# The role of IL-15 in monocyte regulation of T-cell responses to gluten

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## Abbreviations

| | |
|---|---|
| **AA** | Amino acid |
| **Ab** | Antibody |
| **CD** | Celiac Disease |
| **cDC** | Classical DCs |
| **cDNA** | Complementary DNA |
| **CDP** | Common dendritic progenitor |
| **COS** | CV-1 (simian) in Origin, and carrying the SV40 genetic material |
| **CTLL-2** | Cytotoxic T lymphoblastoid line-2 |
| **DCs** | Dendritic cells |
| **DEPC** | Diethylpyrocarbonate |
| **DMSO** | Dimethyl sulfoxide |
| **dNTP** | Deoxyribonucleotide triphosphate |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **FCS** | Fetal calf serum |
| **GMP** | Granulocyte and macrophage precursor |
| **HLA** | Human leukocyte antigen |
| **HS** | Human serum |
| **IFN-γ** | Interferon-γ |
| **IEL** | Intraepithelial lymphocyte |
| **IL-15** | Interleukin-15 |
| **IL-15-LSP** | IL-15 long signaling peptide |
| **IL-15 or IL-2 R** | IL-15 or IL-2 receptor |
| **IL-15-SSP** | IL-15 short signaling peptide |
| **kDa** | Kilo Dalton |
| **JAK** | Janus kinase |
| **LPL** | Lamina propria lymphocyte |
| **mAbs** | Monoclonal antibodies |
| **MDP** | Macrophage and DC progenitor |
| **MPS** | Mononuclear phagocyte system |
| **MHC** | Major Histocomatebility Complex |
| **pABs** | Polyclonal antibodies |

| | |
|---|---|
| **PBMCs** | Periferal blood mononuclear cells |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PDCs** | Plasmacytoid DCs |
| **PHA** | Phytohemaglutinin |
| **qPCR** | Quantitative/real time PCR |
| **RA** | Retinoic acid |
| **RT-PCR** | Revers transcriptase PCR |
| **STAT** | Signal transducer and activator of transcription |
| **TBE buffer** | Tris/Borate/EDTA buffer |
| **TCC** | T-cell clone |
| **TG2** | Transglutaminase 2 |
| **TGF-beta** | Transforming growth factor beta |
| **Th1,2 or17** | T helper cell 1, 2 or 17 |
| **Tregs** | T regulatory cells |

## Abstract

**Background:** Celiac disease (CD) is a chronic T-cell mediated inflammatory disorder of the small intestine. Overexpression of interleukin-15 (IL-15) and interferon-γ (IFN-γ) is important components of the immunopathology in CD, but the function of these cytokines is not fully explored. Furthermore, retinoic acid (RA) has been shown to function as a co-adjuvant together with IL-15 to drive inflammatory immunity against dietary antigens. We have recently found that monocyte-derived $CD14^+CD11c^+$ cells are selectively increased in the active celiac lesion. Here we investigated the capacity of monocytes to activate gluten specific $CD4^+$ T-cell clones (TCCs) in response to IL-15 and IFN-γ, and explored the potential co-adjuvant effect of RA.

**Methods:** Isolated $CD14^+$ monocytes from human leukocyte antigen-$DQ2^+$ (HLA-$DQ2^+$) individuals were pretreated with gluten and different combinations of IFN-γ, IL-15, transforming growth factor-β (TGF-β) and RA, and subsequently incubated with gluten-specific intestinal-derived $CD4^+$ TCCs to assess their stimulatory potential. Moreover, the monocyte responses to such pretreatment were characterized by T-cell proliferation assays, quantitative PCR (qPCR) and flow cytometry, and the T-cell cytokine production in response to pretreated monocytes was analyzed.

**Results:** Pretreating $CD14^+$ monocytes with IL-15 or IFN-γ significantly increased their capacity to induce the proliferation of gluten-specific TCCs, but no significant co-adjuvant effect of RA was detected. Although monocytes did not appear to respond directly to IL-15-stimulation, IL-15-treated $CD14^+$ cells showed membrane bound expression of IL-15, indicating that IL-15 is trans-presented to the T-cells. Interestingly, IFN-γ was found to induce monocyte IL-15 expression at the transcriptional and protein level, and blocking IL-15 in the monocyte-T-cell co-cultures significantly reduced T-cell proliferation.

**Conclusion:** We have demonstrated that IL-15 and IFN-γ strongly enhance the capacity of $CD14^+$ monocytes to activate gluten-specific $CD4^+$ T cells; and the increased T-cell proliferation caused by IFN-γ stimulated monocytes was mostly augmented through IL-15. Our results suggest that intestinal $CD14^+$ cells, by trans-presentation of exogenous or endogenous IL-15 in response to proinflammatory factors, may play an important role in the activation of gluten-reactive $CD4^+$ T cells in the celiac lesion.

# 1. Introduction

## 1.1 General introduction

### 1.1.1 Celiac disease

CD is a common chronic inflammatory disorder of the small intestine, caused by an inappropriate immune response to dietary gluten proteins from wheat, barley and rye in genetically susceptible individuals, resulting in destruction of intestinal epithelium and remodeling of the intestinal mucosa. Gut lesions of most celiac disease patients completely normalize when gluten is excluded from the diet, but symptoms reappear after eating gluten again, thus indicating that gluten is the driving factor. CD occurs in children and adults with a prevalence of about one in 100 [1], and shows a strong HLA association. All patients are either HLA-DQ2 or HLA-DQ8, and patients, but not healthy subjects, have DQ2- or DQ8-restricted gluten-reactive CD4+ T cells in the intestinal mucosa. Although CD4+ T cells are important players in the disease process, T lymphocytes do not recognize protein antigens in its native form. Exogenous antigens, such as gluten, are first captured and processed by other immune cells, so-called antigen presenting cells (APCs), which then present processed antigens for the T cells [2]. Recent studies showed influx of monocytes in the small intestine lamina propria of treated CD patients following gluten challenge [3]. In fact, the increased recruitment of monocytes preceded the appearance of inflamed intestinal lesions, indicating that the monocytes participate in the initiation of the disease. However, knowledge of how the intestinal microenvironment affects the recruited monocytes is scarce. This study focused on the cytokines IL-15 and IFN-γ, both of which are highly upregulated in celiac lesion (1.3.5), and how they affect the capacity of monocytes as APCs

### 1.1.2 Homeostasis in the gut

The human intestine constitutes the “inner body surface”, and this huge surface area, consisting of more than 100 $m^2$ mucosa, is in direct contact with the outside environment. The intestinal mucosal surface is coated by a thick mucus layer, with a single layer of epithelial cells beneath, separating the body and its immune cells from the vast number of commensal bacteria (microbiota) in the gut. The intestinal organ

contain the highest number of leukocytes, thus, to maintain homeostatic conditions the activity of the intestinal immune system must be tightly regulated by several layers of control mechanisms. When maintaining immunological homeostasis we must tolerate the high antigen load of food proteins and the normal gut microbiota which is constantly present in the gut. Otherwise chronic intestinal inflammation would be the result. One important mechanism to maintain homeostatic condition is the mechanism of oral tolerance (1.2.5) which ensures that we do not react inappropriately to soluble proteins.

Like all barrier tissues, the intestine contains an extensive mononuclear phagocyte-network of bone marrow-derived APCs – so-called dendritic cells (DCs) and macrophages. DCs are cells of the immune system, which are specialized to differentiate between microenvironmental cues, like pathogenic or commensal bacteria and harmless antigens. After maturation, DCs migrate to draining lymph nodes, where they stimulate T cells in the context of peptide-MHC molecules. In response to this, naïve T cells have the capacity to differentiate into functionally distinct subsets of T helper cell type (Th) 1, 2, 17 and regulatory T cells (Tregs), depending on the signals received from DCs (Figure 1). Tregs are abundant and important for maintaining immune tolerance in the small intestine, whereas Th1, Th2 and Th17 cells are essential for the elimination of intracellular bacteria and viruses, parasites, and extracellular bacteria and fungi, respectively. Mucosal DCs have a crucial role in generating and shaping immune response to a given stimuli; either by inducing Tregs and promoting immune tolerance under homeostatic conditions, or initiating a productive immune response against infectious agents – triggering Th1, Th2 or Th17 responses depending on the nature of the pathogen [4].

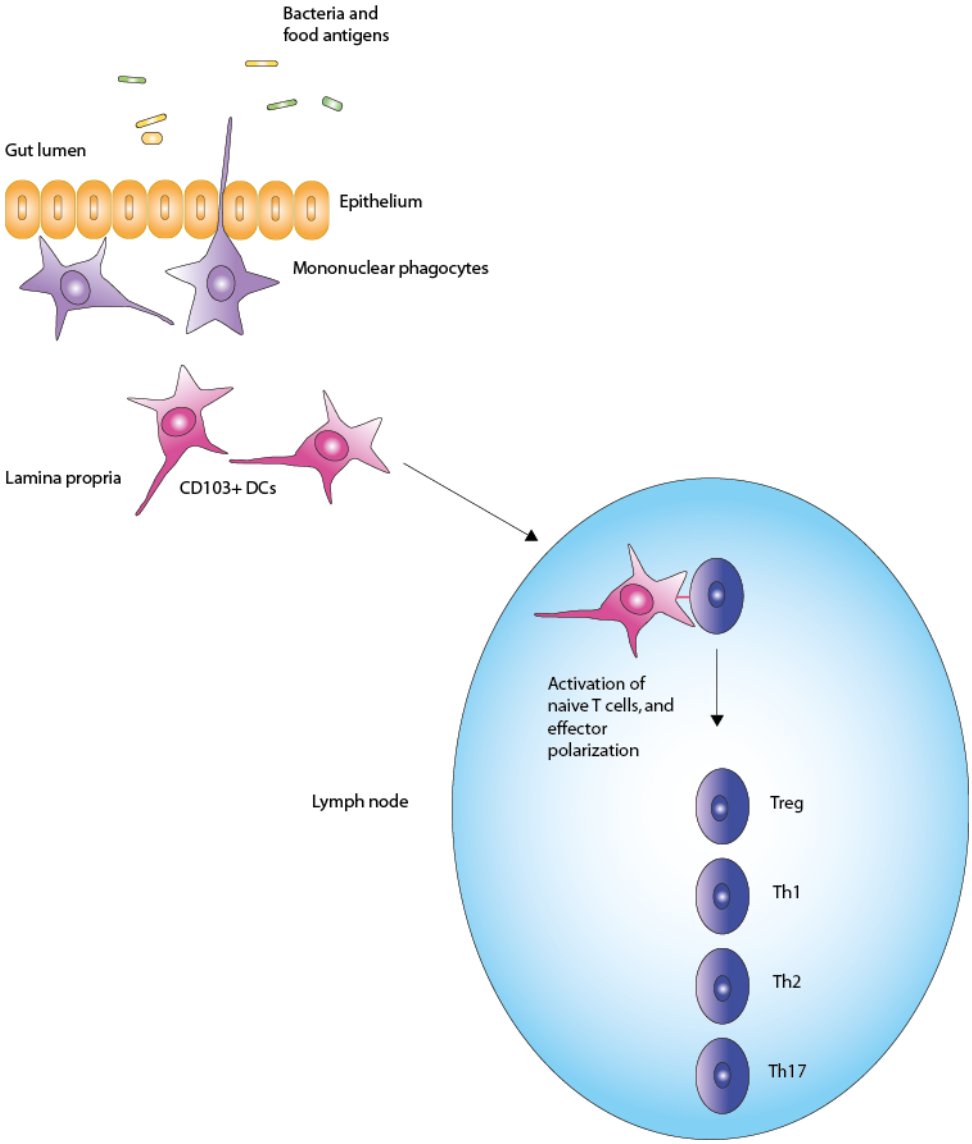


**Figure 1: Priming naïve T-cells.** Mononuclear phagocytes samples antigen from the gut lumen, and dendritic cells (DCs) migrate to draining lymph nodes. Dependent on the micro-environmental stimuli, antigen loaded CD103+ DCs shapes the T-cell response by regulating differentiation of naïve T cells into T regulatory cells, T helper cell type 1 (Th1), Th2 or Th17 cells**.**

## 1.2 The mononuclear phagocytes system

The mononuclear phagocyte system (MPS) is composed of monocytes, macrophages and DCs, and differentiation is determined at the stage of macrophage and DC progenitor (MDP) (Figure 2). MDPs are a subset of proliferating cells in the bone marrow that is devoid of lymphoid, erythroid and megakaryocytic potential, and can either differentiate into monocytes or common dendritic progenitors (CDPs). CDPs can further give rise to plasmacytoid dendritic cells (PDCs) or classical DCs (cDCs), and monocytes may differentiate into to macrophages or inflammatory DCs [5, 6].

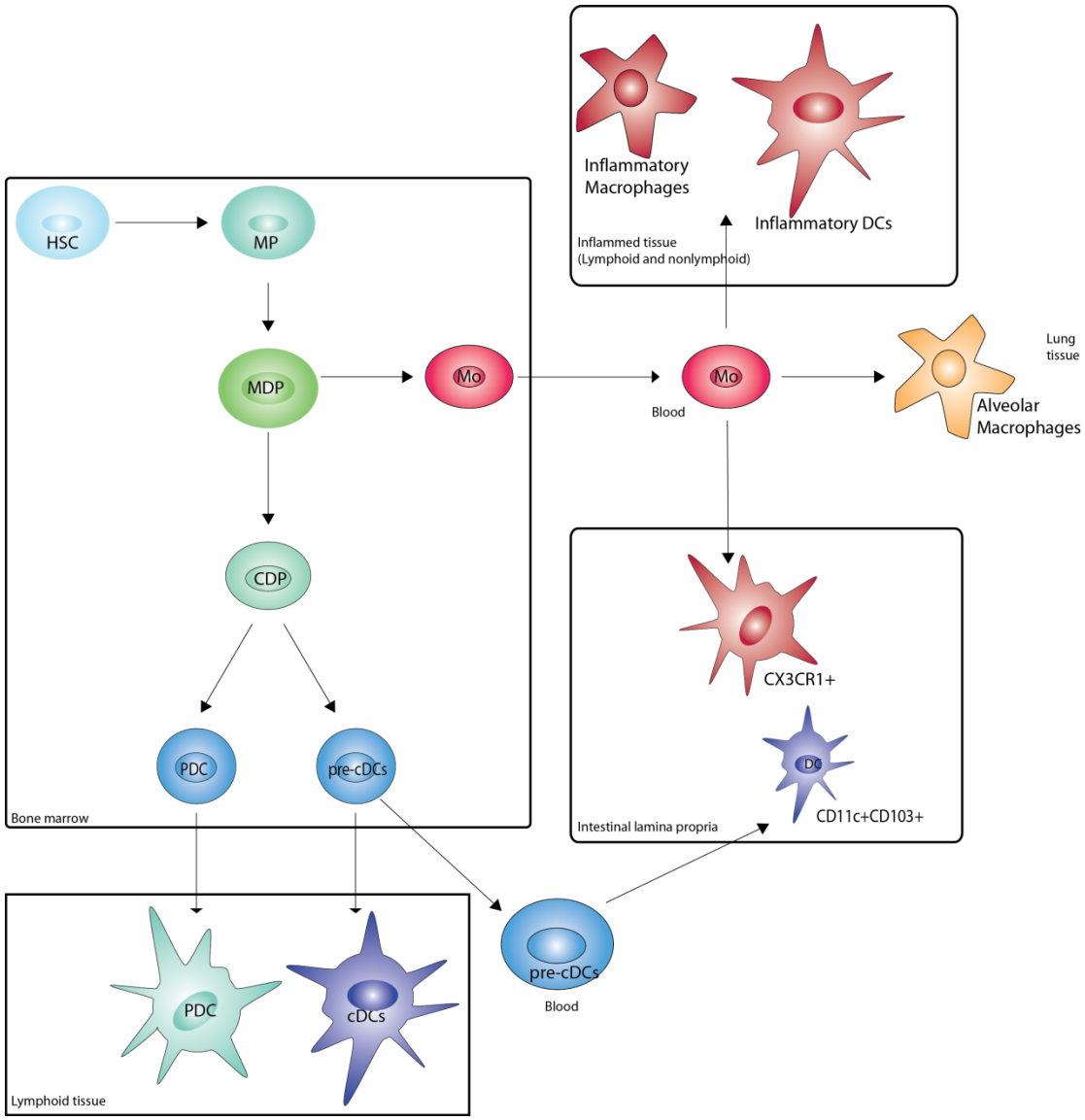


**Figure 2: Differentiation of monocytes, DCs and macrophages in mice.** Hematopoietic stem cells (HSC) produce myeloid committed precursor (MP), giving rise to macrophage and DC precursor (MDP) which may further give rise to monocytes and common dendritic progenitors (CDPs). CDPs give rise to pre-classical dendritic cells (pre-cDCs) or plasmacytoid dendritic cells (PDCs), but have lost the ability to differentiate to monocytes (Mo). Pre-cDCs are found in the blood, bone marrow and spleen. Pre-cDCs may enter lymph nodes from the blood, where they acquire a mature cDC phenotype (CD8+ or CD8-), and intestinal lamina propria, where they give rise to CD11c+CD103+DCs. PDCs also migrate from blood to lymphoid tissue. MDP-derived monocytes circulate in the blood, bone marrow and spleen giving rise to subsets of macrophages and DCs, but not to cDCs and PDCs. Under homeostatic conditions monocytes can give rise to alveolar macrophages, or lamina propria CX3CR1 antigen presenting cells. During inflammation monocytes may differentiate to inflammatory-DCs and macrophages within the tissues[7] .

### 1.2.1 Dendritic cells

DCs are defined by their ability to prime naïve T-cells after migrating to draining lymph nodes [8, 9]. In the mouse intestine, two main populations of cDCs, derived from pre-cDCs, have been described: $CD103^+$ $CD11c^+CD11b^+$ and $CD103^+$ $CD11c^+CD11b^-$ DCs [10]. Monocytes also have the potential to differentiate to DCs, which is well established for intestinal $CX3CR1^+$ DCs and inflammatory DCs in the skin, gut and airways. However, at steady state only the subpopulation of $CD103^+$ DCs emigrates from the lamina propria to the mesenteric lymph nodes, where they effectively present antigens to naïve T cells. Such cDCs are antigen-processing and presenting cells, with high phagocytic activity as immature cells, and high cytokine producing activity as mature cells. In addition, they are highly migratory and can migrate from tissue to lymphoid organs. $CX3CR1^+$ APCs are generally poor T-cell stimulators, and whether they should be defined as DCs or macrophages is controversial [7]. However, a recent study showed that in situations of intestinal bacterial dysbiosis, such cells may serve classical DC functions [11].

### 1.2.2 Monocytes

Monocytes are found circulating in the blood, bone marrow and spleen, and may give rise to subsets of macrophages and inflammatory DCs after recruitment to tissues. Monocytes share many phenotypic functions with DCs, such as their ability to process and present antigen to T cells. They also possess phagocytic function, and the ability to produce inflammatory cytokines [7].

Monocytes were conventionally divided into two subpopulations: classical monocytes expressing HLA-$DR^+$, $CD14^{hi}$, $CD11c^{hi}$, $CD11b^{hi}$ and $CX3CR1^{low}$, or CD16 monocytes expressing HLA-$DR^+$, $CD14^{low}$, $CD11c^+$, $CD11b^+$, $CD16^{hi}$ and $CX3CR1^{hi}$ [12, 13]. More recently, monocyte nomenclature has been further subdivided into three subsets, segregating CD16 positive cells into two populations, based on CD14 expression. thus the current nomenclature consists of $CD14^{++}CD16^-$ classical, $CD14^{++}CD16^+$ intermediate, and $CD14^+CD16^{++}$ non-classical monocytes [14].

Another phenotypic monocyte marker is the chemokine receptor CCR2, which has been shown to be essential for the recruitment of mice classical monocytes, classified as $CCR2^+Ly6C^{hi}$ [15], to inflamed tissues. In the tissues, $Ly6C^+$ monocyte derived

cells express CCR7, and in mice with an inflamed gut such cells were found to be present in lymphatic vessels indicating that they are migratory. Thus it was suggested that monocytes can differentiate into migratory APCs capable of priming naïve T cells [16]. By analogy, $CD14^{+}CD11c^{+}$ monocytes were found to migrate into the small intestine celiac lesion, where they may further mature into potent APCs [3]. However, it has not been directly demonstrated that monocytes can differentiate into migratory DCs in humans.

### 1.2.3 Macrophages

Macrophages from various tissues, such as kuppfer cells, epidermal langerhans cells and microglia have been shown to be derived from the fetal yolk sac, and are maintained by local proliferation [17]. In contrast, the recruitment of monocytes into the small intestinal lamina propria appears crucial for maintaining distinct populations of differentiated tissue macrophages at this site, and these intestinal macrophages has been reported to be non-proliferative [18] [19]. In the steady state intestine of mice, $LyC6^{high}$ monocytes give rise to both inflammatory macrophages ($CXCR1^{low/int}$) and resident macrophages ($CXCR1^{high}$), in comparison with $LyC6^{low}$ monocytes which have little or no potential to replenish these macrophages in the non-inflamed intestine. Hence, $LyC6^{high}$ monocytes give rise to all macrophages, adopting a resident or pro-inflammatory phenotype dependent on the intestinal local environment [20].
Direct evidence for monocyte origin of human intestinal macrophages is lacking. However, although early studies claim that intestinal macrophages are negative for CD14 [21-23], more recent studies indicate that the small intestine contains a population of $CD14^{hi}$ $CD11c^{hi}$ monocytes as well as mature macrophages expressing intermediate levels of CD14 [24, 25]. Tissue macrophages expressing CD14 is compatible with a model in where they are derived from recruited monocytes.

### 1.2.4 Mounting an appropriate immune response

An appropriate immune response depends on the ability of effector and regulatory lymphocytes to migrate from lymph nodes to the site of infection or injury.
In mouse models it has been shown that $CD103^{+}$ DCs in the gut have the ability to induce the gut-homing receptors CCR9 and α4β7-integrin in responding T-cells, and

thereby induce lymphocyte migration to the intestine where antigen was originally encountered [26].

The induction of T-cell gut-homing receptors depends on the capacity of gut DCs to synthesize RA through their expression of the retinal dehydrogenase (RALDH) enzymes. It has been shown in mice that $CD103^+$ DCs express RALDH, and that the activity of RALDH appears to be dependent on RA derived from the intestinal epithelium or the bile [27].

DCs express mRNA for Aldh1a2, the gene encoding RALDH2, and the expression of this gene is particularly high in the $CD103^+$ intestinal DC subset. It has also been reported that induction of Treg development by $CD103^+$ DCs is RA dependent. Generation of Tregs is important for induction of oral tolerance. Indeed, purified intestinal mouse $CD103^+$ DCs have been shown to induce Tregs without the addition of exogenous factors. Blocking the action of TGF-β and RA abrogated their ability to induce Tregs, underlining the importance of RA and TGF-β as anti-inflammatory factors [27].

### 1.2.5 Oral tolerance

Pabst and Mowat [28] have proposed a multistep model of oral tolerance where antigen-loaded $CD103^+$ DCs migrate from the small intestine to the mesenteric lymph nodes; in the mesenteric lymph node, RA produced by these DCs and local stromal cells induce expression of T-cell homing receptors, CCR9 and α4β7, and together with TGF-β promote differentiation of $Foxp3^+$ regulatory T-cells (Tregs). Such Tregs then home back to the small intestinal lamina propria, where they undergo secondary expansion in response to IL-10 produced by tissue macrophages. Some of these Tregs may then exit the lamina propria via the lymph and promote systemic oral tolerance.

In line with this, Coombes et al showed that the induction of tolerogenic T cells by $CD103^+$ DCs require TGF-β and RA acting as a cofactor for inducing this regulatory phenotype. However, TGF-β can together with $CD103^+$ DCs induce Foxp3 expression in naïve T-cells, where increasing amounts of TGF-beta correlate with a more abundant Treg polarization [29].

### 1.2.6 In vivo sources of RA and TGF-β

Vitamin A is obtained from the diet either as all-trans-retinol, retinyl esters or β-caroten. All-trans-retinol and β-caroten can then be oxidized to all-trans-retinal by ubiquitously expressed enzymes, like alcohol dehydrogenases or short chain dehydrogenase reductases. All-trans-retinal can then be further oxidized by RALDH to all-trans-retinoic acid [30]. Thus, generation of RA is RALDH dependent, and many cell types, including intestinal epithelial cells and gut-associated DCs express RALDH [31-33].

There are several possible sources of TGF-β in the small intestine, one of them is $CD103^+$ DCs, which express tgfb2, tissue plasminogen activator (TPA) and latent TGF-β binding protein 3 (ltbn3) mRNA, which are involved in secretion and activation of TGF-β [29], in addition TGF-β is expressed by the small intestinal epithelium in mice [34].

## 1.3 Celiac disease

### 1.3.1 Pathophysiology of celiac disease

CD is triggered by ingestion of gluten proteins, where gluten peptides crossing the epithelium into the lamina propria are deaminated by transglutaminase 2 (TG2) (see 1.3.4), taken up by HLA-$DQ2^+$ or HLA-$DQ8^+$ APCs and presented to gluten specific $CD4^+$ T-cells. Gluten-reactive T cells are primarily Th1 cells [35], and their local production of IFN-γ may act directly on enterocytes, trigger amplified cytotoxicity of intraepithelial lymphocytes (IELs) against enterocytes, and block the function of Tregs. This leads to villous atrophy and crypt hyperplasia (Figure 3) [36] [37].

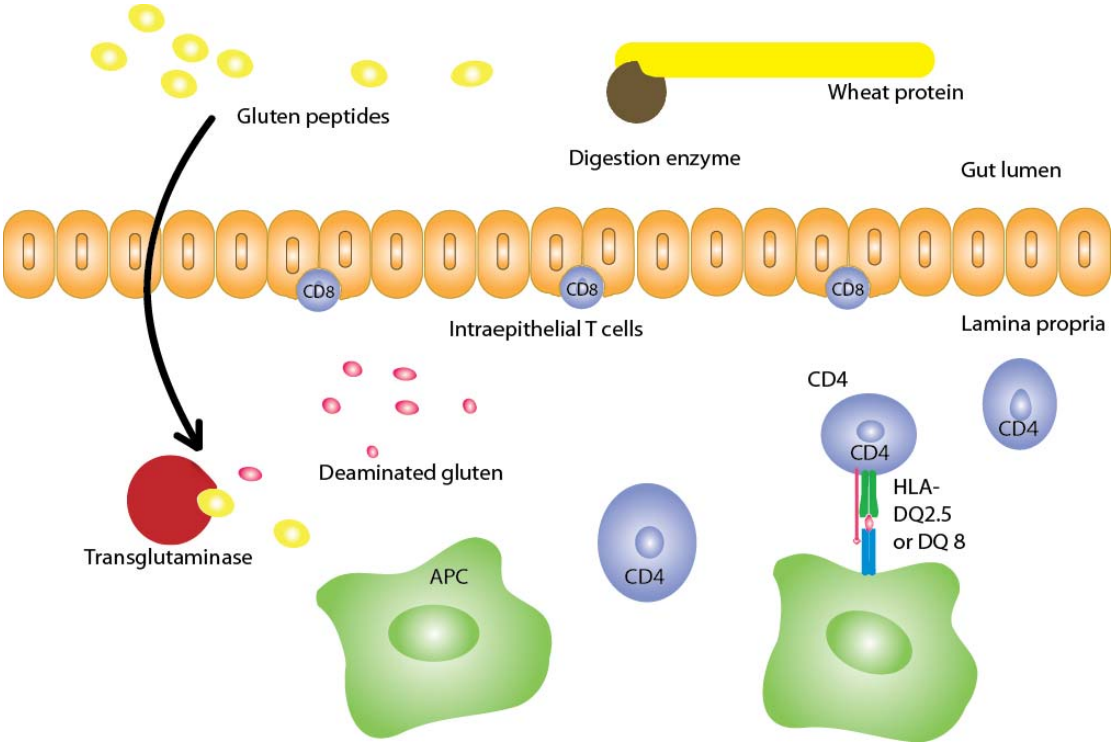


**Figure 3: Pathophysiology of celiac disease.** Celiac disease is a T-cell mediated inflammatory response to gluten peptides in the small intestine lamina propria with an increased content of intraepithelial lymphocytes and lamina propria lymphocytes. Gluten peptides is taken up in the small intestine lamina propria from the gut lumen, and deaminated by transglutaminase 2. Deaminated gluten peptides is further taken up by antigen presenting cells (APCs), presented to gluten specific CD4+ T cells, inducing inflammation and a T helper cell type 1 response [38].

### 1.3.2 HLA association in celiac disease

Genome-wide association studies have demonstrated that CD is a polygenic disorder with involvement of many genes; the HLA locus is the most important genetic factor contributing to ~40% of the genetic variance of the disease.

The general structure of HLA class II proteins is one α- and one β-chain, each consisting of two domains – a polymorphic α1- and β1- region and a highly conserved α2- and β2-region. The polymorphic α1- and β1- region form the antigen binding groove, and can bind various peptides of 14-25 amino acids, depending on the affinity[39]. Furthermore, these classical HLA class II molecules are expressed on the surface of APCs and there is almost a thousand different alleles, encoded by HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA and HLA-DRB1[40]. The nomenclature for naming these genes begins with HLA and the gene name and up to four sets of digits separated by colons. The first set describes the allele group, and can

be determined by antibody recognition. The next set of digits describes the different subtypes, and represents different amino acid (aa) sequences of the encoded protein. The last two sets of digits describes any synonymous mutations in exons and introns, respectively [41].

Approximately 90% of celiac disease patients express HLA-DQ2.5, and the majority of the remaining CD patients express HLA-DQ8. HLA-DQ2.5 and HLA-DQ8 is encoded by DQA1*05:01/DQB1*02:01 and DQA1*03:01/DQB1*03:02, respectively [42]. The functional explanation of the strong association between HLA-DQ2.5 and HLA-DQ8 is their ability to bind and present modified gluten peptides to $CD4^+$ T-cells [43-45], and is well established that CD T-cell antigens preferentially bind to HLA-DQ2.5, as well as to HLA-DQ8 [46]. Interactions between negatively charged glutamate residues in deaminated gluten peptides preferentially interacts with anchor position p4, p6 and p7 in the HLA-DQ2.5 binding groove, or p1, p4 or p7 for HLA-DQ8 [47, 48]. Thus, high affinity binding between the HLA-DQ2.5 and –DQ8 molecule and modified gluten peptides help mediate the immunological synapse between APCs and CD4+ T-cells. The gluten peptides recognized by intestinal T cells are very rich in proline and glutamine residues, thus influencing immunogenicity of gluten. Proline residues prevent breakdown of gluten peptides by digestive enzymes, and TG2 deaminates glutamine residues into glutamate residues with increased affinity to HLA-DQ2 and -DQ8 molecules (1.3.4) [38]. A 33-mer gluten peptide, capable of activating HLA-DQ2-restricted TCCs and gluten specific T-cell lines, has been found to be resistant against breakdown by all gastric, pancreatic and intestinal membrane proteases. Furthermore, this 33-mer contain high amounts of proline and glutamine residues, possessing the potential for reacting with TG2, additionally, homologs have been found in all food grains that are toxic for CD patients [49].

### 1.3.3 Non-HLA association factors in celiac disease

There is also a strong genetic association outside the HLA locus, illustrated by concordance rates of approximately 75 % in monozygotic twins [50], and at least 39 non-HLA CD risk loci are known [51]. Out of these known genetic risk loci, some of the genetic regions has been shown to play a role in regulating T cells (CD28 and IL-2) and B-cells (ICOS and IL-21) in mesenteric lymph nodes, activation and differentiation of proinflammatory T cells (e.g., IL12A, TLR7/8, IRF4) and cell migration (e.g. chemokine receptors like CCR2) [50].

### 1.3.4 Transglutaminase 2 in the context of celiac disease

TG2 is a 78-kilodalton (kDa), calcium dependent enzyme, with multifunctional biochemical properties, including the ability to post-translationally modify glutamine to negatively charged glutamate [52]. In CD pathogenesis, TG2 mediate its effect through deamination of gliadins, increasing the affinity of deaminated gliadin peptides for HLA-DQ2 and -DQ8, which can be recognized by gut derived T cells. TG2 is mainly expressed in the subepithelial region of the mucosa, and at higher levels in CD patients. Further, the effect of TG2 on pepsin-trypsin digested gliadin has been illustrated by performing T-cell proliferation assays with different APCs, resulting in increased T-cell proliferation in response to TG2, independently of the specific APC type [53].

### 1.3.5 Intestinal cytokine response in celiac disease patients

The intestinal cytokine profile in CD patients was partly described by Nilsen et al in 1998 [54]. All CD specimens expressed IFN-γ; in addition IL-2, IL-4, IL-5, IL-6 and TNF-α was detected in some specimens. Untreated celiac disease specimens expressed elevated levels of IFN-γ in comparison with normal controls, and in vitro gluten stimulation of treated CD specimens resulted in up-regulated IFN-γ expression. TGF-beta was also highly expressed, but expression levels was unchanged in treated or untreated CD specimens and non-CD specimens [54]. Additionally, IL-15 [55] and IL-17A [56, 57] has been reported to be over-expressed in lamina propria of patients with active CD.

In the lamina propria of CD patients, there is an increase in the Th1 lymphocyte subpopulation, demonstrated by the upregulated expression of T-bet and IFN-γ in biopsies taken from untreated CD patients [35, 58].

Nielsen et al [59] investigated the cytokine production of gluten specific T-cells ($CD25^{+}CD4^{+}$ TCCs) isolated from celiac lesions [60], and found that all the gluten specific TCCs analyzed secreted IFN-γ, in addition, some also secreted IL-4, IL-5 IL-6, IL-10, TNF and TGF-β. These cytokines were shown to be up-regulated on a transcriptional level by semi-quantitative polymerase chain reaction (PCR) in four TCCs after pre-incubation with APCs and gluten peptides. However, only TGF-β was constitutively transcribed by the TCCs independent of gluten stimulation, although all four TCCs transcribed IL-10 and INF-γ after gluten stimulation. Additionally, some of the gluten specific TCCs contained mRNA for TNF-α, IL-2, IL-4, IL-5 and IL-6 [59].

A more recent study showed that activated gluten specific TCCs secrete IFN-γ and IL-21, but not IL-17 [61].

Another subpopulation which is increased in small intestine of active celiac disease patients are $CD4^+CD25^+FOXP3^+$ T regulatory cells. These cells were shown to be present in the subepithelial layer of the lamina propria, and to be functionally suppressive [62]

### 1.3.6 Increased $CD14^+CD11c^+$ APCs in celiac lesions after gluten challenge

It was recently reported by Beitnes et al. that HLA-$DQ^+$ APCs in healthy human duodenal mucosa can be divided into four subsets: $CD163^+$ $CD11c^-$ macrophages, and $CD11c^+$ DCs expressing either: CD163, CD103 or CD1c. [63]. Consequent studies by Beitnes et al [3] showed that the number $CD11c^+CD14^+$ APC increased after gluten challenge in celiac lesions of CD patients, and that most of these cells were $CCR2^+$. These findings indicate that the $CD11c^+CD14^+$ APC subpopulation originated from blood monocytes. In addition, they also showed that $CD14^+$ monocytes from HLA-$DQ2^+$ donors were capable of activating gluten specific T-cell proliferation upon pre-incubation with gluten peptides.

### 1.3.7 Intraepithelial lymphocytes in the celiac lesion

IELs of the small intestine represents a heterogeneous population consisting mainly of $CD8^+$ memory-effector T-cells residing within the epithelial layer [64]. Early findings on the relative IEL cell count in non-CD controls and CD patients, on a gluten free or gluten-containing diet, showed increased levels in both groups compared to non-CD controls [65]. Subsequent studies has shown that IELs are important for CD immunopathology [66], and it has been proposed that the licensing of IELs to kill requires two factors; activated gluten specific $CD4^+$ T cells, and a stressed epithelium expressing high levels of IL-15 and non-classical MHC class 1 molecules. Epithelial cells expressing these two factors can be targeted and destroyed by IELs in CD, showing the importance of IL-15 and non-classical MHC class 1 [2].

In addition, IELs from untreated CD patients express IL-17A, IFN-γ and IL-10 [56, 67] as well as $FasL^+$, and has the ability to kill $Fas^+$ enterocytes by Fas/FasL interaction [36].

## 1.4 Interleukin-15 and IL-15 receptor complex

### 1.4.1 Identification of IL-15

Interleukin-15 was first discovered as a T-cell growth factor, purified from the monkey kidney cell line CV-1/EBNA. Based on the N-terminal sequence data, cDNA encoding the cytokine was isolated and expressed in mammalian cells, and the 14-15 kDa glycoprotein was designated IL-15 [68, 69]. Due to the similarities between IL-2 and IL-15 in receptor binding and functional responses, the possibility that IL-15 utilizes components of the IL-2 receptor (IL-2R) complex was investigated by Giri et al [69]. They revealed that IL-2R-β and the common γ subunits are required for IL-15 binding and signaling, but the third component of the IL-2R, the IL-2Rα subunit, was not needed for binding or signaling of IL-15. In addition, IL-15 receptor (IL-15R) were found to be expressed by a range of different cells types, including lipopolysaccharide-stimulated monocytes, PHA-stimulated PBMCs, vascular endothelial cells, NK cells and T-cells [69].

### 1.4.2 Cellular sources of IL-15

In patients with active celiac disease, IL-15 is massively up-regulated in the small intestine lamina propria, and can also be detected in intestinal epithelial cells [55]. Doherty et al [70] showed that IL-15 mRNA can be isolated from several sites, including spleen, liver and lung, in addition, macrophages were found to be a major source of IL-15 in mouse lymphoid tissues. Human blood-derived DCs has also been shown to produce functionally active IL-15 following stimulation [71], and monocytes up-regulate IL-15 expression upon IFN-γ stimulation [72].

### 1.4.3 Short and long isoforms of IL-15

There are two IL-15 splice variants, differing in the signaling region, and are designated long signal peptide (LSP) and short signal peptide (SSP) IL-15. The LSP consist of 48 amino acids (aa) and the SSP consist of 21-aa, with only 11 aa residues shared between these signal peptides. The SSP-IL-15 isoform is mostly located in the

cytoplasm and nucleus, in contrast to the LSP-IL-15 isoform that has been observed in the ER, and thus is considered secretable. Supernatant of COS cells transfected with LSP-IL-15 or SSP-IL-15 expression constructs, where tested with a biological assay using IL-2 dependent CTLL-2 cells. These IL-2 dependent cells did not proliferate in the presence of supernatants from SSP-IL-15 transfected COS cells, whereas CTLL-2 cells subjected to supernatant from LSP-IL-15 responded by proliferating [73]. Since LSP-IL-15 is the only secretable isoform, it's assumed that SSP-IL-15 do not affect adjacent target cells. However, Bergamaschi et al [74] presented evidence that SSP-IL-15 actually can be secreted and bioactive as long as IL-15Rα is co-expressed.

### 1.4.4 IL-15 Receptor complex

The biological activity of IL-15 is similar to IL-2 in its ability to stimulate proliferation of the T-cell line CTLL.2, and PHA stimulated PBMCs. It thus follows that IL-15 utilize components of the IL-2R complex, as demonstrated by Giri et al in 1994 [69]. In fact, they showed that IL-15 is both dependent on IL-2Rβ and the common-γ chain, and in addition suggested that IL-15 can induce dimerization of soluble β and γ IL-2R subunits. Furthermore, it was demonstrated that the functional IL-2R complex is not sufficient for IL-15 binding, and that IL-2R α is not involved in the binding of IL-15.

Since IL-2Rβ and the common-γ chain is not sufficient for IL-15 signaling alone, there had to be another component involved in binding IL-15. This component is structurally related to IL-2R α-chain, and is known as IL-15R α-chain [75].

The components of the IL-15R complex have different affinities for IL-15 dependent on how they are combined, hence, an intermediate ($Ka \approx 10^{-9}$ M) affinity for the IL-15R β-/γ-chain complex [76] and high affinity ($Ka \geq 10^{-11}$M) for the IL-15R α-chain [75]. It has also been shown that soluble IL-15/IL-15Rα complex have an increased affinity for IL-15R β-chain compared to soluble IL-15 alone [77]. However, when investigating dynamics of IL-15 and IL-15Rα binding, it was demonstrated that IL-15 can be trans-presented (Figure 4) and undergo an IL-15Rα mediated recycling process. In line with this, cells transfected with IL-15 and IL-15Rα secrete low amounts of IL-15 relative to cells transfected with IL-15 alone, and biological active IL-15/IL-15Rα complexes has been detected on the plasma membrane of IL-15 producing cells [78].

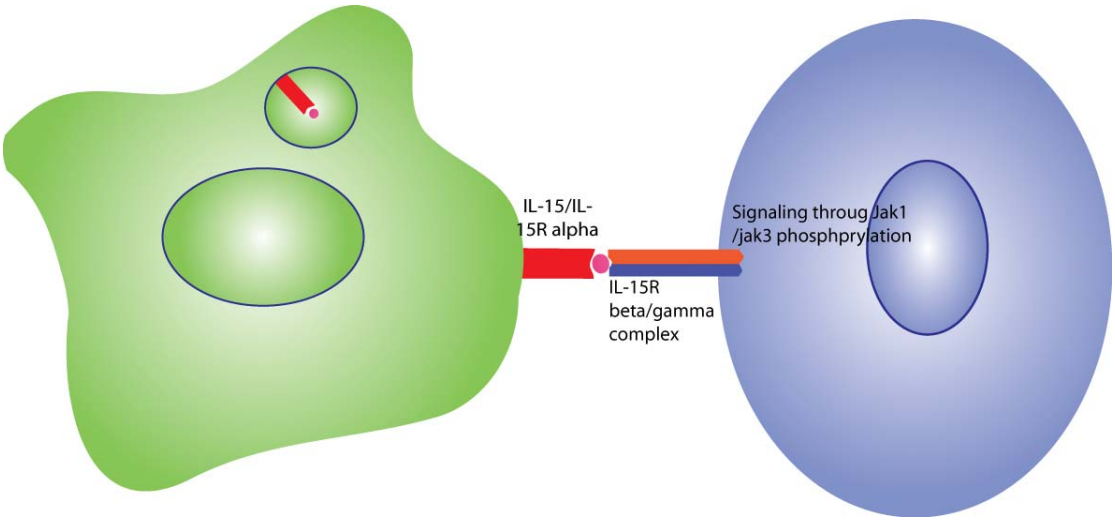


**Figure 4: IL-15 trans-presentation.** IL-15 can be trans-presented by IL-15Rα expressing cells to cells expressing IL-15Rβ and common gamma chain. IL-15 is co-expressed with IL-15Rα and presented to a neighboring cell expressing IL-15 Rβ/γ complex, this results in phosphorylation of jak1 and jak3 [79].

### 1.4.5 IL-15 signaling

IL-15 binds to the IL-15R complex and signals through the IL-15Rβ chain and common γ chain [69] by inducing tyrosine phosphorylation of both JAK1 and JAK3. Cytokines which activate JAKs also activate tyrosine phosphorylation of transcription factors known as Signal Transducer and Activator of Transcription (STAT), and the STATs shown to be phosphorylated upon IL-15 signaling are STAT3 and STAT5 [80].

## 1.5 Co-adjuvant effect of RA and IL-15

A possible association between RA (isotretinoin), used to treat acne, and inflammatory bowel disease has been reported [81]. This finding is compatible with recent mice studies, which showed that RA has a co-adjuvant effect together with IL-15, affecting T-cell differentiation and activation. In the presence of IL-15, RA acted as an adjuvant to promote inflammatory Th1 and Th17 responses, preventing Treg induction. This effect was mediated through DCs, responding to RA and IL-15 by inducing phosphorylation of JNK, thus producing IL-12p70 and IL-23. [82].

## 2. Project description

CD is a T-cell mediated inflammatory response to gluten in the small intestine mediated by APCs [83]. Based on a recent study by Beitnes et al [3], where they showed an increase of $CD14^{+}CD11c^{+}$ APC in small intestine lamina propria after challenging celiac disease patients with gluten, it is suggested that newly recruited blood monocytes are involved in the immunopathology of CD by presenting gluten peptides to gluten-specific T cells in the lamina propria. In active CD, IFN-γ and IL-15 is up-regulated in small intestine lamina propria [55]. Moreover, IFN-γ stimulated monocytes have been shown to up-regulate IL-15 expression [72], and RA together with IL-15 has been shown to have a co-adjuvant effect on immune responses to dietary antigens [82]. Based on this information the aims of this study were as follows:

1. Assess whether IL-15 and IFN-γ affect HLA-DQ $2^{+}$ monocytes ability to activate gluten specific T-cell proliferation
2. Assess whether RA functions as an adjuvant together with IL-15 in the activation of monocytes
3. Investigate by which mechanisms monocytes respond to these cytokines

# 3 Materials and Methods

## 3.1 Materials

All reagents were from Sigma-Aldrich unless otherwise noted. The suppliers of antibodies and primers used in the present study are listed in table 1 and table 2.

### 3.1.1 Biological material

The cells used in this study were $CD4^+$ gluten specific TCCs, $CD14^+$ blood monocytes from HLA-DQ $2^{+/-}$ donors and peripheral blood mononuclear cells (PBMCs). The gluten specific TCCs were provided by the research group headed by Professor Ludvig Sollid (Center of Immune regulation, Oslo, Norway). Monocytes and PBMCs were isolated from buffy coats, ordered from Oslo blood bank (Oslo University Hospital).

### 3.1.2 Culturing reagents

Cell culturing reagents used in this study were BioWhittaker RPMI 1640 without L-glutamine (Lonza), L-glutamine (Lonza), Pen strep (Lonza), fetal calf serum (FCS) , human serum (HS) (IMMI), recombinant human TGF-β1 (rhTGF-β1) (R&D Systems), rhIL-15 (R&D Systems), all trans-Retinoic Acid, LF-33 (p1502.4, gliadin) (GL Biochem (Shanghei)), rhIFN-γ (R&D Systems), $^3H$ thymidine (HartmannAnalytic) and Brefaldin-A. To expand TCCs, Dynabeads CD3/CD28 T-Cell Expander (Invitrogen), IL-2 (PeproTech), IL-15 (R&D Systems), PHA and β-mercaptanol was used.

### 3.1.3 Isolating and freezing cells

Reagents used to isolate cells were lymphoprep (Axis-shield), Dynabeads® Untouched™ Human Monocytes (Invitrogen) and CD14 MicroBeads (Miltenyibiotec).

Reagents used for freezing down cells were dimethyl sulfoxide (DMSO) (Merck), and FCS.

### 3.1.4 Antibodies

The antibodies used in this study are listed in the table below (table 1)

| Antigen | Conjugated | Host | Dilution | Source | Method |
|---|---|---|---|---|---|
| **hCD14** | APC-Cy7 | Mouse | 5:100 | Biolegend | Flow cytometry |
| **hCD11c** | FITC | Mouse | 10:100 | Biolegend | Flow cytometry |
| **hIL-15** | APC | Mouse | 10:100 | R&D Systems | Flow cytometry |
| **hIL-15** | | Mouse | 0.2µg/mL | R&D Systems | Neutralization |
| **Isotype for neutralizing hIL-15 ab** | | Mouse | 0.2µg/mL | Sigma-Aldrich | Neutralization |
| **hCD19** | PacB | Mouse | 10:100 | Biolegend | Flow cytometry |
| **hHLA-DQ2** | FITC | Mouse | 1:250 | IMMI | Flow cytometry |
| **Isotype-hHLA-DQ2** | FITC | Mouse | 20:100 | BD bioscience | Flow cytometry |
| **hpSTAT5** | | Rabbit | 1:100 | CellSignaling | Flow cytometry |
| **hpSTAT6** | | Rabbit | 1:100 | CellSignaling | Flow cytometry |
| **Anti-rabbit** | PE | Goat | 1:500 | Southern biotechnology | Flow cytometry |
| **IL-2** | Pecy7 | Rat | 5:100 | Biolegend | Flow cytometry |
| **IL-17A** | pe | Mouse | 5:100 | Biolegend | Flow cytometry |
| **IFN-γ** | A488 | Mouse | 5:100 | Biolegend | Flow cytometry |
| **CD3** | APC | Mouse | 10:100 | BD bioscience | Flow cytometry |

*FITC:* Fluorescein isothiocyanate, APC: Allophycocyanin, PE: phycoerythrin, PE-Cy7: phycoerythrin- cyanine7 APC-Cy7: Allophycocyanin -cyanine7, PacB: pacific blue

### 3.1.5 Flow cytometry reagents

Flow cytometry reagents used in this study were Fixable Viability Dye eFluor 450 (eBioscience), paraformaldehyde and methanol (Merck). Staining buffer used to wash and stain cell suspensions contained PBS supplemented with 10% FCS and 0.1% Na azid. For compensation, PBMCs or oneComp eBeads (eBioscience) were stained with fluorochrome conjugated antibodies.

### 3.1.6 Storing and isolating RNA

The reagents used in this study to isolate RNA were Glycogen (Ambion), TRI reagent (Ambion), Ethanol, isoproanol (Fluka Analytical), 1-Bromo-3-Chloropropane (BCP), RNeasy mini kit (Qiagen).

### 3.1.7 PCR

The primers used in this study for PCR are listed in the table below (table 2)

**Table 2 Primers**

| Description | Sequence | Concentration | Source |
|---|---|---|---|
| **hLSP IL-15.for** | TTGGGCTGTTTCAGTGCAGGG | 100 nM | Invitrogen |
| **hLSP IL-15.rev** | GTTCCTCACATTCTTTGCATCCAG | 100 nM | Invitrogen |
| **hSSP IL-15.for** | TGGCCCAAAGCACCTAACCT | 100 nM | Invitrogen |
| **hSSP IL-15.rev** | GTTCCTCACATTCTTTGCATCCAG | 100 nM | Invitrogen |
| **hHRPT. For** | AATACAAAGCCTAAGATGAGAGTTCAAGTTGAGTT | 100 nM | Invitrogen |
| **hHRPT. Rev** | TTAGGAATGCAGCAACTGACATTTCTAAAGTAC | 100 nM | Invitrogen |
| **hHRPT. Rev** | CTATAGGCTCATAGTGCAAATAAACAGTTTAGGAAT | 100 nM | Invitrogen |
| **Oligo dT** | CTGAATTCTTTTTTTTTTTTTTT | 20pmol/µl | Invitrogen |

hLSP-IL-15: human long signaling peptide interleukin 15, hSSP-IL-15: human short signaling peptide interleukin 15, hHRPT: human hypoxantine phsophoribosyltransferase.

.

#### *3.1.7.1 Reverse transcriptase PCR*

Reagents used for reverse transcriptase PCR (RT-PCR) was SuperScript$^{TM}$ III RT (Invitrogen), DTT (Invitrogen), First strand buffer (Invitrogen), deoxyribonucleotide triphosphate (dNTP) (GE Healthcare (illustra)), Rnasin (Promega) and Diethylpyrocarbonate (DEPC) water.

#### *3.1.7.2 Real time PCR*

Reagents used for Real time PCR were dNTP (GE Healthcare (illustra)), HotStarTaq DNA Polymerase (QIAGEN), PCR Buffer (QIAGEN), $MgCl_2$ (QIAGEN), EVA Green (BIOTIUM).

#### *3.1.7.3 Gel electrophoresis*

The agargel was made from LE agarose (SeaKem), ethidium bromide and 1x TBE buffer. Reagents used to run samples were 6x Xylene Cyanole gel loading buffer (Bio-Rad), 100 bp DNA ladder (New England Biolabs) and 1x Tris/Borate/EDTA (TBE) buffer.

## 3.2 Methods

### 3.2.1 Cell culture and treatment

Monocytes were cultured in RPMI medium supplemented with 1% L-glutamine, 1% P/S and 1 or 10% FCS. Monocytes, to be used in different experimental setups, was pre-treated with combinations of 1.7 μM gliadin peptide, 100 mM RA, 40ng/mL TGF-β, 250 ng/mL IL-15, and 100 U/mL IFN-γ.

Monocytes-T-cell co-cultures were cultivated in RPMI medium supplemented with 1% L-glutamine, 1% P/S and 10% HS.

During T-cell expansion, cells were cultured in RPMI medium supplemented with 1% L-glutamine, 1% P/S, 0.0007 % 2β-mercaptoethanol and 10% HS.

All cell cultures were incubated at 37 degree Celsius in a humidified 5% $CO_2$, 95% air incubator, and all cell culturing work were performed in sterile hoods to prevent cell culture infections.

#### *3.2.1.1 Proliferation assay*

Monocytes from HLA-DQ $2^+$ donors were pretreated with or without 1.7 μM gliadin and combinations of 250 ng/mL IL-15, 100 U/mL IFN-γ, 100 nM RA, 40 ng/mL and TGF-β over night. The next day monocyte cultures were washed 3 times with PBS and then co-cultured with gluten specific TCCs for 4 days, adding 20 μL $^3H$ thymidine solution (0.05 mCi/mL) per 150 μL medium one day before harvesting the cell cultures. At day 5, cell cultures were harvested on a Harvester 96 (Tomtec) and then measured using MicroBeta plate counter (PerkinElmer).

#### *3.2.1.2 Analyzing STAT phosporylation*

Cells were equilibrated in complete 10% FCS RPMI medium at 37 degrees for approximately 1 hour before activation with 250 ng IL-15 or 20 ng/mL IL-2 in 200 μL for 15 minutes, followed by fixation with 1 mL 1% PFA and incubating at room temperature for 10 minutes. Cells were then washed before resuspending in cold methanol and incubated at 4 degrees for 10 minutes. For primary antibody staining, cells were washed with staining buffer and then resuspend in staining buffer containing desired primary antibodies (table 1), and incubated for 15 min in the dark

at 4 degrees for 15 min. For the secondary staining, cells were washed, resuspend in staining buffer containing desired secondary antibody (table 1), and incubate for 15 min in the dark at 4 degree Celsius for 15 min. Finally, cells were washed, resuspended in staining buffer and analyzed as described in section (3.2.4).

#### *3.2.1.3 Freezing and thawing cells*

Cell samples were resuspended in medium containing 90% FCS and 10% DMSO, aliquoted into cryotube vials (Thermo scientific) and frozen at -80 degree C in Nalgene cryo containers (Thermo scientific) before storing in $N_{2(l)}$.

Cells were thawed in a 37 degree water bath until there was almost no ice left in the cryotube vial, and then transferred to new 15 mL falcon tubes containing approximately 8 mL preheated 20% FCS RPMI medium. Following this, tubes containing cell suspensions were centrifuged at 300 g for 5 minutes before resuspending in the desired medium.

#### *3.2.1.4 Viability assay of fresh versus frozen monocytes*

Monocytes, freshly isolated or frozen and thawed, were cultured overnight in complete 1% FCS RPMI medium, harvested and stained with hCD14 specific antibody (table 1), as described in section (3.2.4.1). The viability of freshly isolated versus frozen monocytes was quite similar (data not shown).

#### *3.2.1.5 Proliferative ability of gluten specific T-cells*

$CD4^+$ tissue derived gluten specific TCCs were provided by the Sollid group. The proliferative ability of different TCCs (TCC: 430.1.142, 493.3.4.33, 412.5.28 and TCC: 436.5.3) was tested in a standard proliferation assay as described in section (3.2.1.1).

T-cell response to IL-15 and the neutralizing effect of anti-hIL-15 antibody used during the blocking assay (3.2.1.8) was tested by culturing TCCs (TCC: 378.3.36, 378.3.78) with/or without 0.2 μg/mL anti-IL-15 blocking antibody or an isotype control (table 1) and 100, 30, 10 or 3 ng/mL IL-15 at day 0. 20 μL $^3H$ thymidine solution (0.05 mCi/ml) per 150 μL medium was added at day 2, before harvesting on a

Harvester 96 and then measuring thymidin incorporation using MicroBeta plate counter at day 3 (data not shown).

#### *3.2.1.6 T-cell response to gliadin antigens*

Gliadin peptide, p1502.4, to be used in future experiments, was compared with one old batch of the same protein and gliadin CTTG (Institute of Immunology, *IMMI*, *Rikshospitalet* University Hospital and University of Oslo). HLA-$DQ2^{+}$ monocytes and Epstein Barr Virus-immortalized B-cells (IMMI) were used as antigen presenting cells. APCs were pre-treated overnight with or without peptides and IL-15 before adding gluten specific TCCs. Remaining procedures was performed as described in section (3.2.1.1). APCs incubated with gliadin p1502.4 induced T-cell proliferation equally good as the old batch (data not shown).

#### *3.2.1.7 T-cell monocyte ratio*

Optimal T-cell monocyte ratio was established by cultivating 100 000, 20 000 or 5 000 monocytes, pretreated with/without 250 ng/mL IL-15 and 1.7 mM gliadin peptide, further cultured, harvested and analyzed as described in section (3.2.1.1).

#### *3.2.1.8 Blocking assay*

Blocking assays was used to investigate the involvement of IL-15 in co-cultures with monocytes and TCC cells. 0.2µg/mL of anti hIL-15 blocking antibody or an isotype control (table 1) were added to the monocyte cultures, pretreated overnight with 250 ng/mL IL-15 or 100 U/mL IFN-γ, washed with PBS, and incubated for 15 min at 37 degree C before adding gluten specific TCCs. These co-cultures were then further cultivated, harvested and analyzed as described in section (3.2.1.1).

#### *3.2.1.9 Expanding T-cell clones*

PBMCs from two donors, isolated as described in section (3.2.5.1), were washed twice with PBS, irradiated with 50 gy, centrifuged at 300 G and resuspend in 10 % HS. Two vials of TCC (8 million T-cells) were then thawed, centrifuged at 300 G and resuspend in the solution with irradiated PBMCs. To this solution, 1 µg/mL PHA, 10

U/mL IL-2 and 1 ng/mL IL-15 was added before transferring to a 24 well plate, 0.5-0.7 million cells in 1.3 mL per well. Medium containing 10 U/mL IL-2 and 1 ng/mL IL-15 were later used to split cell cultures after three or four days. At day five, medium containing no cytokines was used to split cell cultures. Finally, cells were harvested, counted and frozen down at day 7 as described in section (3.2.1.3).

### 3.2.2 RNA isolation and Polymerase Chain Reaction

In order to analyze regulation of gene transcription, RNA was isolated and used to synthesizing complementary DNA (cDNA) by reverse transcriptase PCR, which was subsequently used for qPCR.

#### *3.2.2.1 Lysing and storing cell suspension for RT-PCR*

Cell suspensions were centrifuged for 5 minutes at 300 G, resuspended in 500 mL of TRI Reagent, incubated at room temperature for 5 minutes and then frozen at -80 C. At later time points, samples were thawed and RNA was isolated.

#### *3.2.2.2 Isolating RNA*

Frozen TRI reagent solutions, containing lysed cells, were thawed at room temperature, consequently adding 50 µL BCP per 500 µL, incubated at room temperature for 5-15 minutes before centrifuging at 12000 g for 15 minutes at 4 degree C. From this point, RNA was isolated by following Applied Biosystems TRI Reagent solution RNA isolation reagent protocol (Invitrogen) or Qiagen RNesay mini kit. When using Qiagen RNesay mini kit, the clear phase from the TRI reagent/BCP solution was transferred to new tubes and mixed with one volume of 70% ethanol. For the Applied Biosystem RNA isolation protocol (Invitrogen), 1 µL glycogen was added together with isopropanol to visualize the RNA. RNA concentration was measured on a NanoDrop spectrophotometer ND-1000 (Nanodrop Technologies).

#### *3.2.2.3 Reverse transcriptase PCR*

RT-PCR was used to synthesize cDNA, which was further used for qPCR. Each reaction contained RNA (1 µgram or less), 1 µl oligodT (table 2), 0.5 µl 200 U/µl

SuperScript™ III RT, 2 µl 0.1 M DTT, 4 µl 5x first strand buffer, 1 µl 100 µmol dNTP, 0.5 µl 40 U/µl Rnasin and DEPC water giving a total volume of 20 µl.
The RT-PCR consisted of one cycle; 50 degree C for 60 minutes, and 70 degree C for 15 minutes.

#### *3.2.2.4 Real-time PCR*

QPCR was used to quantitatively measure the relative amount of RNA transcripts from different cell populations. For all the qPCR experiments, a Stratagene Mx3005P (Agilent Technologies) was used, and each reaction consisted of 0.5 µl 100 µmol dNTP, 0.125 µl 5000 U/ml HotStarTaq DNA Polymerase, 2.531 µl 10x PCR Buffer supplemented with 1.25 % BSA (QIAGEN), 25mM $MgCl_2$ (amount dependent on primer set used), 0.625 µl 20x EVA Green, 5 µl cDNA (10 X diluted cDNA solution, added at separate bench) and dd$H_2O$ to reach a total reaction volume of 25 µl per sample. All samples were run in triplicates, and the optimal $MgCl_2$ concentration per primer set was titrated from 1.5 mM to 4.0 mM.
The qPCR consisted of up to 40 cycles; 95 C for 30 s, 60 C for 30 s, finishing each cycle with 72 C for 30 s and measuring absorbance. Finally each qPCR ended with a melting curve analysis consisting of one cycle; 95 degree C for 60 s, 57 degree C for 30 s, finishing off with a temperature rise up to 95 degree C while measuring melting points.
The threshold is the basis for all $C_t$ (threshold cycle) values and should therefore be the same across all PCR array runs in one analysis, this threshold value was set according to $RT^2$ Profiler™ PCR Array System (SABiosciences™). To further analyze the relative transcription levels, $C_t$ values were exported and analyzed by the $\Delta\Delta V_t$ method according to to$RT^2$ Profiler™ PCR Array System (SABiosciences™).

### 3.2.3 Gel electrophoresis

Gel electrophoresis was used to establish the optimal $MgCl_2$ concentration for PCR, and product quality per primer set. The agarose gel was made by heating 1 x TBE buffer containing 2% agar in the microwave until boiling, twice, allowing the agar to dissolve properly. 0.5 ng/mL ethidium bromide was added to the agar solution, when temperature was approximately 50 degree C, and consequently poured into a casting tray, with a comb inserted. After the gel cooled down, the

comb was removed and the casting tray was placed in an electrophoresis chamber filled with 1x TBE buffer. Then PCR product, mixed with 6x xc gel loading buffer, and 100 bp DNA ladder was loaded to the desired wells. To finally separate PCR products and visualizing them, the gel was run for approximately 1 hour at 80 V and visualized by UV-light using a gel Doc (Bio-Rad).

### 3.2.4 Flow cytometry

Flow cytometry was used to measure the presence of fluorescently labeled antibodies. All compensations for surface and intracellular staining were done by using oneComp eBeads, or PBMCs stained with desired antibodies. Standard staining buffer (3.1.5) was used during all procedures unless otherwise noted. All samples were run on a 3-laser LSR II (BD), and compensations were either done in FACSdiva (BD) or flowjo (Tree Star Inc), and the final analyzing was done in flowjo.

#### *3.2.4.1 Surface staining with antigen specific antibodies*

To investigate different cell markers and cell functions, surface staining was performed; antibodies used for this procedure are listed in table 1. Cell suspensions were first washed, before re-suspending in buffer containing desired antibodies. These antibodies are fluorochrome conjugated and will fade if exposed to light, thus following this point it is important to keep cell suspensions on ice and in the dark. Antibody stained samples were incubated for 15 min before washing, and resuspended in buffer. 10 µL 5 µg/mL propidium iodine solution was added to each sample right before analyzing by flow cytometry.

#### *3.2.4.2 Intracellular staining with antigen specific antibodies*

All intracellular staining was done according to the standard protocol from BD (BD Cytofix/cytoperm plus Fixation/Permalization Kit) using antibodies listed in table 1. Live/dead staining was performed, using Fixable Viability Dye Cell Staining following standard protocol (eBioscience$^{TM}$), before surface and intracellular staining.

### 3.2.5 Isolating cells

#### *3.2.5.1 Lymphoprep*

Lymphoprep was used to isolate PBMCs from full blood or buffy coat. One buffy coat was first diluted to a total volume of 200 mL with PBS, before aliquoting it in six 50 mL falcon tubes already containing 10 mL lymphoprep each. These tubes were then centrifuged at 800g for 20 minutes. After this, the white phase containing PBMCs were transferred to new tubes, before washing twice with PBS, and resuspending in desired medium.

#### *3.2.5.2 Dynabeads*

Dynabeads were used to isolate cells from PBMC solutions, by negative selection. Most of the $CD14^+$ monocytes (with an exception of donor 16) were isolated from buffy coats according to the Dynabeads $CD14^+$ untouched negative isolation kit standard protocol (Invitrogen).

#### *3.2.5.3 MACS beads*

MACS beads were used to isolate cells from PBMC solutions (only donor 16), according to MACS $CD14^+$ positive isolation kit standard protocol (Miltenyibiotec).

### 3.2.6 Statistical analysis

All statistical analysis was done in GraphPad Prism 5, using paired t-test.

# 4 Results

## 4.1 Screening for HLA-DQ 2+ blood donors

Screening blood donors for the HLA-DQ 2 phenotype was done to obtain monocytes with the ability to present gluten to gluten specific T-cells. Peripheral blood monocytes does not express surface bound HLA-DQ molecules, so to be able to screen for HLA-DQ $2^+$ patients, $CD19^+$ B-cells were stained and analyzed for the presence of HLA-DQ 2.

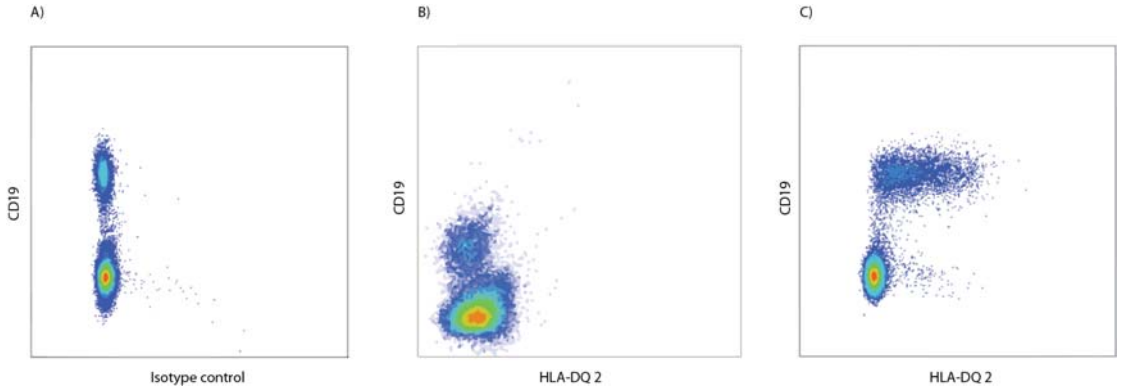


**Figure 1: HLA-DQ 2 screening.** Peripheral blood mononuclear cells (PBMCs) isolated from a buffy coat were stained with CD19- and HLA-DQ 2/isotype control antibodies, and analyzed by flow cytometry, as displayed: (A) Isotype control, (B) HLA-$DQ^-$ donor and (C) HLA-$DQ^+$ donor. Propidium iodide - exclusion was used to detect live cells.

Figure 1 shows both a HLA-DQ 2 positive and negative donor, including an isotype control. $CD14^+$ monocytes from HLA-DQ 2 positive blood donors were further isolated by negative selection, with a typical purity of >90%, and further used in co-cultures with gluten-specific TCC cells.

## 4.2 IL-15 stimulation significantly enhance monocyte activation of tissue derived CD4+ gluten-specific TCC cells

To investigate whether monocytes pre-treated with IL-15 more efficiently activated T-cell proliferation, monocytes were cultured overnight with or without IL-15 and gluten, and further co-cultured with gluten specific TCC cells for 4 days, adding $^{3}H$ thymidine approximately 20 hours before harvesting and measuring thymidine incorporation.

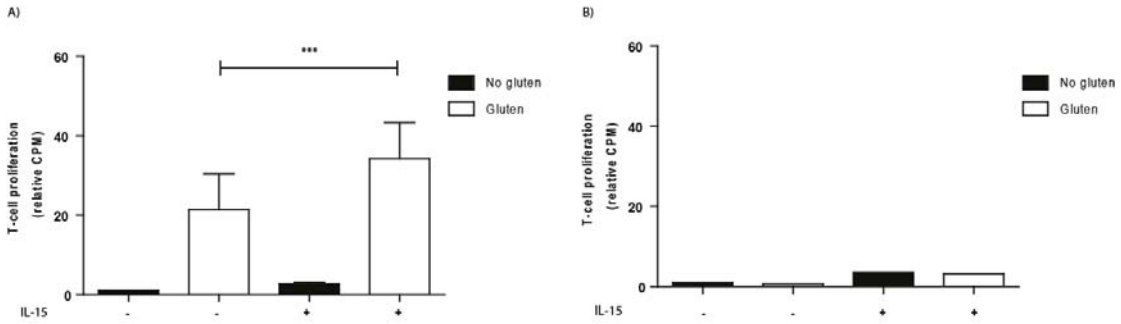


**Figure 2: IL-15 pre-treatment augmented T-cell proliferation.** Monocytes cultured overnight with or without IL-15 and gluten, and further co-cultured with gluten specific T-cell clones for 4 days, adding $^{3}H$ thymidine approximately 20 hours before harvesting and measuring thymidine incorporation, as depicted. A) Activation of gluten specific T-cell clones by monocytes from HLA-DQ $2^{+}$ donors, 21 experiments, 10 monocytes donors and 4 T-cell clones. B) Control experiment with HLA-DQ 2 negative donor revealed no T-cell proliferation, n=1. Statistics were done using paired t test. ***≤0.005

Monocytes pre-treated with IL-15 significantly enhance gluten dependant T-cell proliferation relative to monocytes cultured in absence of this cytokine. This was repeatedly shown using a large number of individual monocyte donors and several different gluten specific TCCs (Figure 2A). Cultures without gluten (Figure 2A) resulted in low levels of T-cell proliferation, showing that the TCC cells are dependent on gluten to proliferate in this system. Further, the proliferation was dependent on HLA-DQ 2 as the proliferation assay using monocytes from HLA-DQ $2^{-}$ donor abrogated the proliferation (Figure 2B).

## 4.3 Monocytes can display surface bound IL-15

The increased proliferation could be the result of trans-presentation of IL-15 by monocytes, so we assessed the binding capacity of IL-15 on monocytes by pre-treating monocytes with IL-15 over night, and subsequently staining with fluorochrome conjugated antibodies specific for IL-15 before analyzing by flow cytometry.

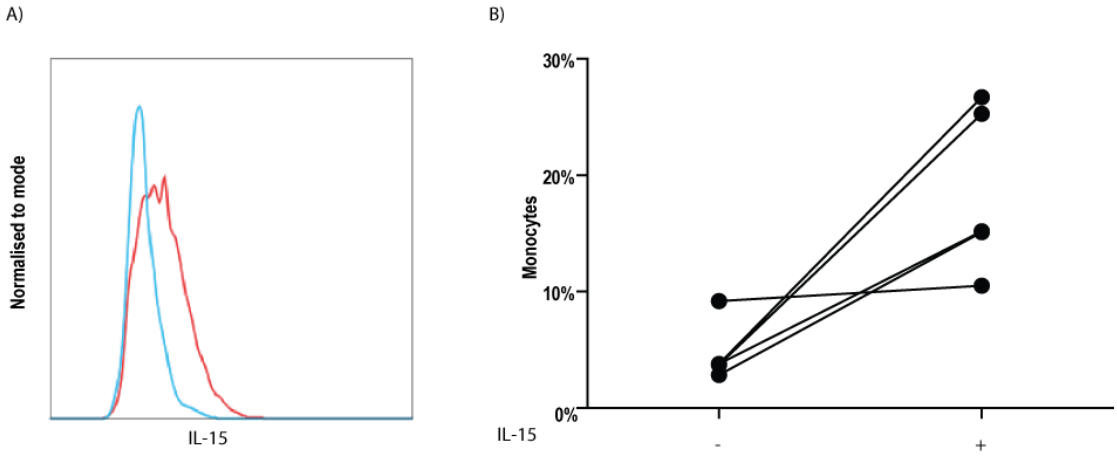


**Figure 3: Monocytes are capable of binding IL-15.** Monocytes were pre-treated over night with/without IL-15 as depicted, stained for surface bound IL-15 and analyzed using flow cytometry. A) Representative flow plot from one monocyte donor pre-treated with (red) or without (blue) IL-15. B) Percentage of IL-15 positive monocytes, treated as depicted. Gating was performed according to CD11c, and Propidium iodide was used to discriminate dead cells. N=5

Results in figure 3A and B shows that monocytes have binding potential for IL-15, suggesting that monocytes exposed to IL-15 have the potential to trans-present the cytokine to target cells expressing surface IL-15R.

## 4.4 Blocking IL-15 abrogates T-cell proliferation

IL-15 pretreated monocytes more efficiently activate T-cell proliferation, and to investigate if this was a direct effect of the cytokine acting on T cells, IL-15 was blocked with a monoclonal neutralizing antibody prior to co-culturing monocytes with gluten specific TCC cells.

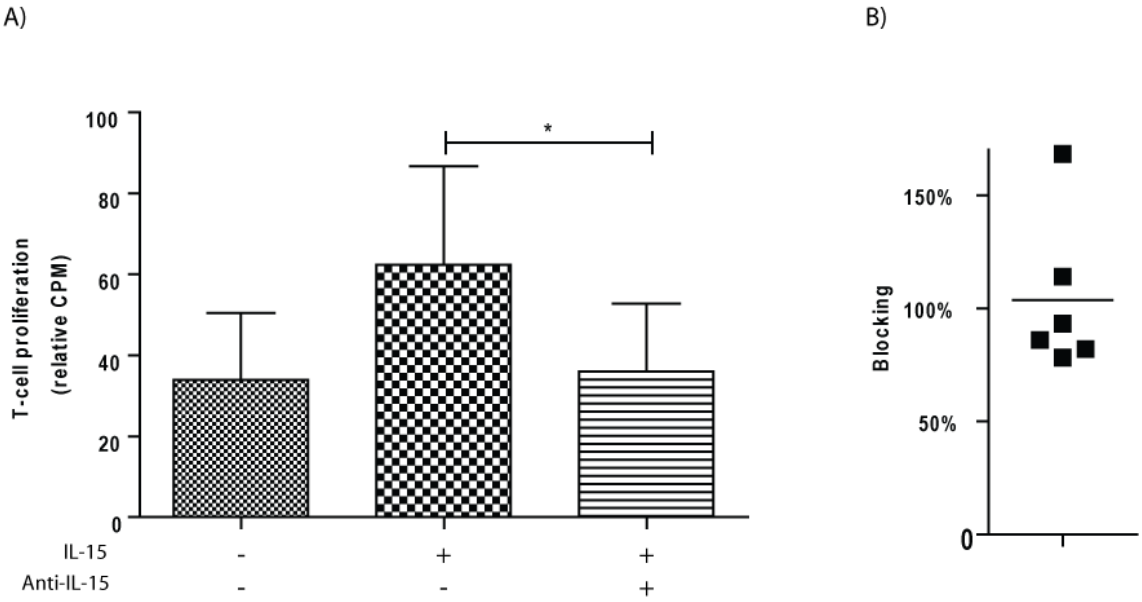


**Figure 4: Blocking IL-15 abrogates T-cell proliferation**. A) Monocytes were pre-treated over night with gluten and with or without IL-15. Anti-IL-15 blocking antibody was added as depicted before co-culturing with gluten specific T-cell clones. B) Percentage of reduced T-cell proliferation caused by blocking IL-15 relative to IL-15 treated monocytes, showing each individual experiment displayed in (A) with the mean value. Statistics were done using paired t test, n=6. *≤0.05

The proliferative effect of IL-15 was significantly reduced by co-culturing monocytes and T-cells together with anti-IL-15 blocking antibody (Figure 4). In fact, co-culturing with anti-IL-15 neutralizing antibody blocked almost 100% of the additional proliferative effect provided by IL-15 (Figure 4 B). This further suggests that IL-15 is either secreted or trans-presented by HLA-DQ $2^{+}$ monocytes to gluten specific TCC cells, and directly stimulating T-cell proliferation.

## 4.5 Monocytes do not exhibit STAT5 phosphorylation following IL-15 stimulation

Binding of IL-15 to IL-15R results in activation of JAK1 and JAK3 by the IL-15Rβ/γ complex leading to phosphorylation of STAT3 and STAT5 (1.4.5). Thus to investigate whether monocytes respond to IL-15, monocytes were cultured for 15 minutes with IL-15 before fixation and intracellular staining for pSTAT5. T-cells are known to respond to IL-15 and IL-2 by phosphorylating STAT5 [80], and were used as positive controls.

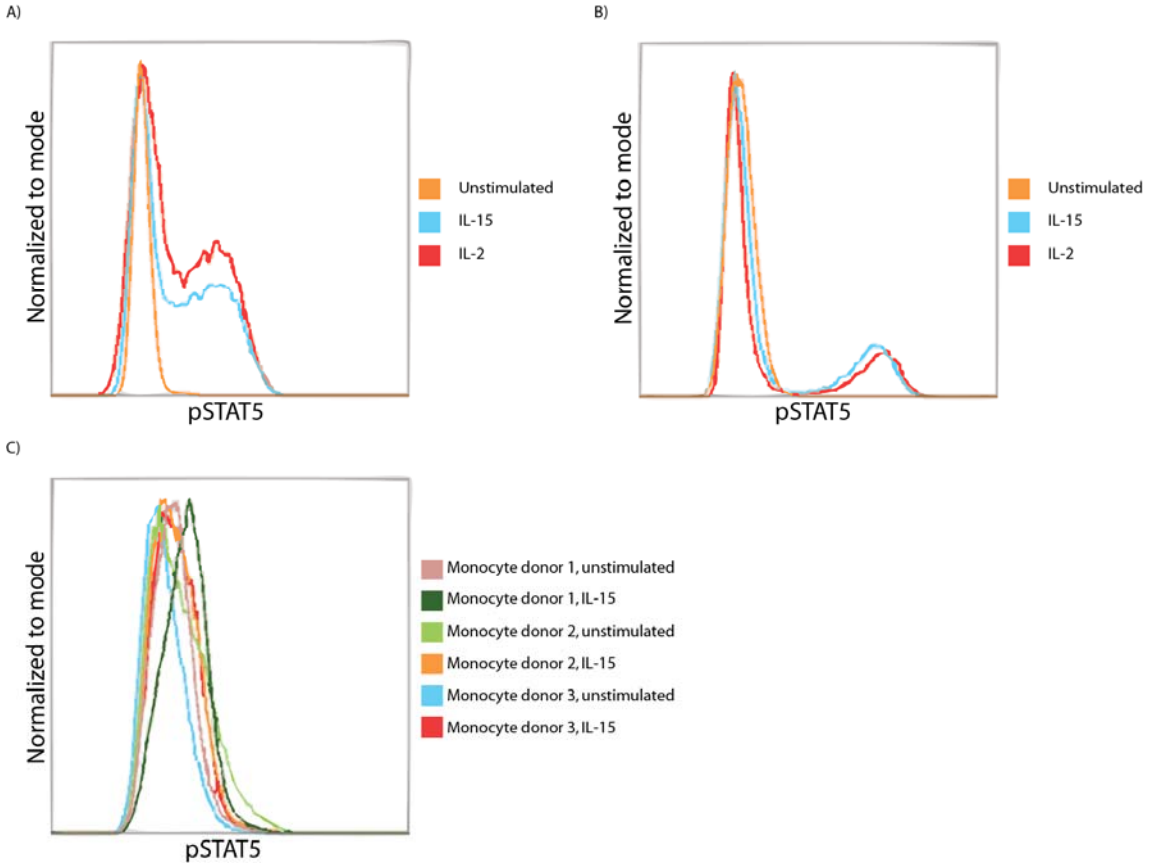


**Figure 5: IL-15 and IL-2 induce phosphorylation of STAT5 in T cells, but not in monocytes.** T-cell clone(A), peripheral mononuclear cells (PBMCs) (B) or three different monocyte donors (C) were cultured for 15 min with or without IL-15 or IL-2 stimulation, as depicted. Gating was performed according to CD3 for PBMC T cells.

Both PBMC T cells and TCCs responded to IL-15 and IL-2 by rapidly phosphorylating STAT5. However, monocytes appeared unresponsive to IL-15 in this manner (Figure 5). On the contrary, monocytes stimulated with IL-4 exhibited robust STAT6 phosphorylation to the same level as PBMCs (data not shown), showing that the monocytes are capable of responding to cytokines in vitro. This further suggest that the increased proliferative effect on T cells by pre-treating monocytes with IL-15,

is caused by surface binding of exogenous IL-15 (Figure 3) and direct presentation to gluten specific TCC cells (Figure 2).

## 4.6 No detectable co-adjuvant effect of RA together with IL-15

Studies have indicated that RA has co-adjuvant effects with IL-15 on APCs, promoting break of tolerance to dietary antigens by overriding the normally tolerogenic combination of RA and TGF-β[82]. To assess whether IL-15 in combination with RA may induce an inflammatory phenotype in monocytes, monocytes were pretreated with combinations of IL-15, RA, TGF-β, and gluten before co-culturing with gluten specific TCC cells.

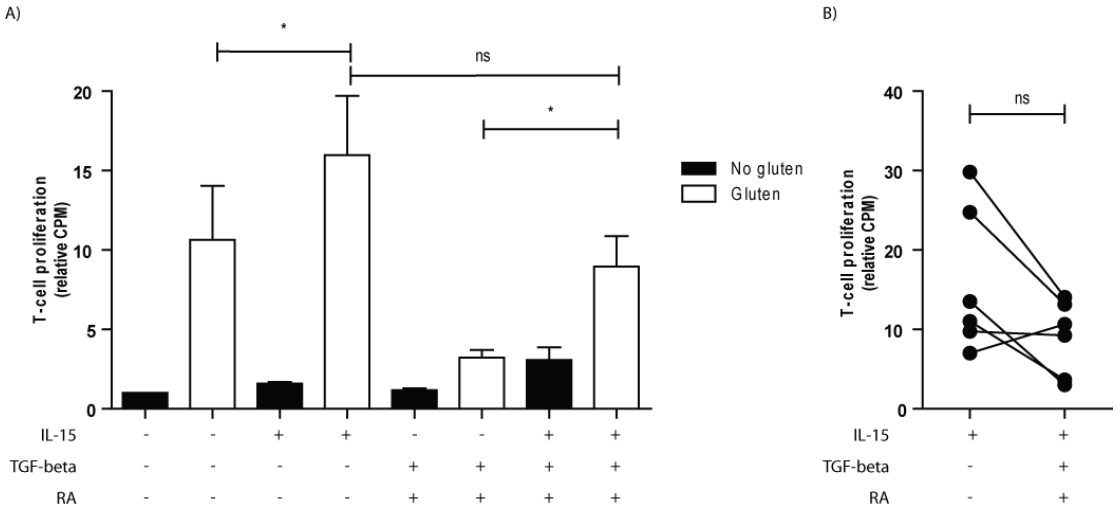


**Figure 6: Retinoic acid (RA) and IL-15 did not exhibit co-adjuvant effects** A, B) Monocytes cultured overnight with or without IL-15, RA, TGF-β and gluten, and co-cultured with gluten specific T-cell clones for 4 days, adding $^{3}H$ thymidine approximately 20 hours before harvesting and measuring thymidine incorporation, as depicted. B) Gluten pre-treated monocytes, treated as depicted, also displayed in A). Statistics were done using paired t test, n=6. *≤0.05, ns=0.065.

Pretreatment of monocytes with RA and TGF-β tended to reduce T-cell proliferation compared to unstimulated monocytes, thus indicating a tolerogenic effect of these factors. However, although adding IL-15 in combination with RA and TGF-β significantly increased proliferation compared to RA and TGF-β alone, the data did not reveal any co-adjuvant effects as the proliferation was not increased compared to IL-15 alone (Figure 5 A). On the contrary, proliferation tended to be reduced, although the data did not reach statistical significance. (Figure 5B)

## 4.7 IFN-γ stimulated monocytes activate T-cell proliferation in an IL-15 dependent manner

Monocytes have been shown to up-regulate IL-15 expression following IFN-γ stimulation [72], and IFN-γ treatment effectively activate gluten specific T-cell proliferation [3]. Therefore, we investigated the effect of IFN-γ on monocytes ability to activate T-cell proliferation, and assessed whether IL-15 has a role in this system. To this end, we pre-treated monocytes with IFN-γ, and blocked IL-15 with a neutralizing antibody before co-culturing with gluten specific TCC cells.

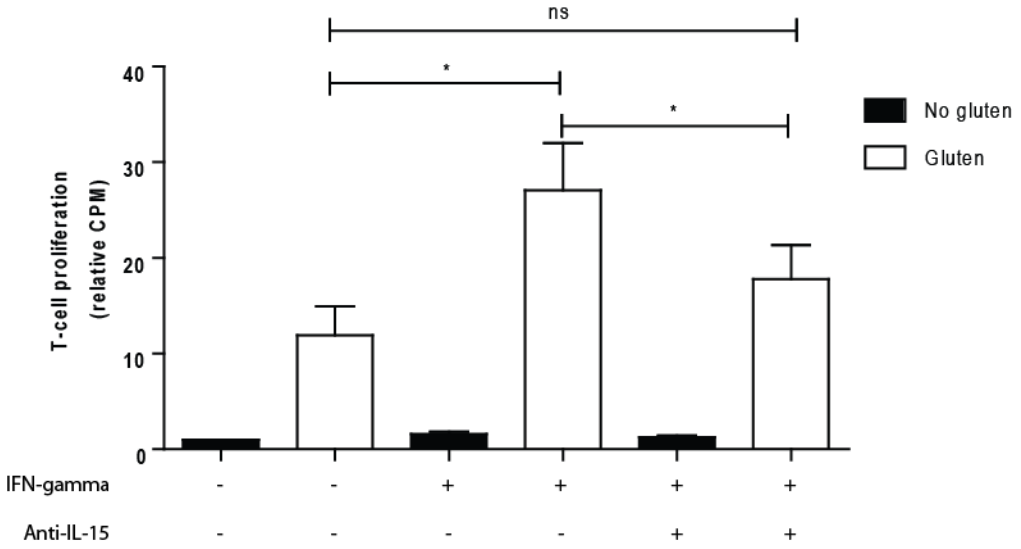


**Figure 7: IFN-γ mediated monocyte activation of T-cell clone (TCC) cells is dependent on IL-15.** Monocytes were pre-treated over night as depicted on the graph. Anti-IL-15 blocking antibody was added before co-culturing with gluten specific T-cell clones. Statistics were done using paired t test *≤0.05, n=6.

T-cell proliferation was significantly increased after pre-treating monocytes with IFN-γ. Strikingly, however, this effect was almost completely abrogated by blocking IL-15 before co-culturing with gluten TCC cells (Figure 7), and was not different from that of untreated monocytes. This indicates that monocytes can secrete or trans-present endogenous IL-15 following IFN-γ stimulation, which further activates gluten specific T-cell proliferation.

## 4.8 Transcriptional regulation of IL-15 in monocytes

IL-15 and IFN-γ stimulated monocytes have an increased ability to induce T-cell proliferation, which was reduced by blocking IL-15. To further dissect the

mechanism, IL-15 transcription levels in monocytes were measured by using qRT-PCR.

To investigate if the increased proliferative stimulation from monocytes were due to increased levels of IL-15 transcription following stimulation with IL-15 or IFN-γ, we cultured monocytes for 6, 15 and 24 hours with the different stimuli before harvesting and isolating RNA for further qRT-PCR analysis.

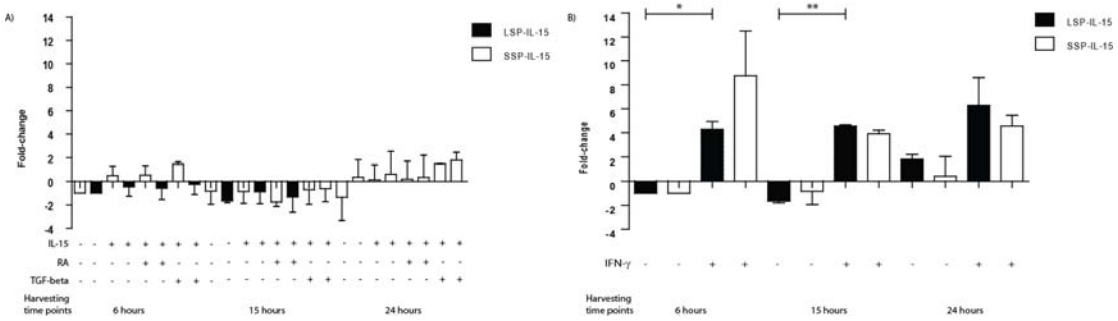


**Figure 8: Only IFN-γ regulates IL-15 transcription.** A) Monocytes were pre-treated over night with or without A) IL-15, RA and TGF-β, or with or without B) IFN-γ, and harvested as depicted. *≤0.05 and **≤0.01, n=3

IL-15 transcription did not appear to be regulated by pre-treating monocytes with IL-15, RA or TGF-β (Figure 8A). However, pretreatment of monocytes with IFN-γ significantly up-regulated LSP-IL-15 and showed an increasing trend for SSP-IL-15 (Figure 8B), suggesting that IL-15 is expressed by monocytes in this system.

## 4.9 Increased IFN-γ-production in gluten specific T cells following antigen stimulation

A hallmark for celiac disease T-cells is an increased IFN-γ production, and thus, we assessed if this is also true for the gluten specific tissue derived TCC cells used in this study. To examine the cytokine production potential to the gluten specific TCC, monocytes were pre-treated with combinations of IFN-γ and gluten over night before co-culture with TCC cells for 24 hours.

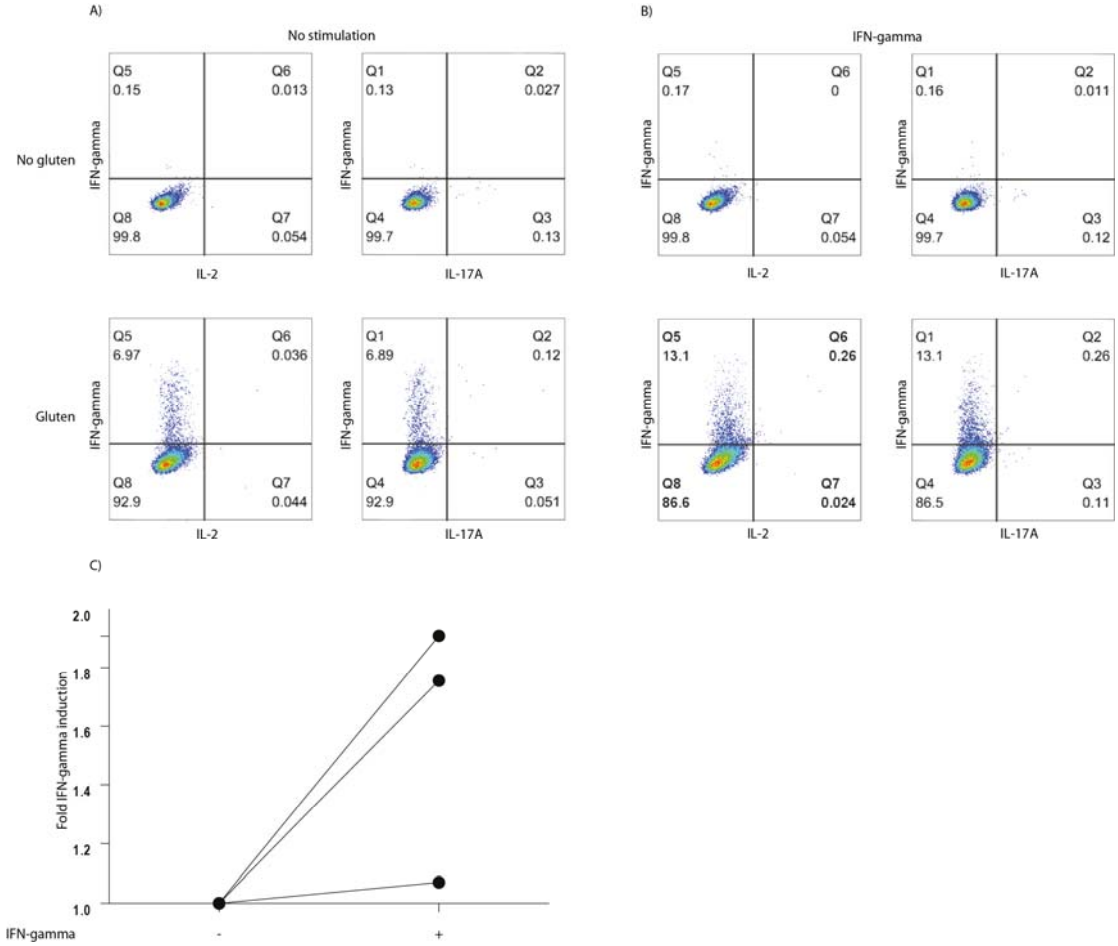


**Figure 9: Activated T-cell clone (TCC) cells produced IFN-γ.** Monocytes were pre-treated overnight with or without gluten and with (A) or without (B) IFN-γ (one representative experiment shown), before co-culturing with gluten specific TCC cells. Gating was done according to live, CD3+ T-cells. C) Fold induction of IFN-γ expression after pre-treating monocytes with IFN-γ and gluten. n=3.

The data revealed that T cells co-cultured with gluten pre-treated monocytes express IFN-γ. Moreover, IFN-γ stimulation of monocytes further increased the number of IFN-γ expressing T cells (Figure 9 C). We did not, however, detect any significant production of IL-2 or IL-17.

# 5. Discussion and future perspectives

## IL-15 enhances the ability of HLA-DQ $2^+$ monocytes to activate gluten specific T-cell clones

As previous showed by Beitnes et al [3], $CD11c^+CD14^+$ monocyte-derived APCs are increased in the small intestine of CD patients. To gain mechanistic insight into the antigen presentation potential of monocytes in the context of CD, monocytes were exposed to factors present the in celiac lesion and assessed for their ability to activate tissue derived gluten specific TCCs.

Blood donors were screened for cells expressing HLA-DQ 2 (Figure 1), which is a prerequisite for presentation of gluten peptides to gluten specific $CD4^+$ T-cell clones [3]. Monocytes from such donors were further pre-treated with factors present in the small intestine lamina propria of celiac disease patients to investigate their antigen presenting ability. Several proinflammatory-cytokines have been shown to be upregulated in celiac lesions, including IL-15 and IFN-γ [55], most likely acting in concert with constitutive intestinal factors like RA and TGF-β [84], and we wanted to understand the interplay between these factors. The results of the present study showed that monocytes pre-treated with IL-15, enhance gluten dependent T-cell stimulation (Figure 2). This proliferative effect on gluten specific T cells was almost completely abrogated by co-culturing with an anti-IL-15 antibody (Figure 4), suggesting that monocytes in response to IL-15 have the ability to either secrete or trans-present IL-15 to gluten specific T-cells, and directly induce T-cell proliferation. In fact, Dubois et al show that IL-15 can be trans-presented when co-expressed with IL-15Rα [78] acting on target cells expressing IL-15Rβ and the common γ-chain involved in IL-15 signaling and intracellular signal transduction [69]. In line with this, IL-15 shows a direct proliferative effect on the gluten specific TCCs (Figure 4), and monocytes were shown to have binding potential for IL-15 (Figure 3), compatible with the notion that IL-15 is trans-presented to gluten specific T-cells.

## Monocytes do not respond to IL-15 by STAT5 phosphorylation

Due to the enhanced T-cell proliferation following IL-15 monocyte stimulation (Figure 2), and results suggesting that IL-15 is trans-presented to T cells causing T-cell proliferation (Figure 4), we investigated whether monocytes exhibited responsiveness to IL-15. STAT5 is one of the key proteins phosphorylated during IL-15 signaling, and can therefore be used to investigate this [80]. Results of the present study shows that monocytes stimulated with IL-15 did not show STAT5 phosphorylation, in contrast to T-cells used as a positive control (Figure 5).

Monocytes stimulated with IL-4 exhibited a robust STAT6 phosphorylation (data not shown) as anticipated [85], showing that monocytes are capable of responding to cytokines in vitro. Based on these findings it is reasonable to suggest that IL-15 has no stimulatory effect on freshly isolated peripheral monocytes.

## RA did not have a co-adjuvant effect on the HLA-DQ $2^+$ monocytes

DePaolo et al has showed that RA has a co-adjuvant effect together with IL-15 on APCs, overriding the normally tolerogenic response of RA and TGF-β to dietary antigens [82].

In the current study, pre-treating monocytes with RA, TGF-β and IL-15 did not reveal any additional increase in T-cell proliferation compared to IL-15 alone, thus any co-adjuvant effect was not apparent (Figure 6). However, there are major differences between these two studies, e.g. their use of in vitro-generated mouse DCs instead of human monocytes and the induction of $Foxp3^+$ cells as readout. In the context of active CD the numbers of DCs are down-regulated in the small intestine, contrary to newly recruited monocytes which are increased. Thus, analysis of the monocytes capacity to regulate T-cell stimulation appears to be more relevant to CD than investigating T-cell regulation by DCs [3].

Additionally, since there are three populations of $Foxp3^+$ cells, resting Tregs, activated Tregs and non-Tregs, where only two of these subpopulations are functionally suppressive [86, 87], the use of $Foxp3^+$ positive cells as a readout for tolerogenic effects could be misleading compared with T-cell proliferation. Thus, we cannot directly compare these two studies, nor did the findings of the present study support claims that RA has an adjuvant effect together with IL-15 on APCs found in the gut.

## Increased T-cell proliferation by IFN-γ stimulated monocytes depend on IL-15

IFN-γ has been shown to induce IL-15 expression in monocytes [72, 78] and to enhance their ability to induce T-cell proliferation [3]. In accordance with this, we found that IFN-γ stimulated monocytes from HLA-DQ $2^+$ donors enhanced gluten specific T-cell proliferation and IFN-γ production (Figure 7 and 9). However, the increased T-cell proliferation was almost completely abrogated by blocking IL-15 with a monoclonal neutralizing antibody during co-culturing of monocytes with gluten specific T cells. These findings suggested that IL-15 is responsible for the increased T-cell proliferation following IFN-γ stimulation of monocytes, and that such monocytes either trans-present or secrete endogenous IL-15 to T-cells.

To further dissect the role of IL-15 during activation of gluten specific T-cell proliferation, we investigated how the different stimuli used in the current study affected transcriptional regulation of IL-15 with qRT-PCR. IL-15 transcription did not appear to be regulated by pre-treating monocytes with IL-15, RA or TGF-β. On the contrary, pre-treating monocytes with IFN-γ resulted in a significantly enhanced transcription of LSP-IL-15 (Figure 8), but not SSP-IL-15, although SSP-IL-15 transcription showed a tendency to be upregulated following IFN-γ stimulation.

Based on these findings, the source of monocyte-associated IL-15 in this system following pre-treatment with IL-15 or IFN-γ was most likely exogenous or endogenous, respectively. These findings suggests that monocyte-derived APCs in celiac lesions can bind exogenous IL-15 and activate gluten specific T-cell proliferation directly by trans-presenting the cytokine [55]. Furthermore, monocyte-derived APCs may produce IL-15 in response to IFN-γ present in celiac lesions, further amplifying their T-cell stimulation capacity.

## Concluding remarks/summary

Monocytes from HLA-DQ $2^+$ donors (Figure 1) pre-treated with gluten and IL-15 or IFN-γ had a significantly enhance ability to induce gluten specific T-cell proliferation (Figure 2 and 7). In the latter situation we also demonstrated that increased proliferation was paralleled by increased IFN-γ production (Figure 9).

Monocytes did not respond to IL-15 by exhibiting STAT5 phosphorylation (Figure 5) or upregulating LSP- or SSP-IL-15 (Figure 8), suggesting that IL-15 pre-treatment of monocytes enhance T-cell proliferation by IL-15 trans-presentation.

Importantly, monocytes were capable of producing IL-15 following IFN-γ stimulation (Figure 7 and 8), and IL-15 appeared to be responsible for mediating the additive effect of IFN-γ on monocyte-driven T-cell proliferation.

One can hypothesize that triggering inflammation in CD could be caused by epithelial stress factors, viruses, bacteria or gluten peptides, which respond by producing IL-15 [2]. Monocyte derived APCs loaded with deaminated gluten peptides bind exogenous IL-15, furthermore activating gluten specific T cells initiating the initial burst of T-cell activation, IFN-γ production and proliferation. T-cell produced IFN-γ could further stimulate APCs to produce and present more IL-15 to the T cells – thus additionally amplifying the inflammation. Monocyte derived APCs could by this mechanism disturb homeostatic conditions and maintain inflammation in the small intestine of CD patients on a gluten containing diet.

However, IL-15 in vivo has been shown to be secreted in a heterodimer with IL-15Rα [88], and whether this complex can bind to IL-15Rα and be further trans-presented to IL-15Rβ/γ expressing cells is not known.

## Future perspectives

We could not detect a co-adjuvant effect of RA together with IL-15 - on the contrary, T-cell proliferation tended to be reduced following pre-treatment of monocytes with RA and IL-15. To further assess if RA can have a co-adjuvant effect on monocytes, investigating if monocytes express IL-15R β-chain, which is necessary for IL-15 signaling (1.4.5), would demonstrate if they actually have the ability to directly respond to IL-15 or not; hence, possess the ability to respond in a co-adjuvant manner to IL-15 and RA.

IL-15, in the context of celiac disease was the main focus of this study, and investigation of how monocytes from the small intestine lamina propria respond to IL-15 is important. In fact, preliminary results shows that IL-15 pre-treated $CD14^+$ cells isolated from small intestinal lamina propria of HLA-DQ $2^+$ donors, co-cultured with gluten specific TCC cells, enhance T-cell proliferation (data not shown). However, the full range of IL-15 effects on $CD14^+$ lamina propria derived APCs should be assessed by T-cell proliferation assays, transcriptional analysis and analyzing the response to IL-15 by the presence of intracellular pSTAT5.

Since RA has an adjuvant effect on mice DCs responding to IL-15 [82] it would be interesting to further explore the mechanisms behind RA on APCs from human small intestinal lamina propria of HLA-DQ $2^+$ donors, responding to IL-15.

One approach could be assessed by performing T-cell proliferation assays, and investigating transcription and expression levels of IL-12p70 and IL-23, which was produced by RA and IL-15 stimulated mice DCs [82].

IL-15 is also highly expressed in intestinal autoimmune disorders referred to as inflammatory bowel disease (IBD) [89], which is much more complicated to manage then CD [90]. In addition, IL-15 has been shown to induce T-cell activation and proinflammatory cytokine production [89]. Therefore, treatment targeting blocking of IL-15 in IBD patients may be beneficial as a therapeutic agent to reduce inflammation.

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