2 and hBD-3 as had been observed in the mixed cell cultures. Analysis of IL-2 secretion by purified T cells using ELISA shows that addition of hBD-2 and 3 to cultures induces increased IL-2 production by CD4\(^+\) T cells \textit{in vitro}. These elevated levels of IL-2 may promote the increased levels of T cell proliferation mentioned above. Characterisation of intracellular FoxP3 expression showed an increase in FoxP3 expression in CD4\(^-\)CD127\(^-\)CD25\(^+\) Tregs following co-incubation of hBD-2 or hBD-3 for 18hrs. These data suggested that co-culture with hBD-2 and hBD-3 may induce Treg differentiation by 18hrs \textit{in vitro}. However, this effect is apparently reversed by 42hrs in culture. At this later time point, we found that FoxP3 expression decreased in the CD4\(^+\)CD127\(^-\)CD25\(^+\) Treg population. These data indicate that Treg suppressive function may be potentially defective following the co-incubation of purified T cells with either hBD-2 or hBD-3 for 42hrs \textit{in vitro} due to the apparent loss of FoxP3 expression. It has been extensively demonstrated in the literature that Treg-mediated suppression of Teff cell activation and
proliferation depends on the stability of FoxP3 expression within the Treg compartment, especially in conditions using αCD28 for co-stimulation in vitro. Thus, the data presented in this chapter demonstrate a role for hBD-2 and 3 in driving human CD4$^+$ T cell activation and proliferation. These results also reveal that co-incubation of T cells with hBD-2 and 3 in vitro may exert an effect on Treg cell function and, possibly, plasticity thereby reducing the ability of Treg cells to mediate their suppressive function and, as a consequence, allowing for an increase in activation and proliferation.

Recent studies have demonstrated that hBD-2 treatment in vitro induces cytokine production in human CD3$^+$ T cells (Kanda et al., 2011). In the presence of hBD-2, secretion of IFN-γ was found to be up-regulated in the activated CD3$^+$ T cells using αCD3/28 (Kanda et al., 2011), suggesting that hBD-2 may promote a Th1 polarizing environment. Thus, we hypothesized that hBD-2 may push CD4$^+$ T cells to differentiate towards a Th1 phenotype and away from a Treg phenotype. In Chapter 5, we characterised the role of hBD-2 and hBD-3 in driving human CD4$^+$ T cells polarisation. Our in vitro data suggests that treatment with hBD-2 and hBD-3 can not only induce Teff differentiation into RORγt$^+$T-bet$^+$ (Th17/Th1) cells, but can also trigger the differentiation of regulatory T cell (Treg) expressing RORγt and T-bet rather than the master controller of Treg function, FoxP3. This apparent plasticity of T cell phenotype allowing them to convert from Treg to Th1/17-like effector T cell occurred following 18hrs in culture and in the presence of the activation stimulus provided by αCD3/28. Interestingly, by 42hrs in culture, treatment with hBD-2 and hBD-3 induced both Teff cell and Treg cell differentiation towards the Th17-like phenotype. Compared with treatment with hBD-2, treatment with hBD-3 induced a more pronounced effect in terms of Treg cells expressing increased levels of RORγt in CD4$^+$ T cells. This elevated expression may, in turn, be responsible for the induction of higher IL-17A secretion.
Consistent with this idea, it was found that treatment with hBD-3 but not hBD-2 was capable of inducing the higher level of secretion of IL-17A. Additionally, treatment with hBD-3 induced an increased expression of IL-6. IL-6 is a cytokine, which is described as having the ability to drive the differentiation of naïve T cells to IL-17 secreting Th17 cells. Thus, this study explored the function of hBD-2 and hBD-3 in regulating CD4⁺ T cell plasticity which could potentially result in altered Treg and Teff cell differentiation and function.

It is of interest that a number of papers have been published documenting a loss of FoxP3 in Tregs in the clinical environment such as in the case of psoriasis (Soler and McCormick, 2011, Joller and Kuchroo, 2014, Lowes et al., 2014). Psoriasis is a chronic inflammatory skin disease mediated by T cells and dendritic cells. Activated T cells contained within psoriatic lesions discretely secrete IFN-γ, IL-17 and IL-22, suggesting that Th1 and Th17 cells are involved in the development of pathology of psoriasis (Lowes et al., 2014). Clinical studies showed that Tregs isolated from patients with psoriasis appeared to exhibit an enhanced RORγt expression whilst simultaneously losing FoxP3 expression. These cells were capable of differentiating to become IL-17A-secreting cells on ex vivo stimulation (Bovenschen et al., 2011). It has also been suggested that the numbers of both FoxP3⁺ Treg cells and Th17 cells are increased in patients with psoriasis, however, the suppressive function of Treg cells in these patients was found to be decreased (Furuhashi et al., 2013, Zhang et al., 2015), indicating the dysfunction of Tregs under these inflammatory conditions. Accordingly, FoxP3⁺ Tregs in psoriatic lesions can be induced to differentiate to become FoxP3 and RORγt dual-expressing cells that converge on the IL-17-expressing Tregs, potentially polarizing in certain situations towards the pathogenic Th17 lineage (Soler and McCormick, 2011). Interestingly, the IL-17-expressing Tregs were still found to display potent suppressive
function as well as the expression of IL-17. The presence of Tregs in the skin is beneficial to the maintenance of the balance between host immunity and commensal bacteria under non-pathogenic conditions (Mercer et al., 2014). However, under pathogenic conditions, such as in the case of psoriasis, IL-23, expressed in the inflamed lesion can down-regulate FoxP3 expression and promote Treg cells to differentiate into pathogenic Th17 cells (Soler and McCormick, 2011, Lowes et al., 2014), leading to chronic inflammation. Our data presented in this chapter demonstrated that upon treatment of hBD-3, CD4\(^+\) T cells may become dual expressing Treg cells with the induction of ROR\(\gamma\)t expression in the FoxP3\(^+\) Treg population. There is also a simultaneous enhancement of IL-17A secretion. These findings lend support to the hypothesis that treatment with hBD-2 and 3 may cause aberrant polarisation of the T cell compartment leading to the onset of pathology associated with psoriasis.

The level of IL-6 was also found increased in the hBD-2 treated CD3\(^+\) T cell culture activated by \(\alpha\)CD3/28 co-stimulation, whilst the level of IL-17 decreased (Kanda et al., 2011). However, in CD4\(^+\) T cell culture treated with hBD-2, we did not find significant change of the levels of IL-6 and IL17. Because CD3\(^+\) T cell population includes CD4\(^+\) T cells, CD8\(^+\) T cells, NKT cells and HLA DR\(^+\) T cells (Orru et al., 2013), it is possible that the other T cell subsets can respond to hBD-2 treatment to secret IL-6 but also induce an alternative pathway to restrict Th17 differentiation to limit IL-17 production. Future studies should investigate the effect of hBD-2/3 treatment on cytokine production by non-CD4\(^+\) T cells, such as CD8\(^+\) cell cytokine production.

As we had reported the loss of FoxP3 expression and the gain of ROR\(\gamma\)t expression in CD4\(^+\) T cells by 42hrs in the presence of hBD-2, we hypothesized that treatment with hBD-2 may cause Treg cells to lose their suppressive function as they lose expression of
FoxP3 and as a consequence will not be able to control Teff proliferation. Additionally, with the emergence of RORγt+ Teff cells, these effector T cells may become resistant to the suppressive action of Tregs. Thus, in this chapter, we aimed to test these two possibilities in vitro by culturing together FACS-sorted Treg (CD4+CD127−CD25+) and Teff (CD4+CD127+CD25+) cell populations isolated from human peripheral blood samples. Our data suggested that treatment of Treg cells with hBD-2 may dampen down their ability to induce suppression of Teff cell activity. Interestingly, co-culture with hBD-2 would also appear to increase Teff cell resistance to Treg immunoregulation in vitro.

The data reported so far suggests that hBD-2 has the capacity to regulate human CD4 T cell differentiation and so it became important to try to characterise some of the molecular events that mediate this regulation.

To this end, we performed microarray gene analysis in order to investigate the difference between the transcriptome profile of stimulated human CD4+ T cells with or without treatment of hBD-2. This analysis demonstrated, firstly, that Ccl1 is a potential gene responding to hBD-2 treatment. The neutralization of CCL1 has been reported to inhibit Treg communication to Teff cells, leading to the reduced suppressive function in tumour-associated Treg cells (Hoelzinger et al., 2010). The down-regulation of this gene by hBD-2 treatment may therefore be involved in mediating the impaired suppressive function of Treg cells in our in vitro culture. Secondly, further analysis using principle component analysis (PCA) followed by validation using qPCR demonstrated that hBD-2 treatment down-regulated Ctl4 gene expression in CD4+ T cells whilst up-regulating Il21 gene expression. These results are consistent with our findings that hBD-2 regulates Treg function and Th17 differentiation. In contrast to
hBD-2 treated cells, the preliminary data from hBD-3 treated cells revealed a different pattern of gene expression which shows that hBD-3 may also up-regulate Il23r gene expression, leading to a pathogenic Th17 phenotype differentiation.

8.2 Future directions

8.2.1 Using naïve T cells

In the periphery, naïve T cells are activated and polarized in response to their cognate antigen to become different subsets, such as Th1, Th2, Th17 and Treg cells (Lazarevic et al., 2013). In our experiments, we used purified CD4+ T cells, which include memory T cells, naïve T cells as well as Treg cells. This mimics the in vivo conditions in the periphery when T cells are involved in immune response. However, memory T cells are antigen experienced T cells which can be activated more quickly and become matured effector T cells. The response by these memory T cells therefore can alter the differentiation and plasticity of naïve T cells by changing the cytokine components in the cell culture. Because of the variation in human peripheral T cell samples, the memory T cells compartments from each participant will have different antigen experience. In future studies, we could focus on purified naïve T cells in order to characterize more effectively the differentiation and plasticity of only naïve CD4+ T cell subsets in the presence of hBDs. The characterization of naïve T cell differentiation can be performed using single-cell RNA sequencing in order to profile the transcriptome in different subsets from the heterogeneous T cell population.
8.2.2 Further investigation on CCL1 signaling

Our microarray data suggested that expression of CCL1 was down-regulated in the presence of hBD-2 which may lead to the impaired Treg-mediated immunosuppression of Teff proliferation. Future studies should further characterise whether hBD-2 and hBD-3 treatment can also reduce the level of CCL1 secretion in to the cell culture. In addition to the detection of secreted CCL1, it would be interesting to know if this defective Treg function can be prevented or overcome by CCL1 treatment, and it is equally important to clarify the mechanisms and the factors involved in the down-regulation of Ccl1 expression by hBD-2. This information can help to develop novel therapeutic agents to restrict the symptoms in a number of autoimmune diseases in which overexpression of hBDs have been reported, such as psoriasis.

Interestingly, the neutralization of CCL1 has been reported to be a potential therapy to limit Treg suppression in cancer immunity (Hoelzinger et al., 2010), hBD-2 and hBD-3 treatment may provide an alternative pathway to down-regulate CCL1 production in order to deactivate Treg cells and promote the effector T cell response. In vivo studies focusing on the effect of hBD-2 and 3 on T cell development in experimental models of cancer, such as melanoma, could be investigated. These experimental mouse models could be treated with defensins to see if the tumour development is inhibited. In addition to the control of cancer cell proliferation, Treg development should also be investigated at the same time. β-defensin knockout mice could be an important model to investigate the role of defensins in tumour immunology.
8.2.3 Further validation of the other genes found in Microarray analysis

Besides Ccl1, we also found other genes differently regulated in hBD-2 treated human CD4+ T cells. Some of them are involved in the biological process, including RNA binding in mitochondria (Mt-td, Mt-tm), DNA replication (Hist1h2aa, Cdrt15l2), cell apoptosis (Ddx46), NFκB signalling (Tnip3), non-coding RNA (Rasa2-itI) and pseudogenes (Rnu4, Rnu6). Although the roles of these genes in human T cells are still not clear, further investigation should be expanded in order to validate the expression of these genes after hBD-2 or hBD-3 treatment using RT-PCR.

8.2.4 Characterization of β-defensins using in vivo models

In this project, our experiments were performed using an in vitro system. So, we should ask the question whether we would observe a similar effect in vivo and how both sets of data could be applied to the understanding of the mechanism of actions of hBD-2 and 3. In vivo, T cells are activated by interacting with MHC-antigen complexes expressed on APCs. The participation of APCs could result in different effects being mediated from those observed using αCD3/28 activation, because Treg can also interact with APCs to inhibit their activities and the secretion of IL-10 by Tregs can limit the level of inflammation occurring in local tissues. However, these processes will not be recapitulated in our in vitro cell cultures. Therefore, we can speculate that, using an in vivo system, following β-defensin treatment, the induction of FoxP3+ Treg phenotype by hBD-2 or hBD-3 might be prolonged or more stable than that observed using our in vitro cultured Tregs. Indeed, recent studies by Navid et al. (2012) and Bruhs et al. (2015) using mBD-14 verify that in vivo treatment of mBD-14 induced the Treg-mediated suppression of contact hypersensitivity (CHS) and experimental autoimmune encephalomyelitis (EAE), respectively. Additionally, our data collected at 42hrs may reflect a scenario whereby there is chronic inflammation induced in which the inflamed
epithelial tissue is continuously secreting β-defensins. The extended treatment of hBD-2 or hBD-3 may lead to a more pro-inflammatory condition causing tissue damage and a higher secretion of IL-6. Thus, future studies could involve the use of animal models displaying more chronic inflammation, such as psoriasis or IBD to characterise the role of continuous secretion of hBD-2 and hBD-3.

8.2.5 DefbΔ9/DefbΔ9 mice

There are many murine β-defensin knockout models. The research group led by Prof. Julia Dorin from University of Edinburgh has developed a mouse model, so called DefbΔ9/DefbΔ9 mice, in which the whole cluster of beta-defensin on chromosome 8 containing 9 mouse β-defensins is knocked-out. Their recent studies mainly focus on the sperm dysfunction and infertility in KO mice. Because β-defensin genes are clustered in a group and respond to the stimuli at the same time, the loss of one beta-defensin can be compensated for by the presence of the other orthologs in the same clusters. Therefore, data collected from these knockout models may show no significant difference between the single gene knockout mice and the wild-type control (Navid et al., 2012). Thus, knocking out the whole cluster can solve this problem. Zhou et al. reported that the partial deletion of β-defensin cluster in chromosome 8 resulted in sperm dysfunction, suggesting the essential role of β-defensins in sperm maturation (Zhou et al., 2013, Dorin, 2015). However, very few studies have used this model in immunology related studies. Also, the knocking out of the whole cluster may create another possibility which is to identify the function of each individual beta-defensin gene, as each of the 9 beta-defensin peptides may be added back one by one. It would be of great interest to use this model to study the role of beta-defensin in the pathology of autoimmune disease. We could potentially induce psoriasis or IBD in this KO model
to see if the pathogenic symptoms can be limited or even worsened. Also, this model can be used to study β-defensin-induced T cell trafficking.

8.2.6 Epigenetic/post-translational level study

Epigenetic regulation in T cell differentiation and function is also a rapidly growing area of research. Treg function is strictly controlled by demethylation of CpG motifs in the TReg cell-specific demethylated region (TSDR) of the foxp3 locus to obtain the normal suppressive function. Recently, Bruhs et al. (2015) revealed that treatment with mBD-14 induces FoxP3 expression in CD4+CD25− murine T cells via demethylation of the CpG sites of the foxp3 promoter.

Additionally, the development of Treg lineage can be regulated by microRNAs. It is reported that microRNA-15b/16 can enhance Treg differentiation (Singh et al., 2015). Recent studies by Okoye et al. (2014) demonstrated that Treg cells can produce exosomes containing microRNA which are capable of suppressing the differentiation of pathogenic Th1 cells. It is interesting to identify whether hBDs can regulate these pathways to alter the translation of other genes which play a key part in Treg function, such as Cell.

8.2.7 Allergy

In Chapter 3, we demonstrated that hBD-2 and hBD-3 treatment resulted in the appearance of of T cells with a Th1-like phenotype following 18hrs in culture. This finding suggests that hBD-2 and hBD-3 may induce a bias microenvironment towards Th1 cell differentiation. This environment can also induce Th2 cells to differentiate into a Th1 phenotype. It has been demonstrated that allergen-specific Th2 cells are involved in the pathology of allergic diseases, such as asthma (Chung, 2015). It is worth
investigating whether this Th1 polarization effect induced by treatment with hBD2 can drive Th2 cells to differentiate into Th1 cells thus reducing the symptoms of these Th2-mediated allergic diseases.

8.3 Concluding remarks

In conclusion, we demonstrated that the treatment of hBD-2 and hBD-3 in vitro induces human CD4⁺ T cell activation and proliferation in both whole PBMCs culture and purified CD4⁺ T cell culture. Additionally, our results reveal a role of hBD-2 and hBD-3 in CD4⁺ T cell differentiation on the point of view of T cell plasticity. The presented data suggest that hBD-2 and hBD-3 treatment up-regulates the percentage of FoxP3⁺ Treg in culture. However, the treatment of hBD-2 and hBD-3 is also capable of inducing T-bet and RORγt expression in both Treg cells and Teff cells, leading to Th1-like and Th17-like phenotype differentiation. Compared with hBD-2, hBD-3 induced Treg expressing more RORγt which induces IL-17A expression. Our Treg suppression assay suggests that hBD-2 may dampen down Treg ability to suppress at the same time that it causes an increase in Teff resistance to Treg mediated immunoregulation in vitro. These findings reveal a role of hBD-2 and hBD-3 in the pathology of T-cell-derived autoimmune diseases, such as psoriasis. Further characterisation using the microarray assay revealed Ccll as potential target genes responding to hBD-2 treatment, providing a useful target for regulating hBD-2 related CD4⁺ T cell immune response or tissue inflammation.
References


'Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation', *J Immunol*, 190(5), pp. 2252-62.


Appendix

Appendix 2.1 NHS Ethical Approval

23 April 2013

Mr. Dawei Chen
School of Health, Sport & Bioscience
UH301 Stratford Campus
Water Lane
University of East London
E15 4LZ

Dear Mr. Chen

Study title: Crosstalk between components of the host innate and adaptive immune system in response to infection by pathogenic and commensal bacteria in the gut.

REC reference: 13/LO/0296
IRAS project ID: 124826

Thank you for your letter of 16 April 2013, responding to the Committee’s request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter.

Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Tom Lucas, tom.lucas@UHBristol.nhs.uk.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.
Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.cofforum.nhs.uk

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centres"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Covering Letter</td>
<td></td>
<td>12 February 2013</td>
</tr>
<tr>
<td>Evidence of insurance or indemnity</td>
<td></td>
<td>26 September 2012</td>
</tr>
<tr>
<td>Investigator CV</td>
<td>2. January 2007</td>
<td>12 February 2013</td>
</tr>
<tr>
<td>Other: CV - David Rowley</td>
<td></td>
<td>12 February 2013</td>
</tr>
<tr>
<td>Other: CV - John George</td>
<td></td>
<td>12 February 2013</td>
</tr>
</tbody>
</table>
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document “After ethical review – guidance for researchers” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/LG/0296 Please quote this number on all correspondence

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hr-a-training/

With the Committee’s best wishes for the success of this project.
Yours sincerely

Pp

Dr David Slovick
Chair

Email: nrescommittee.london-cityroadandhampstead@nhs.net

Copy to: Mr. Merlin Harries
Appendix 2.2  The UREC Sponsorship Confirmation Letter

18 February 2014

Dear Dawei,

<table>
<thead>
<tr>
<th>Project Title:</th>
<th>Crosstalk between components of the host innate and adaptive immune system in response to infection by pathogenic and commensal bacteria in the gut.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Researcher(s):</td>
<td>Dawei Chen</td>
</tr>
<tr>
<td>Principal Investigator:</td>
<td>Dr Susan Outram</td>
</tr>
</tbody>
</table>

I am writing to confirm that the application for the aforementioned NHS research study reference 13/LO/0296 is sponsored by the University of East London.

Please note as a condition of your sponsorship by the University of East London your research must be conducted in accordance with NHS regulations and any requirements specified as part of your NHS ethical approval.

With the Committee’s best wishes for the success of this project.

Yours sincerely,

Catherine Fieulleteau  
Ethics Integrity Manager  
For and on behalf of  
Professor Neville Punchard  
University Research Ethics Committee (UREC)  
Research Ethics Office  
Email: researchethics@uel.ac.uk
Appendix 2.3 Quality Control of Affymatrix Microarray analysis

The figure below shows the profile of hybridization controls analysed using GeneSpring GX 13.1 software. As it is shown in this figure, the profiles of 8 control probes are close and the curves of 4 samples are similar. The profiles reflect the staggered concentration of these 8 probes. The similarity of the curves indicate good hybridization and washing of the arrays.
Appendix 2.4 Calculation for qRT-PCR

The figure below shows a worked example of the calculation of relative expression using the relative standard method. Standard curves are generated for both the gene of interest (in this example, Btk) and the house keeping gene, Hprt. The standard curve is shown below. A 1:10 serial dilution of the genes was carried out. The standard curves for both genes were generated by plotting the Ct values against the Log\textsubscript{10} concentrations of the gene.

The table below shows the average Ct value of the different samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Ct</th>
<th>Average Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{Hprt1}</td>
<td>\textit{Ccl1}</td>
</tr>
<tr>
<td>Stim Co. 18hrs</td>
<td>21.96</td>
<td>25.42</td>
</tr>
<tr>
<td>Stim + hBD-2 18hrs</td>
<td>21.36</td>
<td>26.22</td>
</tr>
<tr>
<td>Stim Co. 42hrs</td>
<td>21.07</td>
<td>29.04</td>
</tr>
<tr>
<td>Stim + hBD-2 42hrs</td>
<td>21.32</td>
<td>29.73</td>
</tr>
</tbody>
</table>
The equation obtained from the standard curve for \textit{Hprt1} and \textit{Ccl1} was used to calculate the gene concentration in the different samples. The table below shows the mean value in Log$_{10}$ of different samples from triplicates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration $\text{(Log}_{10}\text{)}$ \textit{Hprt1}</th>
<th>Concentration $\text{(Log}_{10}\text{)}$ \textit{Ccl1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stim Co. 18hrs</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>Stim + hBD-2 18hrs</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Stim Co. 42hrs</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Stim + hBD-2 42hrs</td>
<td>0.09</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Relative gene expression in each sample was obtained by dividing the concentration of the gene of interest (\textit{Ccl1}) by the housekeeping gene (\textit{Hprt1}). The mean value of relative expression from triplicates is shown below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative expression $\text{Ccl1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stim Co. 18hrs</td>
<td>2.06</td>
</tr>
<tr>
<td>Stim + hBD-2 18hrs</td>
<td>0.89</td>
</tr>
<tr>
<td>Stim Co. 42hrs</td>
<td>0.18</td>
</tr>
<tr>
<td>Stim + hBD-2 42hrs</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Appendix 2.5 Melting curve

An additional programme for melting curve analysis was carried out in order to ensure the product of primer was the expected size and not the result of primer dimer. The image below shows an example of a melting curve.

![Melting Curve Diagram](image)

**Figure 1.** The image of melting curve.

Changes in fluorescence (y axis) with increasing temperature (x axis) was detected. When the temperature increases, the two strands of the amplicon breaks to form single stranded DNA triggering the fluorescent intercalating dye to dissociate from the DNA and stop fluorescing.
Appendix 3.1  Gate strategy for FoxP3 staining in CD4⁺CD25<sup>High</sup> T cells in human PBMCs.

PBMCs were stained and analysed using flow cytometry. To exclude dead cells, a plot of forward scatter (FSC-A) vs. side scatter (SSC-A) plot was set at the beginning of analysis. Lower granularity on SSC-A and FSC-A indicates dead cells. Hence, the viable cells are gated on higher SSC-A and FSC-A signal (upper left). In a mixed cell population, to identify CD4⁺ T cells, CD4 was used as the first marker and it is plotted against the SSC gated in R1 (upper middle). Cells are then analysed for the expression of T cell activation markers, CD25 and CD69 in the CD4⁺ T cell gate (upper right). To identify Foxp3 expression, CD4⁺ T cells were gated with the CD25 (middle). The population was then separated into three fractions. The expression of Foxp3 in each fraction was analyzed (lower). As it is reported, higher amounts of Foxp3 expressed in CD4⁺CD25<sup>High</sup> T cells (Bilate and Lafaille, 2012). Thus, we analyze Foxp3 expression in the fraction of CD4⁺CD25<sup>High</sup> T cells.
Appendix 7.1  A table of 24 genes differentially expressed in hBD-2 treated human CD4\(^+\) T cells at 18hrs.

<table>
<thead>
<tr>
<th>Transcripts Cluster Id</th>
<th>Genes</th>
<th>Regulation in hBD-2 treated samples</th>
<th>Fold change</th>
<th>Genedescription</th>
</tr>
</thead>
<tbody>
<tr>
<td>17100659</td>
<td>Mt-td</td>
<td>down</td>
<td>-2.970</td>
<td>Mitochondrially encoded tRNA aspartic acid</td>
</tr>
<tr>
<td>16843309</td>
<td>Ccl1</td>
<td>down</td>
<td>-1.988</td>
<td>Chemokine (C-C motif) ligand 1</td>
</tr>
<tr>
<td>17016296</td>
<td>Hist1h2aa</td>
<td>down</td>
<td>-1.550</td>
<td>Histone cluster 1, H2aa</td>
</tr>
<tr>
<td>17100651</td>
<td>Mt-tm</td>
<td>down</td>
<td>-1.525</td>
<td>Mitochondrially encoded tRNA methionine</td>
</tr>
<tr>
<td>16832227</td>
<td>Cdrt15l2</td>
<td>down</td>
<td>-1.514</td>
<td>CMT1A duplicated region transcript 15 - like 2</td>
</tr>
<tr>
<td>16786964</td>
<td>Unknown gene*</td>
<td>up</td>
<td>1.517</td>
<td>Unknown</td>
</tr>
<tr>
<td>16946426</td>
<td>Rasa2-it1</td>
<td>up</td>
<td>1.540</td>
<td>RASA2 intronic transcript 1 (non-protein coding), novel transcript</td>
</tr>
<tr>
<td>17009424</td>
<td>Unknown gene*</td>
<td>up</td>
<td>1.548</td>
<td>Unknown</td>
</tr>
<tr>
<td>16989367</td>
<td>Ddx46</td>
<td>up</td>
<td>1.552</td>
<td>DEAD (Asp-Glu-Ala-Asp) box poly peptide 46</td>
</tr>
<tr>
<td>17117545</td>
<td>Loc100131541</td>
<td>up</td>
<td>1.558</td>
<td>Unknown</td>
</tr>
<tr>
<td>16792068</td>
<td>Unknown gene*</td>
<td>up</td>
<td>1.571</td>
<td>Unknown</td>
</tr>
<tr>
<td>16742636</td>
<td>Rnu6-126p</td>
<td>up</td>
<td>1.600</td>
<td>RNA,U6 small nuclear 126, pseudogene</td>
</tr>
<tr>
<td>16942038</td>
<td>Rnu6-1181p</td>
<td>up</td>
<td>1.610</td>
<td>RNA,U6 small nuclear 1181, pseudogene</td>
</tr>
<tr>
<td>16937793</td>
<td>Rnu6-377p</td>
<td>up</td>
<td>1.614</td>
<td>RNA,U6 small nuclear 377, pseudogene</td>
</tr>
<tr>
<td>16938269</td>
<td>Rnu4-85p</td>
<td>up</td>
<td>1.634</td>
<td>RNA,U4 small nuclear85, pseudogene</td>
</tr>
<tr>
<td>16703431</td>
<td>Unknown gene*</td>
<td>up</td>
<td>1.640</td>
<td>Unknown</td>
</tr>
<tr>
<td>16736266</td>
<td>Rnu6-593p</td>
<td>up</td>
<td>1.690</td>
<td>RNA,U6 small nuclear 593, pseudogene</td>
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<tr>
<td>16979444</td>
<td>Tnip3</td>
<td>up</td>
<td>1.692</td>
<td>TNFAIP3 interacting protein 3</td>
</tr>
<tr>
<td>16770254</td>
<td>Unknown gene*</td>
<td>up</td>
<td>1.721</td>
<td>Unknown</td>
</tr>
<tr>
<td>17109675</td>
<td>Unknown gene*</td>
<td>up</td>
<td>1.754</td>
<td>Unknown</td>
</tr>
<tr>
<td>16979466</td>
<td>RNU6-948P</td>
<td>up</td>
<td>1.847</td>
<td>RNA,U6 small nuclear 948, pseudogene</td>
</tr>
<tr>
<td>16970859</td>
<td>RNU6-531P</td>
<td>up</td>
<td>1.861</td>
<td>RNA,U6 small nuclear531, pseudogene</td>
</tr>
<tr>
<td>16694232</td>
<td>Unknown gene*</td>
<td>up</td>
<td>2.012</td>
<td>Unknown</td>
</tr>
<tr>
<td>16914935</td>
<td>Unknown gene*</td>
<td>up</td>
<td>2.021</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* The information of the gene relevant to this probe ID has not been updated in the database (presented as “<N/A>” in Figure 7.4).