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Regulatory B and T cell responses in patients with autoimmune thyroid disease and healthy controls.

Birte Kristensen

This review has been accepted as a thesis together with three previously published papers by the University of Southern Denmark on 15th of October 2014 and defended on 24th of April 2015.

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The three original papers include:

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2. Kristensen B, Hegedüs L, Lundy S, Brimnes MK, Smith TJ, Nielsen CH. Characterization of Regulatory B cells in Graves' Disease and Hashimoto's Thyroiditis. PLOS One 2015; 10: e0127949.
3. Kristensen B, Hegedüs L, Madsen HO, Smith TJ, Nielsen CH. Altered balance between self-reactive Th17 cells and Th10 cells and between full-length FOXP3 and FOXP3 splice variants in Hashimoto's thyroiditis. Clin Exp Immunol 2015; 180: 58-69.

Introduction

Autoimmunity

Autoimmunity is the breakdown of immune self-tolerance (1) and can occur in an organ-specific or systemic manner. Organ-specific autoimmunity is characterized by a T-cell or antibody mediated attack on a specific organ, whereas systemic autoimmunity is an uncontrolled immune response towards ubiquitous self-antigens (1,2). Under homeostatic conditions, central and peripheral tolerance aid in eliminating auto-reactive T and B cells. While in the thymus or bone marrow, T cells and B cells, respectively, undergo checks to determine their self-reactivity (3). Central tolerance is the elimination of auto-reactive T cells and B cells, in the thymus and bone marrow, respectively; it recognizes self-antigens with a strong affinity (2,4,5). The main mechanism of central tolerance is negative selection. Negative selection allows the elimination of developing T cells and B cells if the corresponding receptor on T cells (TCR) or on B cells (BCR) recognizes a self-antigen with high affinity (4–7). Additionally, B cells undergo receptor editing to avoid deletion. Receptor editing is the re-arrangement of genes that encode the BCR and thus allow the expression of a BCR with low affinity towards self-antigens (5,8). T cells and B cells that are able to recognize self-antigens with a low affinity are able to leave the thymus and bone marrow, respectively (5). At this point, peripheral tolerance including anergy as well as regulatory T cells and B cells steps in and aids in the regulation of auto-reactive cells (9–13).

Human T lymphocytes

Within the T cell group, multiple subsets exist, including CD4+ T helper (Th), CD8+ cytotoxic T cells (CTL), natural killer T cells (NKT cells) and regulatory T cells (Tregs) (14). All these subsets play an important role within the immune system. However, only CD4+ T cells and regulatory T cells will be discussed here.

Th1 / Th2

To stimulate a naïve CD4+ T cell (Th0), both the TCR and the co-stimulatory molecules are needed to be stimulated. This occurs by interaction between antigen presenting cells (APC) and Th0 cells (14). However, it is the local cytokine milieu that will determine whether a Th0 cell will become a Th1 or a Th2 cell (14–16). Figure 1 summarizes the differential pathways of a naïve CD4+ T cell. It was Mosmann et al that initially coined the term 'Th1 and Th2' (17,18). Classically, Th1 cells will produce interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2), whereas Th2 cells are known to secrete IL-4, IL-5, IL-10, and IL-13 (15,17–20). The presence of IL-12 and IFN-γ will activate signal transducer and transcription activator 4 (Stat4) and Stat1 signalling pathways, respectively, which induces T-bet expression, thus promoting Th1 cellular differentiation (21–23). For Th2 differentiation, exogenous IL-4 induces GATA3 via the Stat6 signalling pathway (22,24,25). IL-2 acting via Stat5 signalling pathway is also needed for full Th2 differentiation (22,26).

Once fully differentiated, IFN-γ and IL-4 are needed to amplify and augment pre-existing Th1 and Th2 cellular populations, respectively (15,22,27). This will allow for a Th1 or Th2 dominated immune response. It is known that Th1 and Th2 cells can cross-regulate each other and that this cross-regulation can occur on a cytokine and transcriptional level (22). IFN-γ secreted by Th1 cells can suppress the expansion and effector function of Th2 cells. Additionally, IL-4 produced by Th2 cells can exert the same regulatory function on Th1 cells (15,22,28). On a transcriptional level, T-bet can suppress Th2 differentiation by preventing GATA3 from binding to the Th2 cytokine gene locus and inhibit cytokine production, which would lead to Th2 differentiation (22,29). On the other hand, GATA3 has been shown to downregulate Stat4, which is crucial for Th1 development (22,30). One study by Usui et al has

suggested that Th1 differentiation occurs because T-bet suppresses GATA3 instead of amplifying the IFNG gene (31).

Th1 and Th2 cells are functionally distinct. Where Th1 cells aid in the combat of intracellular bacteria, such as mycobacterial infections, and viruses, Th2 cells help with extracellular parasites such as helminths (15,18,20,22,23). However, if these responses are not regulated, exaggerated Th1 responses have been linked with several organ-specific autoimmune diseases and exaggerated Th2 responses have been shown to play a crucial role in the development of allergic inflammation and asthma (20,32).

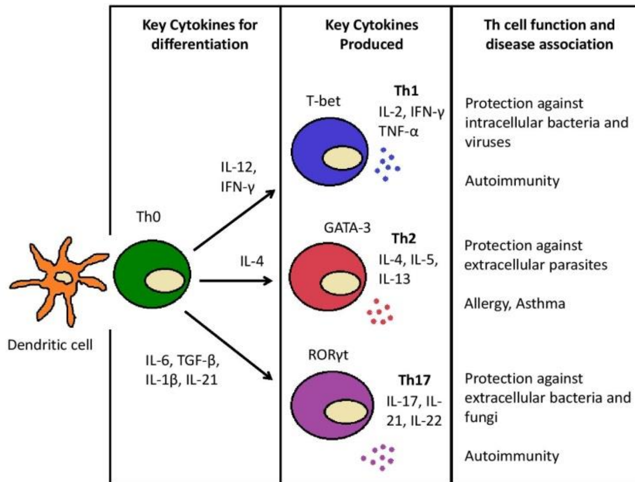

Figure 1 Summary of the differentiation pathways of a naïve CD4+ T cells.

Th17 cells

The discovery of a third Th subset, namely Th17, caused a shift in the classical Th1 and Th2 paradigm. The third Th cell subset is termed 'Th17' due to its ability to produce the cytokine IL-17A and IL-17F (33,34). These cells also produce IL-21, IL-22, and IL-26 (35). IL-17 is thought to be a pro-inflammatory cytokine that is able to induce local inflammation by stimulating the production of IL-6 as well as amplifying local inflammation by synergizing with other pro-inflammatory cytokines such as IL-1β, IFN-γ and TNF-α (36). The cytokines produced by Th17 cells have effects on numerous immune and non-immune cells, some of which include epithelial cells, NK cells, B cells, macrophages, and neutrophils (35). The primary function of Th17 cells is to provide protection to the host by aiding in the clearance of extracellular bacteria and fungi (37). However, Th17 cells have also been linked to various autoimmune diseases, such as rheumatoid arthritis (RA), psoriasis, inflammatory bowel disease, multiple sclerosis, and autoimmune thyroid disease (38–41).

The differentiation of human Th17 cells from Th0 cells is still unclear. Several cytokines including IL-1β, IL-6, IL-21, IL-23, TNF-α and transforming growth factor-beta (TGF-β) have been involved in the differentiation of human Th17 cells either in combination or alone (42–46). The exact effect of IL-23 on human Th17 cells is not yet clear. In mice, it is known that IL-23 is upregulated only after cellular activation (33) and may have a role within Th17 expansion and pathogenicity, but in humans its central role may only be to direct Th17 differentiation (16,46).

The role of TGF-β in Th17 differentiation is truly fascinating. Until recently, TGF-β has been associated with regulatory T cells (Tregs) and not Th17 differentiation. Several groups have reported that TGF-β is crucial to Th17 differentiation (42,44,46,47). However, TGF-β may not have a direct role in Th17 differentiation, but instead it may limit Th1 differentiation and thereby allow the differentiation of Th17 cells (48). Further investigation is needed to clarify the role of TGF-β in Th17 differentiation.

Human Th17 cells express the transcription factor retinoic acid receptor-related orphan receptor C (RORC) (49,50), which distinguishes these cells from the prototypical Th1 and Th2 cells (51). RORC is the human ortholog to the mouse RORγt (37).The cytokines IL-1β, IL-6, IL-23, and TGF-β are all able to induce the expression of RORC in Th17 cells (37,44,45,52).

Regulatory T cells

Tregs are crucial in maintaining homeostasis within the immune system (13). Tregs help to prevent an immune response against self-antigens as well as to suppress an immune response against exogenous antigens before they can become a danger to the host (10,53). Regulatory T cells consist of a heterogeneous population of cells including CD4+ T cells, CD8+ T cells and NK cells (54). However, only natural and inducible Tregs which are CD4+ will be discussed here.

Natural Tregs (nTregs) are developed in the thymus and migrate to the periphery (54). These Tregs are characterized by the surface markers CD4, CD25 (9), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and glucocorticoid-induced tumor-necrosis-factor-receptor-related protein (GITR) (54,55). Additionally, the transcription factor, forkhead box protein 3 (FOXP3), is important in the identification and development of nTregs (56–58). The cytokine TGF-β is reportedly able to enhance the expression of FOXP3, and is thus vital in the maintenance of nTregs (54). The specificity of the TCR on nTregs is towards self-antigens that are present in the thymus (53).This allows nTregs to suppress auto-reactive T cells and B cells by cell-contact dependent mechanisms (53,59), and one of the core methods of suppression is the expression of CTLA-4. CTLA-4 regulates the co-stimulatory markers CD80/CD86 (59–61) and by binding to CD80/CD86, CTLA-4 gives the inhibitory signals which will prevent T cell activation (59,61). Normally, CD80/CD86 binds to CD28 and provides the necessary co-stimulatory signals to allow for T cell activation (62). In addition, nTregs may act like a 'sink' for the available IL-2 in the micro-environment, which will result in the apoptosis or downregulation of effector T cells. This is because IL-2 is needed for effector T cell survival and growth, but also for the activation of nTregs and the upregulation of FOXP3 expression (54,59,63–65).

Inducible Tregs (iTregs), also known as adaptive Tregs, differentiate from effector (CD4+CD25-) T cells into iTregs in the periphery (10,53,66). iTregs are identified based on their ability to secrete IL-10 and TGF-β (10,67,68). There is speculation that iTregs are not one heterogeneous population, but are sub-divided into two populations named Tr1 and Th3, the difference being the cytokines that they secrete. Tr1 cells are thought to secrete mainly IL-10, whereas Th3 cells secrete predominately TGF-β (66,69). The conversion of effector (CD4+CD25-) T cells into iTregs is achieved after antigen recognition and with help from CTLA-4, TGF-β or IL-10 (54,68,70–72) and after stimulation through CD46 (73). Unlike nTregs, the suppression method for iTregs is cytokine dependent and is carried out via the secretion of IL-10 and TGF-β (10,53,70,72). The suppressive effects of IL-10 and TGF-β are multifaceted. Indirectly, iTregs, via IL-10 and TGF-β, can affect the function, cytokine production, and co-stimulatory molecule expression of APC, which would subsequently affect the cytokine production and proliferative capability of CD4+ T cells. Directly, iTregs, via IL-10 and TGF-β, can affect the cytokine production of CD4+ T cells (74–78)(10,68). Although, the main suppressive mechanism of iTregs is cytokine-dependent, there is some data

that indicate that iTregs may upregulate inhibitory receptors that could inhibit APC or CD4+ T cells on a cell-to cell contact basis (10,53).

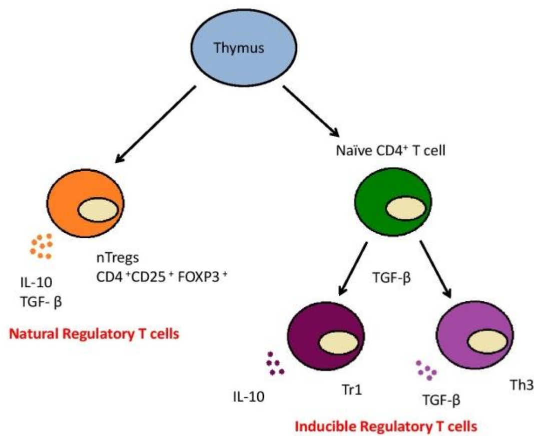

Figure 2 Regulatory T cell subsets.

Difficulty arises when trying to discern nTregs from iTregs or even from effector (CD4+CD25-) T cells, due to the similarities in the expression of certain surface markers. iTregs have been shown to acquire the expression of CD25 and FOXP3 (79), while nTregs, normally cytokine independent, can induce and secrete IL-10 and/or TGF-β (80–82). Studies have also demonstrated that effector (CD4+CD25-) T cells in the periphery can acquire the expression of FOXP3 and CD25, as well as regulatory T cell activity (83–86).

The transcription factor FOXP3 is a key component in the suppressive function, development, and cell lineage commitment of regulatory T cells (56–58). Mutations in FOXP3 may lead to dysfunction of or a lack of Tregs. If this occurs in humans, the condition called immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome may arise, which includes uncontrolled cytokine production and proliferation (87,88). However, recent evidence suggests that there are three isoforms of human FOXP3. These three isoforms are full length FOXP3 and the two truncated splice forms: FOXP3Δ2, which lacks exon 2, and FOXP3Δ2Δ7, which lacks exons 2 and 7 (89–91). It is not known whether these FOXP3 isoforms are co-expressed or are expressed in different Tregs, but it is conceivable that all three isoforms are functionally different. Full length FOXP3 functions as a repressor of NF-kB, NF-AT, RORγt and ROR-α (92–96). However, FOXP3Δ2 isoform is unable to functionally repress RORγt (95), ROR-α (94), and NF-kB (96,97).

Th17 and Treg plasticity

Human Th17 cells are potentially not locked into one phenotype but are able to exhibit plasticity. Annunziato et al were among the first to show that a proportion of human Th17 cells were able to produce both IL-17 and IFN-γ; they are called the 'Th17/Th1 cells'. In the same study, they also showed that human Th17 cells were able to differentiate into Th1-like cells and produce IFN-γ in the presence of IL-12 (98). These cells were called 'non-classical Th1' or 'Th17-derived Th1 cells'. The expression of the IL-12 receptor is important for the differentiation into Th1 cells (98,99). Additionally, depending on the stimulation Th17 cells can produce IFN-γ or IL-10. Zielinski et al showed that stimulating with Candida albicans Th17 cells were able to produce IL-17 and IFN-γ, while stimulating with Staphylococcus aureus, Th17 cells were able to produce IL-17 and IL-10 (100).

In addition to Th17 cells having the ability to exhibit plasticity, there is a growing notion that Tregs can also exhibit plasticity. It has been demonstrated that CD4+CD25hi Tregs can express the transcription factors FOXP3 and RORγt, concurrently (101,102). These CD4+CD25hiFOXP3+ Tregs have also been shown to produce IL-17 after PMA/ionomycin stimulation with or without IL-1β and IL-6 present (101–104). The presence of pro-inflammatory cytokines, such as IL-1β and IL-6, might enhance IL-17 production in FOXP3+ Tregs. This might be important at sites of inflammation or in autoimmune diseases where these pro-inflammatory cytokines are in abundance. However, there are conflicting results as to whether or not these IL-17+FOXP3+ Tregs lose their suppressive function after secreting IL-17. Voo et al demonstrated that IL-17+FOXP3+ Tregs are still capable of inhibiting proliferation of CD4+ T cells, whereas Beriou et al observed a diminished suppressive activity of IL-17+FOXP3+ Tregs in terms of inhibiting IFN-γ production (103,104). A closer and more complex relationship between CD4+CD25hi Tregs and Th17 cells could exist than initially thought.

Human B lymphocytes

Human B cells are developed in the bone marrow from hematopoietic stem cells (105) and have multiple functions within the immune system. They are able to produce antibodies, cytokines, and function as antigen presenting cells (106–109). B cell subsets, such as B1 B cells, transitional B cells, marginal zone B cells, and follicular B cells, have been more extensively investigated in mice than humans. Therefore, what is currently known about B cell subsets in mice and humans is summarized below.

B1 B cells

In mice, B1 B cells are located in the peritoneal and pleural cavities (110,111) and are able to spontaneously produce natural antibodies that provide the first line of defense against pathogens (14,112,113). Additionally, these B1 B cells are self-replenishing (110,114) and are able to efficiently present antigens and induce differentiation of effector CD4+ T cells (115). In mice, B1 B cells are comprised of two subsets, B1a and B1b, and these subsets are defined on the basis of CD5 expression (105,110,114). Similarly, in humans, CD5+ B cells play a protective role in the host by the production of natural antibodies (116,117). In several autoimmune diseases, such as RA and systemic lupus erythematosus (SLE), it has been observed that patients have an increased frequency of CD5+ B cells and that these B cells are able to produce autoantibodies (118–120). However, the existence of B1 B cells and use of CD5 as a phenotypic marker in humans is still under great debate. CD5 is expressed on the majority of B cells during childhood as well as being used a pan-T cell marker (121,122). It has also been speculated that CD5 is an activation marker and that the expression of CD5 can be induced or upregulated after stimulation with phorbol esters - making it unsuitable for the identification of a distinct cellular subset (116,123). It has been proposed that the human B1 subset can be identified by the phenotype CD19+CD27+CD43+ instead of CD5+ (124). Similar to their murine counterparts, these human B1 B cells are able to spontaneously produce IgM as well as induce stimulation of T cells (124).

Transitional B cells

The term transitional B cells was first coined in mouse studies by Carsetti et al (125) and describes B cells that are developmentally situated between immature B cells in the bone marrow and

fully mature B cells in the peripheral blood (126). In mice, transitional B cells have been divided into IgMhiCD21negCD23negIgDneg transitional 1 (T1) or IgMhiCD21hiCD23hiIgDhi transitional 2 (T2) B cells (122,127). The expression of the developmental marker, CD23, highlights the difference in the development between the two subsets (128). These CD23hi T2 subsets are located primarily in the spleen and are more capable than T1 B cells in terms of proliferating, differentiating and surviving BCR-induced activation, thus allowing for development into mature B cells (128–131). T1 B cells are located in the bone marrow, blood and spleen and readily die via apoptosis after BCR-induced activation. It has also been suggested that T1 B cells are the precursors for T2 B cells. However, T1 B cells may also develop directly into mature B cells (130).

In humans, earlier observations characterized transitional B cells, located in the peripheral blood or bone marrow, as CD19+CD24hiCD38hi using the two developmental markers CD24 and CD38 (122,131). Additionally, studies have shown that the human bone marrow has a greater proportion of B cells expressing the CD24hiCD38hi transitional phenotype, and that these transitional B cells were able to reconstitute the peripheral blood after hematopoietic stem cell transplantation (126,132). However, more recent studies have indicated that humans, like mice, have two subsets of transitional B cells, namely T1 and T2 (133,134). Human T1 and T2 transitional B cells have been subdivided based on the low or high expression of CD21 or IgD (126,134). The difference in the expression of CD21 or IgD may indicate a difference in the developmental stage of the transitional B cells (126,134). Similar to CD23 in mice, CD21hi T2 B cells in humans are better at proliferating and secreting immunoglobulins than CD21low T1 B cells, indicating that T2 B cells are more developmentally mature than T1 B cells (134). Additionally, human T1 B cells are more prone to apoptosis than T2 B cells, which is comparable to their murine counterpart (131).

Marginal zone and Follicular B cells

Marginal zone (MZ) and follicular zone (FO) B cells are two subsets of the splenic B cell subset (135), which may be derived from transitional T2 B cells (136). MZ B cells are located in the marginal zone of the spleen whereas FO B cells are primarily located in the lymphoid follicles of the secondary lymphoid organs (111,136,137). In mice, the phenotype for MZ B cells is IgMhiIgDlowCD21hiCD23low whereas for FO B cells it is IgMlowIgDhiCD21intCD23hi (138). FO B cells are able to circulate, capture and present T cell-dependent antigens to CD4+ T cells present in the lymphoid follicles in the white pulp (111,136). Additionally, FO B cells may have a role in the production of IgM antibodies in a T cell-independent manner in the bone marrow. This indicates that FO B cells may reside in both the spleen and bone marrow and carry out different functions (111,136,139). MZ B cells are able to rapidly produce antibodies against T cell-independent antigens, such as bacterial antigens, making these cells crucial as the first line of defense against blood-borne pathogens (14,137). In a similar way to FO B cells, studies have shown that MZ B cells can capture and present T cell-dependent antigens to CD4+ T cells and induce antigen-specific differentiation and proliferation (111,138,140). It has been proposed that MZ B cells are more capable of presenting antigens to T cells than FO B cells due to their high expression of co-stimulatory molecules (111,138,140). Another important function of MZ B cells is the shuttling of antigens from the marginal zone to the lymphoid follicles in the spleen. MZ B cells were initially thought of as being sessile however, a study by Cinamon et al demonstrated that MZ B cells are able to pick up antigen and transport it to the follicles in the spleen (141,142). In humans, MZ B cells have been found and may exhibit similar functions as their murine counterparts. There is evidence that human MZ B cells are important in processing and presenting T cell-independent antigens and providing first line of defense (143). Tables 1 summarizes the different B cell phenotypes discussed in this thesis, and are associated with B1 B cells, transitional B cells, MZ, and FO B cells as well as regulatory B cells.

Table 1 Summary of B cell phenotypes associated with different B cell subsets.

	Possible Phenotypes	
B cell subset	Human	Mice
B1	CD27+CD43+	B1a: CD5+ B1b: CD5-
Transitional	CD24hiCD38hi T1: CD21low T2: CD21hi	T1: IgMhiCD21negCD23negIgDneg T2: IgMhiCD21hiCD23hiIgDhi
Marginal		IgMhiIgDlowCD21hiCD23low
Follicular		IgMlowIgDhiCD21intCD23hi
Potential Breg cells	CD5+, CD25hi, TIM-1+, CD27+CD43+, CD27+CD43+CD11b+, CD24hiCD27+, and CD24hiCD38hi	

Regulatory B cells

It has long been believed that B cells may have a suppressive capacity. In the murine model for multiple sclerosis, namely experimental autoimmune encephalomyelitis (EAE), Wolf et al have demonstrated that mice deficient in B cells due to a genetic fault have a greater degree of disease severity and suffer from chronic EAE (144). What role these B cells play in the pathogenesis of EAE was unclear until Fillatreau et al demonstrated that murine B cells were capable of producing IL-10. It was precisely the production of IL-10 that suppressed the Th1-type/pro-inflammatory responses and allowed the EAE mice to recover, proving that B cells did have a regulatory function (11,145). A regulatory role for B cells and IL-10 has subsequently been demonstrated in other murine models of intestinal inflammation (146,147) and autoimmunity including collagen-induced arthritis, a model for RA (148,149). In murine studies, IL-10 produced by murine B cells are able to suppress Th1 and Th17 cells, thereby reducing the production of pro-inflammatory cytokines and responses (147–149). In a study by Carter et al, IL-10-producing B cells were actually capable of inducing iTregs (149).

The existence of regulatory B cells (Bregs) has also been demonstrated in humans. In some studies, Bregs may also be called B10 cells; however, these B10 cells normally denote IL-10-producing B cells. Currently, no one particular surface marker or transcription factor has been pin-pointed to identify Bregs. Therefore, the ability to produce IL-10 is the best marker to date. Several phenotypes have been proposed to identify Bregs, but currently there is no consensus. Some of the prevailing phenotypes include CD5+, CD25hi, TIM-1+, CD27+CD43+, CD27+CD43+CD11b+, CD24hiCD27+, and CD24hiCD38hi (121,124,150–161). A suppressive quality, in terms of inhibiting cytokine production, proliferation or differentiation, has also been demonstrated in human Bregs. Human CD24hiCD27+ Bregs have the ability to inhibit pro-inflammatory cytokine production such as TNF-α and IFN-γ from monocytes and CD4+ T cells, respectively, in an IL-10-dependent manner (154,156). Predominately human B10 cells with the phenotypes CD25hi, TIM-1+, CD24hiCD27+ and CD24hiCD38hi, have been shown to suppress

CD4+ T cell activation or proliferation as well as inhibit differentiation of naïve CD4+ T cells into Th1 or Th17 cells (151,157,158,161,162).

Additionally, a number of phenotypic subpopulations such as CD24hiCD27+ and CD24hiCD38hi have been able to convert effector (CD4+CD25-) T cells into Tregs (CD4+CD25hi) with an intact functional suppressive capacity (158–160). Kessel et al demonstrated that CD19+CD25hi Bregs were able to enhance the expression of FOXP3 and CTLA-4 in Tregs. However, this was not dependent on IL-10 but instead partially dependent on TGF-β and cell-to-cell contact (157). Intriguingly, Bregs have also been shown to inhibit TNF-α production from CD4+ T cells via an IL-10-independent pathway (156). This contributes to the theory or speculation that Bregs may induce suppression via cytokine-independent methods.

In summary, this indicates that B10 cells may have multiple phenotypes and may have an array of suppressive activities. Currently, there is no definitive B10 cell phenotype (see appendix I for a table reviewing some of the current Breg/B10 literature).

Autoimmune thyroid disease

Autoimmune thyroid disease (AITD), which encompasses Hashimoto's thyroiditis (HT) and Graves' disease (GD), are classical examples of organ-specific autoimmunity (2). These two diseases are clinically diverse because GD is primarily a humoral disease where autoantibodies are generated against the thyroid stimulating hormone receptor (TSHR) leading to hyperthyroidism, whereas in HT, T cells aid in the destruction of the thyroid epithelial cells (thyrocytes) and thyroid epithelial structure leading to hypothyroidism (2,163–165). However, these diseases still share several immunological features. These features include lymphocytic infiltration of the thyroid gland as well as auto-reactivity against three thyroid auto-antigens which are thyroglobulin (TG), thyroid perioxidase (TPO) and TSHR (166,167). Figure 3 outlines the immuno-pathogenesis of HT and GD.

Clinical diagnosis of GD and HT patients

Diagnosing GD or HT is dependent on measuring the levels of thyroid stimulating hormone (TSH), serum freeT4 (FT4) and freeT3 (FT3) as well as measuring the autoantibody levels against TSHR and TPO. Individuals diagnosed with GD have suppressed levels of TSH with elevated levels of FT4 and/or FT3 along with elevated anti-TSHR antibody levels. An ultrasound of the thyroid demonstrating diffuse hypoecchogenicity can be used to confirm the GD diagnosis (168). The presence of suppressed TSH, but normal FT4 or FT3 may indicate subclinical hyperthyroidism (169). In contrast to GD, HT patients have a raised TSH level, a decreased level of FT4 or FT3 and the presence of anti-TPO Ab and/or anti-TG Ab (165,170). If HT patients have a raised serum TSH level, but normal FT4 level, that indicates subclinical hypothyroidism (165). Approximately 95% of all HT patients have anti-TPO antibodies and about 60-80% of HT patients have anti-TG antibodies (170). The presence of anti-TPO Abs is a clinical marker for HT, while anti-TSHR Ab is a clinical marker for GD (170,171). However, anti-TPO Abs and anti-TG Abs are not unique to HT patients because these antibodies are detectable in the majority of GD patients (168).

Epidemiology, genetic and environmental factors of GD and HT patients

Both GD and HT are among the most common autoimmune diseases (168,170). Approximately, 2% of the general population will develop either GD or HT (172,173). In Denmark, the prevalence of female HT patients was 0.4% (174) whereas the prevalence for female GD patients was 1.2% (175) These diseases have a strong female preponderance, which could in part be due to the hormone estrogen (164,176). Parity, the number of times a women has given birth, and skewed X-chromosome inactivation can help to explain the predominance of GD or HT in females (176,177).

GD and HT are complex diseases caused by a combination of genetic and environmental factors (173,174,178,179). Few susceptibility genes have been discovered and can roughly be divided into immuno-regulatory (HLA-DR, CTLA4, CD40, PTPN22, CD25, and FOXP3) and thyroid-specific genes (TSHR and TG) (164,168,171,176,180,181). However, not all of these susceptibility genes are a causative factor for both GD and HT development because the susceptibility genes CD25, CD40 and TSHR are specific only for GD patients (181). The environmental factors such as dietary iodine, stress, smoking, and alcohol have all been associated with autoimmune thyroid disease (176). Stress or having a stressful daily life may be a risk factor for developing GD, but surprisingly, not for the development of HT (176). In Denmark, iodine was added to table salt and bread in order to prevent countrywide iodine deficiency. As a result of iodine fortification in Denmark, the incidence of hypothyroidism increased, but the prevalence of hyperthyroidism remained the same (182–185). It has been speculated that smoking is associated with the development of AITD (186). Studies from Denmark have shown that cigarette smoking has a protective effect for development of HT/hypothyroidism, but is a risk factor for GD development (173,187,188). Surprisingly, Danish population-based case-control studies have revealed that alcohol may have a protective effect and prevent the development of GD and HT (189,190). However, the mechanisms by which these environmental factors drive AITD pathogenesis is still not clear.

Graves' disease

As mentioned earlier, GD is an autoantibody-mediated disease and often, but not always, is lymphocytic infiltration present in GD patients (167,191). However, the lymphocytic infiltration detected in GD patients may not be as severe as HT or destroy the thyroid architecture (192). In GD, the lymphocytic infiltrate consists mainly of CD4+ and CD8+ T cells as well as CD19+ B cells. As a result of this lymphocytic infiltration into the thyroid gland, ectopic germinal centers (GC) can be formed (191,193). These ectopic GC are secondary lymphoid follicles, which contain autoreactive B cells and allows the affinity maturation of autoreactive B cells. This can potentially lead to the production of autoantibodies (2,194,195). GC might be the source of autoantibodies in the pathogenesis of GD as well as HT.

It is a well-known fact that pathognomonic antibodies are produced against the TSHR in GD, which leads to the over-activation of the thyroid gland and hyperthyroidism (168,196). These TSHR autoantibodies, also called TRAb, primarily belong to the immunoglobulin (Ig) G subtype (197). The types of TSHR auto-antibodies includes thyroid stimulatory (TSAb), TSH blocking (TBAb) and neutral auto-antibodies, which all are regarded to have different biological activity (196,198–200). For example, TSAb will bind and stimulate the TSHR, which induces proliferation as well as increases the thyroid hormone production and consequently leads to hyperthyroidism. Conversely, TBAb, will lead to hypothyroidism because these autoantibodies block the TSHR and reduce thyroid hormone production (198,199,201). The exact function of the neutral TSHR autoantibodies is not yet clear since it seems to

neither stimulate nor block the TSHR (196). The TSHR belongs to the G-protein coupled receptor family and is made up of an ectodomain subunit A and a transmembrane subunit B (164,201). Uniquely to the TSHR, this receptor undergoes intramolecular cleavage in the ectodomain resulting in the shedding of the subunit A (196,201). It has been suggested that the 'free or shed' subunit A is the immunogen that induces the production of TSHR autoantibodies by B cells (163,202,203). This is because recent studies have shown that monoclonal TSHR Abs of both human and murine origin have a higher affinity towards the 'free or shed' subunit A rather than the holoreceptor (202,203).

Human CD4+ T cells may have several roles within GD pathogenesis. Firstly, the interaction between CD4+ T cells and auto-reactive B cells is required for the production of autoantibodies against the TSHR (2,107,108,164,200). Secondly, CD4+ T cells produce cytokines, predominately Th2-related, including IL-4, IL-5 and IL-10, which may play a protective role in GD (192,204–206). Production of IL-4 and IL-10 may prevent thyrocyte destruction by inducing T cell anergy, preventing IFN-γ production by macrophages, inhibiting cytotoxic responses from CD8+ T cells, and by inducing a phenotypic switch from Th1 to Th2 (2,207,208). IL-4 and IL-10 may also up-regulate the expression of anti-apoptotic proteins from the BCL-2 family, which includes Bcl-2, Bcl-xL, and cFLIP (192). Expression of these anti-apoptotic proteins in thyrocytes hinders the activation of the caspase pathway which induces apoptosis (2,209).

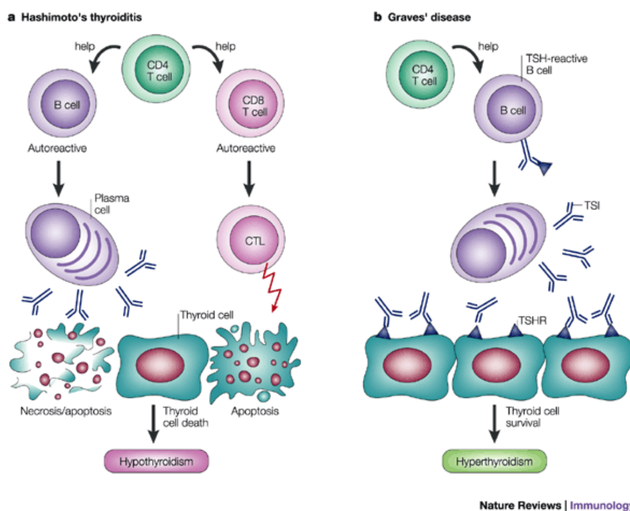

Figure 3. The immune-pathogenesis of Hashimoto thyroiditis and Graves' disease (Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol] (Stassi and De Maria; 2:195–204), copyright (2002) (2)).

Hashimoto's thyroiditis

Hashimoto's thyroiditis (HT) is primarily a T-cell mediated disease where the thyroid parenchyma is destroyed (210). The human thyroid gland is infiltrated with CD4+ and CD8+ T cells, CD19+ B cells, macrophages, and plasma cells leading to destruction of the thyrocytes, fibrosis, impaired thyroid hormone production and eventually hypothyroidism (2,166,192,210). However, what the initial insult is that causes the lymphocytic infiltration into the human thyroid gland is still unclear.

B cells may play multiple roles within HT pathogenesis. Primarily, auto-reactive B cells in HT could be the predominant source for autoantibodies against TG and TPO. These autoantibodies can be produced in various locations including ectopic germinal centers (194,195,210). Additionally, B cells may act as the antigen presenting cell, presenting thyroid self-antigens and thus activating naïve auto-reactive CD4+ T cells (8,165). B cells, which also have the ability to produce cytokines, may be a source for cytokines contributing to inflammation (211,212). However, the exact role of B cells in HT pathogenesis remains unclear.

Primarily, HT is characterized by thyrocyte destruction, and the rate at which thyrocytes are destroyed determines the clinical outcome of the disease (2,213). Thyrocyte destruction can occur via three main mechanisms: cytotoxic T lymphocytes, death receptors, and antibodies (2).

The role of T cells in HT pathogenesis is well-established. A murine study by Flynn et al showed that L3T4 (mouse CD4+ T cells) are responsible for the initiation of experimental autoimmune thyroiditis (EAT). In the same study, Flynn et al showed that Lyt-2+ cells (mouse CD8+ T cells) played a cytotoxic role in EAT pathogenesis (214). The present theory is that auto-reactive CD4+ T cells become activated, which induces the migration of both B cells and cytotoxic CD8+ T cells into the thyroid gland (166,215). Cytotoxic T lymphocytes (CTLs) produce cytotoxic granules such as perforin, granzymes (including granzyme B), and proteoglycans (216). The perforin molecule functions by forming a pore in the cellular membrane of target cells, and granzyme B functions by activating pro-apoptotic molecules such as caspases and cytochrome c (2). The presence of CTLs or perforin-secreting intra-thyroidal T cells has been detected among HT patients and could be one of the causative factors for thyrocyte destruction and hypothyroidism (217,218). A study by Ehlers et al demonstrated that TPO- and TG-specific CD8+ T cells are present in the peripheral blood and in the thyroid gland of HT patients. The study further demonstrates that these TPO- and TG-specific CD8+ T cells were able to cause the lysis of target cells in vitro. Given the cytotoxic ability of these TPO- and TG-specific CD8+ T cells, this could be an important mechanism for thyrocyte destruction in HT (219). Additionally, the presence of IFN-γ in the local environment may be able to promote the expression of several pro-apoptotic genes as well as increase the activity of caspases 3 and 8 and thus perpetuate thyrocyte destruction (2,209).

The second mechanism for thyrocyte destruction is via death receptors. Death receptors including FAS (CD95) have a cytoplasmic death domain that allows the transmission of the apoptotic signal into the cell (220). There is evidence that thyrocytes from HT patients have the expression of both FAS and FAS ligand (213,221–223). A theory is proposed that thyrocytes from HT patients undergo thyrocyte apototosis in a suicide or fraticide dependent manner due to the simultaneous expression of FAS and FAS ligand (2,215,221,224). This theory is proposed to be one of the main mechanisms for thyrocyte destruction. The interaction between FAS and FAS ligand will induce apoptotsis in the cell carrying FAS. Activation of a cell death receptor also enhances the expression of pro-apoptotic genes such as Bid and Bak, which encourages apoptosis in the thyrocyte (192).

It has been suggested that the pro-inflammatory cytokines IFN-γ and IL-1β, are able to enhance the expression of FAS on human thyrocytes, especially in HT patients (192,213,215,221–223,225). It has been theorized that the presence of IFN-γ and IL-1β in the thyroid gland of HT patients may induce or upregulate the expression of FAS. This increased FAS expression may perpetuate thyrocyte destruction (222). Evidence has shown that infiltrating T cells among HT patients had low or lacked any significant expression of FAS ligand indicating that infiltrating T cells did not induce thyrocyte destruction (215,222,224,226). However, infiltrating T cells still might play a role in thyrocyte destruction by providing the cytokines IL-1β and IFN-γ (222).

The third mechanism of thyrocyte destruction is by autoantibodies. Characteristically, patients with HT have autoantibodies against TG and/or TPO (210). Several studies have shown that autoantibodies, especially IgG1 anti-TPO antibodies, may cause thyrocyte destruction by fixing complement and inducing antibody-dependent cell-mediated cytotoxicity (ADCC) (227,228). As a result, the destroyed thyrocytes will then release cytokines such as IL-6, IL-1β, and IL-8, which can either initiate or perpetuate the inflammation by causing more lymphocytes to migrate to the thyroid gland (2,229,230). Our group has shown that TPO-antibodies promote production of pro-inflammatory cytokines by phagocytic cells and T cells, by facilitating binding of TPO/anti-TPO complexes to Fcγ-receptors on antigen-presenting cells (231). However, uncertainty remains as to whether autoantibodies are truly pathogenic or are secondary to the inflammation and destruction occurring in the thyroid gland (232).

It was initially believed that HT was a Th1-mediated disease, and there is ample of evidence to support this theory (233–236). However, recent data is suggesting that Th17 cells may play a more prominent role in HT. In particular, studies by Shi et al, Nanba et al and Figueroa-Vega et al, have shown that HT patients have increased proportions of circulating Th17 cells secreting IL-17, both in the peripheral blood and thyroid gland (39–41). IL-17 is known to be pro-inflammatory and induce the production of other pro-inflammatory cytokines such as IL-1β and IL-6, both of which have a role in HT, and chemokines from neighboring cells (35). Thus, this might perpetuate the inflammation and enhance the migration of lymphocytes into the thyroid gland. IFN-γ, which has a critical role in HT, is a key cytokine for Th1 cells. However, IFN-γ may also be produced by Th17 cells. There is data to suggest that Th17 cells are able to produce both IL-17 and IFN-γ (called Th17/Th1 cells) (98). Additionally, in sites with chronic inflammation, Th17 cells may differentiate into Th1 cells if IL-12 is present (99). Further investigations are needed to determine which Th subset, Th1, Th17 or Th17/Th1, is more important in HT.

Role of thyroid epithelial cells in AITD

Thyroid epithelial cells or thyrocytes may have a much larger role to play in the pathogenesis of HT and GD than initially thought. Thyrocytes have been shown to express MHC class II which interacts with CD4+ T cells (237). The expression of MHC class II, induced by IFN-γ, allows the thyrocyte to act as an antigen presenting cell (237,238). Under normal conditions, the MHC class II expression induces anergy in naïve CD4+ T cells due to the lack of the co-stimulatory molecules CD80/CD86 on the thyrocyte. Therefore, under normal conditions, thyrocytes are able to induce peripheral tolerance (180,239). However, in AITD, auto-reactive memory CD4+ T cells exist and become stimulated and proliferate in response to the auto-antigens presented by MHC class II on the thyrocyte. This will then perpetuate the autoimmune response (180,237). Additionally, thyrocytes, themselves, are able to produce and secrete a whole host of pro-inflammatory cytokines and chemokines. These cytokines and chemokine will stimulate T cells and B cells and increase the migration of lymphocytes to the thyroid gland, thus perpetuating the disease (229,230,240,241). Therefore, thyrocytes may not be the innocent bystander, as once believed, and may, in fact, play an important role in the pathogenesis of AITD.

Aims of the PhD study

The overall aim of this PhD study is to investigate the pathogenesis of autoimmune thyroid disease with respect to thyroid self-antigens and the effect they have on the immune system.

The specific objectives of this PhD study are:

- To investigate the ability of B cells to present AITD-associated self-antigens including TG and TPO to T cells in healthy donors and AITD patients.
- To determine whether TG and/or TPO could drive a pro-inflammatory or regulatory response in B cells and CD4+ T cells in healthy donors and AITD patients.
- To investigate if there is a difference between healthy donors and AITD patients with respect to B cell phenotypes associated with regulatory B cells.
- To investigate whether the regulatory response is impaired or defective in patients with AITD in comparison to healthy donors.

Summary of papers I to III

A summary of the findings from all three articles are outlined below.

Paper I: B-cell exposure to self-antigen induces IL-10 producing B cells as well as IL-6- and TNF-α-producing B-cell subsets in healthy humans.

Material and Methods: Peripheral blood mononuclear cells (PBMC) were isolated from 18 healthy donors. Monocytes or B cells were depleted from isolated PBMC using Dynabeads coated with anti-CD14 or anti-CD19, respectively, followed by CFSE labelling. CFSE-labelled intact PBMC or monocyte-/B-cell-depleted PBMC were plated at a density of 2.5 x 105 cells per well in a 96-well plate and stimulated with TG (30µg/mL) or TT (30µg/mL) for 7 days. Culture supernatants were collected at day 1 and the cytokines measured were TNF-α, IFN-γ, IL-2, IL-4, IL-6, and IL-10 using the Th1/Th2 cytometric bead array kit. Concomitantly, B cells and CD3+ T cells were purified from intact PBMC using Human B cell Enrichment and Human CD3 Positive selection kits, respectively. Purified B cells were preloaded with no antigen, TG (30µg/mL) or TT (30µg/mL). 1.0 x 105 TG- or TT-pulsed B cells were co-cultured with 2.5 x 105 purified CD3+ T cells for either overnight or 7 days. The cytokines TNF-α and IL-10 were measured using anti-CD45/anti-capture antibody beads, whereas IL-6 was detected using intracellular staining and stained with anti-IL-6 PE antibody.

Results: Depletion of monocytes from intact PBMC induced a significant reduction in TNF-α, IL-6 and IL-10 production ($P<0.009$, $P<0.02$, $P<0.04$ respectively). However, depletion of B cells resulted in a significant reduction of IL-10 only ($P<0.05$). TG-pulsed B cells, but not TT-pulsed B cells, were able to induce IL-10 secretion in 1.1 ± 0.5% of B cells ($P=0.01$ versus TT) and 1.0 ± 0.2% of CD4+ T cells ($P=0.006$ versus TT). Additionally, TG also induced secretion of IL-6 and TGF-β. In contrast, TT induced secretion of the Th1-type cytokines IFN-γ and IL-2. Both TG- and TT-pulsed B cells induced TNF-α secretion. The IL-10-secreting B cells detected in this study were significantly enriched with the surface markers CD5 ($P=0.03$ versus non-IL-10 secreting cells) and CD24 ($P=0.02$ versus non-IL-10 secreting cells), and not with CD27 and CD38 surface markers. After 7 days, IL-10 secretion by 3.3 ± 1.0% of CD4+ T cells was still observable after co-culture with TG-pulsed B cells. No IL-10 secretion was detected from TG-pulsed B cells on day 7.

Conclusions: Our findings show that B cells pulsed with the self-antigen TG is able to induce the secretion of IL-10 in both B cells and CD4+ T cells. Additionally, TG-pulsed B cells also induced the production of IL-6 and TNF-α. Also, higher frequencies of the

IL-10-secreting B cells were CD5+ and CD24hi. Together, TG-pulsed B cells are able to drive a protective immune response.

Paper II: Characterization of Regulatory B cells in Graves' Disease and Hashimoto's Thyroiditis.

Material and Methods: Peripheral blood was collected from 12 healthy donors as well as from 12 HT patients and 12 GD patients. For induction of cytokine production, 1 x 106 isolated intact PBMC were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin cell stimulation cocktail (2µL/well) for 4 h. PBMC were stained extracellularly with a combination of the following antibodies: anti-CD19-PerCP, anti-CD5 APC, anti-CD43 FITC, anti-CD27 PECy7, anti-CD24 FITC, anti-CD38 PECy7, anti-CD25 FITC, anti-TIM-1 PE, and intracellularly with either anti-IL-10 APC or anti-IL-10 PE. Concurrently, B cells were isolated and purified using the Human CD19 Positive Cell Isolation kit. Purified B cells (1 x 105 cells) were preloaded with TG (30 µg/mL) or CpG oligodeoxynucleotide (ODN; 10 µg/mL) for one hour. The preloaded B cells were co-cultured with 2 x 105 of the remaining PBMC for 48 h. The co-cultured cells were stained extracellularly and intracellularly with anti-CD19 APC, anti-CD14 FITC, anti-CD4 PerCP, anti-CD8 PECy7, and anti-IL-10 PE.

Results: Among the HT patients, 6.1% and 2.5% of the bulk B cells expressed the surface markers CD25 and TIM-1, respectively, in comparison to 2.9% and 1.6% of the bulk B cells among the healthy donors (P=0.026 and P=0.015 respectively). GD patients did not differ in their frequency of CD25+ or TIM-1+ B cells when compared to healthy donors. No differences were found between each patient group and healthy donors in terms of CD24hiCD38hi and CD27+CD43+ B cells frequencies. However, patients with HT or GD had a lower frequency of CD24hiCD38- B cells than healthy donors (P=0.02 and P=0.0005, respectively). Additionally, GD patients also had lower proportions of CD27+CD43- B cells (P=0.037 versus healthy donors). The cytokine IL-10 was induced by the polyclonal stimuli PMA/ionomycin and CpG ODN and by the antigen-specific stimulus TG. PMA/ionomycin and CpG ODN induced IL-10 secretion from approximately 1% of B cells. TG induced IL-10 secretion in 0.05% of healthy donor and HT patient B cells as well as in 0.18% of GD patient B cells. No differences were detected in the frequency of IL-10-secreting cells between each patient group and healthy donors after stimulation with PMA/ionomycin, CpG ODN or TG. HT patients had a significantly higher proportion of CD25+ and TIM-1+ B10 cells than healthy donors (P=0.0009 in both cases). Similarly, GD patients also had a significantly higher proportion of CD25+ and TIM-1+ B10 cells than healthy controls (P=0.039 and P=0.024, respectively). Similarly to bulk B cells, no differences were found in the proportions of CD24hiCD38hi and CD27+CD43+ B10 cells between patients and healthy donors. HT patients had a significantly lower proportion of B10 cells within the CD24hiCD38- subset than healthy donors (P=0.012). GD patients had a lower proportion of CD27+CD43- B10 cells than healthy donors (P=0.019).

Conclusions: Similar frequency of B10 cells are detected in HT, GD and healthy donors indicating that B cells from patients are not impaired with respect to inducing an immuno-regulatory response. The B10 cells did not segregate into any clearly defined subgroups, but HT patients had increased frequencies of CD25+ and TIM-1+ B10 cells.

Paper III: Altered balance between self-reactive Th17 cells and Th10 cells and between full-length FOXP3 and FOXP3 splice variants in Hashimoto's thyroiditis.

Material and Methods: This study included blood from 10 HT patients, 11 GD patients and 15 healthy donors. Isolated PBMC were plated onto a 96-well plate with a density of 5 x 105 cells per well and stimulated with either TG (30µg/mL), TPO (30µg/mL), E. coli LPS (50ng/mL), anti-CD3/anti-CD28 or left unstimulated. PBMC were stimulated for a total of 18h to measure IL-17 or IL-6 or for 48h to measure IL-10. PBMC were first extracellularly stained with anti-CD4 PerCP, anti-CD45RA FITC, and anti-CD45R0 APC, and then intracellularly stained for either anti-IL-17A PE, anti-IL-6 PE or anti-IL-10 PE. Culture supernatants were assessed for IL-1β, IL-6, and TGF-β1 production by Luminex after 18h. Concurrently, mRNA was extracted, purified and cDNA was synthesized from 5 x 105 PBMC after no stimulation or stimulation with TG, TPO, E. coli LPS or anti-CD3/anti-CD28. Subsequently, total FOXP3 and FOXP3Δ2 isoform was measured. As house-keeping gene, CD4 was utilized due to the stability of its expression irrespective of stimulation or not. Additionally, FOXP3 is expressed by CD4, thus it is preferred to use a house-keeping gene that is specific to the cell population studied.

Results: After stimulating with the thyroid self-antigen TG, no differences in the frequency of IL-17 producing cells were detected between each patient group and healthy donors in either the naïve or memory Th cell compartments. In contrast, TPO and E. coli LPS induced IL-17 production in 2.8 and 11.9 per 10,000 naïve Th cells among HT patients, but failed to do so in GD patients or healthy donors (P=0.016 and P=0.014 versus healthy donors, respectively). The induction of Th10 cells, was uniform between each patient group and healthy donors, irrespective of whether TG, TPO or E. coli LPS was used as a stimulus. However, compared to healthy donors, a significant reduction in the proportion of IL-10-producing cells were observed among HT patients after anti-CD3/anti-CD28 stimulation (P=0.028). After stimulation with TG, naïve Th cells among healthy donors preferentially differentiated towards a Th10 phenotype, whereas the naïve Th cells among patients with HT or GD differentiated towards a Th17 phenotype. HT patients had a higher baseline production of both IL-6 and TGF-β1 than healthy donors (P=0.038 and P=0.0096, respectively) possibly contributing towards Th17 differentiation. Only in the healthy donor group did stimulation with TG or TPO enhance the IL-6 production above the basal level. In contrast, E. coli LPS induced the production of IL-6 above the basal level in all three groups. Stimulation with TG, TPO or E. coli LPS did not alter TGF-β1 expression in any of the three groups. In addition, the baseline expression of total FOXP3 was uniform in all three groups. However, HT patients as well as GD patients had a higher baseline expression of FOXP3Δ2 than healthy donors (P=0.012 in both cases).

Conclusions: An increased frequency of thyroid antigen-specific Th17 cells in the naïve CD4+ T cell compartment is detected in HT patients while the frequency of Th10 cells remains unaltered. This indicates a skewed Th17:Th10 ratio in HT patients. Additionally, an elevated baseline production of IL-6 and TGF-β1 and of mRNA encoding FOXPΔ2 may contribute to the skew towards Th17 differentiation detected in HT patients.

Discussion

The discussion below will deal with IL-10 production by B cells, regulatory B cell phenotypes, the antigen presenting ability of B cells and monocytes, IL-10 and IL-17 production from CD4+ T cells, Th17/Th10 ratio and Th17 differentiation, and finally FOXP3 and Th17 plasticity.

The B cell studies

IL-10 production by B cells

The human cytokine IL-10 is a regulatory cytokine and was initially discovered by Mossman and colleagues who showed that murine IL-10 was able to inhibit cytokine production from Th1 cells (242). The biological activities of IL-10 are far-reaching and have been shown to have immunosuppressive effects on monocytes and macrophages in terms of inhibiting pro-inflammatory cytokine production as well as antigen presentation (74–76,243). Opposed to the effects of IL-10 on monocytes and macrophages, IL-10 has been shown to have immuno-stimulatory effects on human B cells. In this case, IL-10 helps to prevent apoptosis, increases proliferation and enhances antigen presentation by B cells by up-regulating MHC class II expression (74–76).

In our B cell studies, we used the polyclonal stimuli PMA/ionomycin and CpG ODN 2006 (Toll-like receptor 9 ligand) to maximally stimulate the B cells, as well as a more biologically relevant stimulus, the thyroid self-antigen thyroglobulin (TG). The foreign recall antigen tetanus toxoid (TT) was also used.

In paper I, TG was found to be able to induce a significant secretion of the anti-inflammatory cytokine IL-10 in human B cells from healthy donors. This was in contrast to the foreign recall antigen TT, which induced more Th1-type cytokines such as IFN-γ and IL-2, and minimal IL-10. It should be noted that TG also induced the secretion of some pro-inflammatory cytokines including IL-6 and TNF-α from B cells in healthy donors. Similarly, in paper II we were able to induce IL-10 secretion after TG stimulation in healthy donors and in patients with GD or HT. Notably, TG induced similar proportions of IL-10 in all three donor groups. Using TG as the stimulus proved that self-antigens can induce IL-10 secretion from healthy donors and patients. This is novel because IL-10 has been predominately induced by polyclonal stimulation, and TG is more relevant to the pathogenesis of AITD. This indicates that self-antigens induce antigen-specific immuno-regulatory responses by B cells, which may play a role in controlling AITD. Additionally, in paper II, the polyclonal stimuli PMA/ionomycin or CpG were used to induce IL-10 secretion. Such polyclonal stimuli are potent and induce the maximum IL-10 secretion by B cells. CpG stimulates B cells by interacting with its receptor TLR9 and activates NF-κB. This is in contrast to PMA/ionomycin, which diffuses across the cell membrane and activates protein kinase C and NFAT (244–247). We demonstrated that IL-10 secretion was similar between each patient group and healthy donors after PMA/ionomycin or CpG stimulation. In contrast to our results, Zha et al observed a significant decrease in the ability of B cells to secrete IL-10 from new-onset GD patients after stimulation with CpG and PMA/ionomycin (156). An explanation for this discrepancy could be that Zha et al divided the GD patients into two groups based on disease status, active disease and euthyroid, and stated that none of the patients were undergoing anti-thyroid drug treatment before blood collection (156). The possible effects of anti-thyroid drugs on our cytokine production will be discussed below, under the limitations section. The observation that patients with GD or HT were equally as capable of secreting IL-10 as the healthy donors, irrespective of whether TG, PMA/ionomycin or CpG was used, indicates that GD or HT patients do not have a defective immuno-regulation by B cells.

The main difference between the observed B-cell production of IL-10 in paper I and II was the amount of IL-10 secreted after TG stimulation. In paper I, we detected IL-10 secretion from 1.00 ± 0.5% healthy donor B cells, whereas in paper II we detected IL-10 secretion by 0.09 ± 0.1% from healthy donor B cells. In paper II, among patients with GD or HT, we measured 0.32 ± 0.5% and 0.28 ± 0.8% IL-10 from B cells, respectively. The primary reason for this difference could be the method used to detect IL-10. In paper I, a cytokine secretion assay was used, whereas in paper II intracellular staining was used. In the secretion assay, the cytokine is retained on the surface of a cell by a capture antibody during a 45 minute secretion phase (248). There is a risk of false positives, if the cell density is too high, which allows a non-secreting cell to be in close proximity to a secreting cell and thus capture its cytokines (248). However, this has presumably not been the case in our study, since the cell density was kept within the recommended cell density for the assay. For intracellular staining, our studies used the stimulation period that lasted from 4 hours to 48 hours with brefeldin A. Brefeldin A is a fungal metabolite, which blocks the transport of proteins from the endoplasmic reticulum to the Golgi (249,250). It has been shown that brefeldin A affects antigen presentation to both CD4+ T cells and CD8+ T cells by inhibiting the presentation of protein by MHC class II and I, respectively (251,252). If B cell production of IL-10 is dependent upon interaction with CD4+ T cells, then inhibition of antigen presentation by brefeldin A (or interaction between other surface molecules on the two cell types) could be the cause of the lower IL-10 production detected in paper II.

A more trivial explanation for the discrepancy between the IL-10 secretions among the healthy donors in papers I and II is the potential contamination of the TG preparation with LPS. It was discovered in paper II that the bought TG preparation was contaminated with LPS. The contaminating LPS was removed and the preparation purified using the Triton X-114 phase separation technique outlined by Liu et al (253). Triton X-114, a non-ionic detergent, was chosen due to its high protein recovery rate and ability to work with small volumes of the TG preparation. Unfortunately, it cannot be determined whether or not the TG preparation used in paper I was LPS contaminated since the first batch, bought several years earlier, was not tested for LPS contamination. It is well known that LPS, which is a ligand for TLR4 (254–256), is able to induce pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-12, IL-1β, IL-8, and IL-4 (243,255,257,258). However, LPS is also able to induce the production of IL-10 and TGF-β from monocytes and/or macrophages (243,259,260). These cytokines may have had a bystander effect on our cytokine production from our CD4+ T cells and/or B cells. However, in paper I, both CD4+ T cells and B cells were isolated, purified and co-cultured back together which prevents the bystander effect from the cytokine production from the monocytes.

Although LPS induces cytokine production it is not clear, when reviewing the literature, whether LPS has an effect on human B cells. Within the literature there is consensus that TLR4 is expressed on monocytes and/or macrophages and dendritic cells (255). Several studies have reported that B cells from healthy donors express very little or no TLR4 (261–263). However, some recent studies have reported that TLR4 expression is upregulated on circulating human B cells during inflammatory diseases, such as type 2 diabetes and periodontal disease (264,265). It is postulated that cytokines like IL-4, or the antigen-specific interaction between B cells and T cells, or possibly a combination of the two, allows the upregulation of TLR4 on B cells (266,267). The function of TLR4 on B cells is not yet fully understood, but there is speculation that TLR4 may actually reduce the cells' ability to produce IL-10 (267,268). With regard to paper I, if B cells do not express TLR4 then the LPS from the first TG preparation would not have affected our cytokine production by B cells, or by T cells in the B-cell/T-cell co-cultures. However, the possibility that the first bought TG preparation was LPS contaminated, and that this had an effect on the cytokine production from the B cells cannot be ruled out. In subsequent studies, TG was purified and determined

to be LPS-free to avoid speculation. Therefore, the IL-10 secretion detected in B cells in paper II was solely due to TG stimulation.

Currently, stimuli such as CpG ODN 2006 (TLR9 ligand), LPS (TLR4 ligand), PMA/ionomycin, anti-CD40 antibodies, and anti-IgM antibodies have been reported to induce IL-10 production in human B cells (151,154,159,160,162). It is not known how the different B cell subtypes including naïve, memory or transitional B cells respond to the different stimuli, and if the different stimuli induces different subsets or types of Bregs (269,270). TLRs are important in initiating the innate immune response, which aids in protecting the host against pathogens and act as the first line of defense (245,247,255,271). It has been suggested that TLRs such as CpG/TLR9 or LPS/TLR4 induces 'innate-like' Bregs and thus induces innate-like responses. These 'innate-like' Bregs produce IL-10 and may be important in the first line of defense to reduce excessive inflammation (11,269,270). In contrast, stimulated with anti-CD40 antibodies, CD40L or anti-IgM antibodies may induce more 'acquired type' Bregs, which may play a role in the adaptive immune response (11,269,270). TLRs such as TLR9/CpG may also play a role in the adaptive immune system by inducing the expression of co-stimulatory molecules, cytokines and enhancing the antigen presenting ability of APCs (245,255,272). CpG and anti-Ig antibodies are presumably the optimal stimuli for inducing IL-10 in human B cells since they simultaneously stimulate via TLR9 and the BCR (162,269).

The overall message from our IL-10 and B cell studies is that the thyroid self-antigen, TG, is able to induce IL-10 production in B cells from healthy donors as well as from patients with GD or HT. This finding is novel and may have implications for the pathogenesis of GD and HT.

Regulatory B cell phenotypes

Human B cells may have a regulatory role within the immune system through the production of IL-10 or TGF-β (273). There continues to be great interest in phenotyping these potentially regulatory B cells. However, no definitive phenotype has yet been assigned, and the production of IL-10 is still the best functional hallmark or phenotypic marker we have to identify Bregs.

In paper I, the phenotype of the IL-10-producing B cells among healthy donors (also called B10 cells in this thesis) was investigated. It should be noted that the term 'B10 cells' only encompasses the Bregs that produce IL-10, but other Breg subsets may exist (274). In papers I and II, the CD5+ B10 cells did not represent the majority since approximately 75% of the B10 cells were CD5-. However, it should be noted that in paper I, healthy donor B10 cells induced by TG stimulation were more frequently CD5+ than were the non-IL-10 producing B cells. CD5 expression on B cells has been associated with natural antibody production. Certain B cells have the ability to bind to both self and foreign antigens and to secrete poly-reactive antibodies also known as natural antibodies (116,117,275). It has been speculated that these poly-reactive antibody-producing B (PAB) cells are CD5+ (116,275,276). Within the normal immune response, the CD5+ PAB cells and poly-reactive antibodies aid in protecting the host from infections by several mechanism. These mechanism include activating the complement system and forming the lytic complex, by enhancing the phagocytosis of the bacteria and poly-reactive Ab complex by macrophages or by having a direct neutralising effect (112,117,277,278). Additionally, CD5+ B cells and poly-reactive antibodies may play a role in autoimmunity (277). CD5+ B cells have been shown to be the source of autoantibodies against the rheumatoid factor and double-stranded DNA in RA and SLE patients, hinting towards a pathogenic role for CD5+ B cells (118–120).

There is evidence that disease-associated autoantibodies are somatically hypermutated, whereas poly-reactive antibodies are not (117). The presence of high-affinity autoantibodies against double stranded DNA in SLE, and against TG, TPO or TSHR in AITD indicates that somatic hypermutation is crucial in the development of pathogenic autoantibodies (279,280). There is speculation that poly-reactive antibodies could be the precursors to the high affinity pathogenic autoantibodies (277,279,280).

In paper II, when investigating the whole B-cell population, healthy donors had an increased proportion of bulk B cells expressing CD24hi than GD patients, but this was not the case for HT patients. The surface marker CD24 has been associated with memory B cells (122), which could indicate that GD patients had a lower proportion of memory B cells. When investigating the phenotype of B10 cells our initial findings (paper I) showed that healthy donors had a higher frequency of IL-10-producing B cells expressing CD24hi. This correlated with the findings in paper II where healthy donors had increased proportions of CD24hi B10 cells. Additionally, in paper II we demonstrated that patients with GD or HT had a significantly lower frequency of CD24hi B10 cells than healthy donors. CD24 has been found to have a role in controlling B cell differentiation and maturation, controlling activation-induced B cell responses, and in co-stimulation for CD4+ T cell growth (281–283). In summary, this indicates that B10 cells expressing CD24 are memory cells regulating T-cell function.

Possible B10 surface markers and phenotypes were expanded upon in paper II, including TIM-1+, CD25+, CD24hiCD38hi, and CD27+CD43+. HT patients had a significantly higher frequency of bulk B cells and B10 cells expressing the marker TIM-1 than healthy donors. The function of TIM-1 is in control of CD4+ T cell effector differentiation and responses (284,285). TIM-1 was primarily believed to be expressed only on CD4+ T cells and vital for regulating Th2 responses. However, new insights have revealed that TIM-1 can be expressed on multiple cell types, and that it is able to regulate not only Th2 cells but also Th1, Th17, and Tregs (285–287). In mice, TIM-1 was shown to be expressed on B cells and ligation of TIM-1 induced IL-10 secretion from said B cells (153). A study by Liu et al was among the first to show that human B10 cells were TIM-1+, and that these TIM-1+ B10 cells were able to suppress IFN-γ and TNF-α production from CD4+ T cells (161). Given the regulatory function of TIM-1, the expression of TIM-1 on B cells may aid in the secretion of IL-10 as well as in the regulation of auto-reactive T cells in HT pathogenesis.

Similar to TIM-1, HT patients had a higher frequency of bulk B cells and B10 cells expressing the marker CD25 than healthy donors. It should be noted that GD patients had a higher proportion of CD25+ B10 cells but did not have the corresponding expression on bulk B cells. CD25, the alpha chain of the IL-2 receptor (288,289), has been shown to be expressed on human B cells, and is expanded in untreated multiple sclerosis patients (290,291). CD19+CD25+ B cells are able to secrete IL-10 and suppress CD4+ T cell proliferation (157,291). Intriguingly, Kessel et al observed that CD19+CD25+ B cells are able to enhance the expression of both CTLA-4 and FOXP3 in Tregs (157). This indicates that CD19+CD25+ B cells could have some immuno-modulatory/suppressive functions or at least able to enhance immunosuppressive properties in circulating Tregs. In addition, the expression of CD25 on Tregs (CD25hi or CD25++), allows them to be responsive to IL-2 (9). IL-2 is needed for the development of Tregs but also for their suppressive function (59,63). Having a functional IL-2 receptor allows the

Tregs to act like a 'sink' for the IL-2 in the surrounding microenvironment and thereby, prevents the activation and responses of the surrounding T cells (59,64,65,292). It can be speculated that by having an expression of CD25 on B cells, equivalent to Tregs, this may allow the CD19+CD25+ B cells to act like a 'sink' for IL-2 and thereby inhibit the surrounding effector T cells' responses similarly to Tregs. Additionally, expression of CD25 may allow B10 cells to become activated – and regulate the immune response – under circumstances with abundance of IL-2 in the environment, i.e. in presence of activated effector T cells.

It should be taken into consideration that in our data, TIM-1+ and CD25+ B10 cells did not represent the majority of B10 cells; therefore, these markers should not be used to define B10 cells.

In our findings, there were no differences in the proportion of bulk B cells or B10 cells regarding the transitional B cell phenotype CD24hiCD38hi between healthy donors and patients with GD or HT. Our results do not correlate with the findings of Blair et al or Sims et al, who observed that SLE patients had a higher percentage of CD24hiCD38hi B cells than did healthy donors, or with Flores-Borja et al who observed that RA patients had a decreased percentage of CD24hiCD38hi B cells (131,151,158). The B cell phenotype CD24hiCD38hi has been associated with a regulatory function due to its ability to produce IL-10, limit pro-inflammatory cytokine production as well as control Th1 and Th17 differentiation (151,158). CD24hiCD38hi B cells from RA and SLE patients had a lower IL-10 production and were functionally impaired in suppressing Th17 differentiation or production of Th1-related cytokines, respectively (151,158). It should be noted that IL-10 production has also been detected within the CD24intCD38int and CD24hiCD38- B cell phenotypes, indicating that these subsets also have some immunosuppressive capability (151).

The IL-10+ B cells compared to the IL-10- B cells from paper I were not enriched with the surface marker CD27 among healthy donors. Similarly in paper II, no differences were detected in terms of CD27 expression among the bulk B cells or B10 cells in healthy donors or in patients with GD or HT. CD27 in combination with CD43 has been shown to have some regulatory functions. Griffin et al observed that CD27+CD43+CD11b+ B cells were able to secrete IL-10 as well as modulate T cell activation (124,293). However, no differences were detected among the CD27+CD43+ B10 cells between patients and healthy donors. Another phenotype, which might be of importance but was not included in our studies, is CD24hiCD27+. Iwata et al are among the first to demonstrate that B10 cells from healthy donors are found predominately in the CD24hiCD27+ B cell subpopulation (154). These B10 cells had the ability to suppress cytokine production from monocytes in an IL-10-dependent manner (154). Inhibition of cytokine production may be due to the fact that IL-10 is able to inhibit NF-κB, and NF-κB is crucial for the production of pro-inflammatory cytokines including IL-1β, IL-6, IL-8, IL-12 and TNF-α (294–296). B10 cells with the CD24hiCD27+ phenotype have also been detected among GD patients and allergic asthma patients. However, in these cases a lower proportion of CD24hiCD27+ B cells and an impairment of their suppressive function were detected (156,159). This indicates that CD24hiCD27+ B cells do have a regulatory function, and that a decrease or lack of suppressive function may facilitate disease development. Further investigation of this phenotype is required to determine the potential role it has for AITD pathogenesis.

From our findings it is evident that B10 cells do not express a particular surface maker or separate into one clear subpopulation or phenotype. This correlates with Bouaziz et al who also discovered that B10 cells can be found in multiple subpopulations (162). Given the complexity of this field, it will not be sufficient to use only surface markers or even IL-10 to characterize regulatory B cells. A combination of phenotype, function and even genetic profile will be needed to truly define a human regulatory B cell (297).

Antigen presenting ability of B cells and monocytes

Antigen presenting cells (APC) include B cells, monocytes/macrophages and dendritic cells (109,298–300). In paper I, the efficacy of monocytes and B cells to act as antigen-presenting cells were studied. Depletion of monocytes from whole PBMC significantly reduced the secretion of several cytokines such as TNF-α, IL-6, and even IL-10, while B-cell depletion did not have the same effect. With B cell depletion, only a modest reduction in IL-10 was detected. This could indicate that monocytes were a major source of IL-10, TNF-α and IL-6 (74,243,301). Additionally, depleting monocytes dramatically decreased CD4+ T cell proliferation, which was not observed when B cells were depleted. This shows that, in our study, monocytes were more efficient at antigen presentation than B cells. This is in accordance with a study by Beck et al who observed that monocytes were more efficient than B cells at stimulating human CD4+ T cells and are thus a more efficient antigen presenting cell (302). Intriguingly, a study by Constant et al showed that peptides were preferentially taken up by dendritic cells and B cells preferred the uptake of whole proteins (303).

B cells are competent APC in terms of priming CD4+ T cells and may be the predominant antigen-presenting cells under circumstances where the antigen is scarce, due to their ability to up-concentrate antigens on the cell surface via BCR (107,304). In paper I, human B cells were purified and preloaded with the TG antigen and then co-cultured with CD4+ T cells. Subsequently, we were able to measure IL-10 secretion by the co-cultured CD4+ T cells. This indicates that the purified B cells were acting as antigen-presenting cells. A study by Guo et al was the first to demonstrate the existence of TPO-specific B cells and the ability of these B cells to present TPO to T cells, as indicated by the induction of T cell proliferation in murine spleen cell cultures (305). Nielsen et al demonstrated that TG was able to induce proliferation in CD4+ T cells in cultures of human PBMC. The removal of B cells from intact PBMC markedly reduced this proliferation indicating that B cells were acting as antigen-presenting cells in terms of TG presentation (278). In paper I, the TG-preloaded B cells induced a protective response in the CD4+ T cells by the secretion of IL-10. In contrast, B cells preloaded with TT induced a pro-inflammatory response in the co-cultured CD4+ T cells.

In paper III, intact PBMC were investigated instead of purified B cells and CD4+ T cells. Therefore, it could not be determined whether it was the B cells or the monocytes that acted as the main antigen-presenting cell. However, since depleting monocytes had the biggest effect on cytokine production and proliferation, it can be speculated that it was the monocytes that were acting as the main antigen-presenting cells in paper III.

The T helper cell study

IL-10 production by CD4+ T cells

In this thesis, IL-10-producing CD4+ T cells are referred to as Th10 cells. The term 'Th10' has been used in the literature to denote all IL-10-producing Th cells irrespective of their origin (306). As mentioned earlier, the biological activities of IL-10 are far-reaching. IL-10 has also been shown to have both indirect and direct effects on CD4+ T cells. Indirectly, IL-10 can inhibit the production of key cytokines such as IFN-γ or IL-4 from monocytes or macrophages which could affect Th1 or Th2 differentiation and

response, thus regulating Th1 and Th2 responses via antigen presenting cells (74–76). Studies have shown that IL-10 may also downregulate MHC class II expression as well as co-stimulatory molecules on monocytes/macrophages resulting in an impaired capacity to stimulate T cells (243,307). Directly, IL-10 may also suppress the cytokine production and the proliferation of CD4+ T cells, as well as induce Tregs (74–76).

In paper I, a significantly higher proportion of CD4+ T cells in healthy donors were able to secrete IL-10 after stimulation with the thyroid self-antigen TG than after stimulation with the foreign recall antigen TT. On the other hand, TT induced the secretion of Th1-type pro-inflammatory cytokines and minimal IL-10 secretion. Once again in paper III, TG was able to induce IL-10 secretion above the background within CD4+ T cells from healthy donors as well as in patients with GD or HT.

In paper III, CD4+ T cells were divided into naïve (CD45RA+CD45R0-) or memory (CD45RA-CD45R0+) cell compartments and TPO, a thyroid self-antigen, and E. coli LPS, a foreign control antigen, were used to induce cytokine production. Our data show that TPO and E. coli LPS were able to induce IL-10 production above the background in both CD4+ T cell compartments in healthy donors as well as in both patient groups. In addition, no differences were found in the proportion of Th10 cells between the healthy donors and patients with GD or HT after polyclonal stimulation with anti-CD3/anti-CD28 antibodies. Our findings show that similar proportions of Th10 cells were detected between patients with GD or HT and healthy donors thereby, indicating that patients were not impaired in the ability of inducing a regulatory T-cell response. It should be noted that the Th10 cells detected in our studies may in fact encompass both iTregs and nTregs because our staining protocol did not discriminate between these two subsets. In the literature, there are conflicting results in terms of the frequency of Tregs in AITD patients. Glick et al, who grouped GD and HT as AITD, observed that the frequency of CD4+CD25hi Tregs were similar between AITD patients and healthy donors (308). Comparable results were observed by Pan et al in GD patients when compared against healthy donors (309). Notably, these Tregs may be nTregs and were characterized and measured solely on the basis of phenotype. Marazuela et al observed that AITD patients had a significantly higher frequency than healthy donors of CD4+GITR+ and CD4+FOXP3+ as well as CD4+IL-10+ in the peripheral blood, which they claim represents nTregs and iTregs, respectively. (310). Our data showed that patients with GD or HT had an adequate number of circulating Th10 cells, but whether these Th10 cells function as well as healthy donor Th10 cells in suppressing cytokine production or proliferation was not measured in our study. Two studies have demonstrated that CD4+CD25hi Tregs from AITD patients were less able to inhibit proliferation than their healthy donor counterparts (308,310).

The above studies were carried out in the peripheral blood, and the situation may not be the same in the thyroid tissue. A study by Nakano et al, who grouped GD and HT as AITD, showed a lower proportion of CD4+CD25+ Tregs among the intra-thyroidal lymphocytes in the thyroid than among blood lymphocytes (311).

Overall, our results show that thyroid self-antigens are able to induce IL-10 secretion in CD4+ T cells in healthy donors and in patients with GD or HT. Additionally, patients with GD or HT were not impaired or deficient in inducing an immuno-regulatory response as noted by the similar proportions of Th10 cells in healthy donors and both patient groups.

IL-17 production by CD4+ T cells

HT has always been believed to be a Th1-mediated autoimmune disease (205,209), due to the increased production of IFN-γ, IL-2 and TNF-α detected among HT patients (233–236). However, many autoimmune diseases such as RA, inflammatory bowel disease, multiple sclerosis and possibly even HT (37,38) may instead be driven by Th17 cells and their production of IL-17 rather than Th1 cells and IFN-γ.

After incubation of intact PBMC with anti-CD3/anti-CD28 antibodies, in paper III, the proportion of Th17 cells did not differ between each patient group and healthy donors. However, incubation with the thyroid self-antigen TPO did induce higher proportions of Th17 cells in the naïve CD4+ T cell compartment of HT patients than of healthy donors or GD patients. This finding is novel, as Th17 induction by thyroid self-antigens has not been reported previously. This indicates that HT patients have a higher frequency of self-antigen specific Th17 cells than healthy donors, even though the frequency of dedicated Th17 cells, in general, is not elevated in patients.

In contrast to our results, Nanba et al and Figeuroa-Vega et al showed that a higher proportion of Th17 cells were detected in peripheral blood from HT patients than in healthy donors or GD patients after polyclonal stimulation with PMA/ionomycin (40,41). Shi et al also observed a higher baseline mRNA level of IL-17 among PBMC from HT patients than among PBMC from healthy donors (39).

It should be noted that the increase in the proportion of Th17 cells was not restricted to the self-antigen TPO. E. coli LPS, used as a foreign control antigen, was also able to increase the proportions of both naïve and memory Th17 cells in HT patients, compared to those of healthy controls or GD patients. This correlated well with McAleer et al who observed that E. coli LPS expanded pre-committed Th17 cells instead of de novo induction of Th17 differentiation (312,313).

Our data indicates that irrespective of the stimulus, TPO or E. coli LPS, CD4+´T cells from HT patients are more prone to differentiate into IL-17-producing cells than CD4+ T cells from healthy donors.

Th17/Th10 ratio and Th17 differentiation

Th10 cells and Th17 cells have been investigated separately in AITD, but this study is the first to analyze their relative frequencies together. In paper III, we observed that Th0 cells among the healthy donors preferentially differentiated into Th10 cells, whereas for patients with GD or HT, Th0 cells preferentially differentiated into Th17 cells. The preferential differentiation of Th0 cells into Th17 cells in AITD patients could be a result of the surrounding microenvironment. Therefore, we quantified IL-1β, IL-6 and TGF-β1 in the culture supernatants knowing that the local cytokine environment is crucial to Th17 differentiation. However, which cytokines are the most important for Th17 differentiation is still under debate. To date, several cytokines have been implicated in Th17-cell differentiation, including IL-1β, IL-6, IL-21, IL-23, TNF-α and TGF-β (37,42–46,314). We found that HT patients had a higher baseline concentration of IL-6 and TGF-β1 in unstimulated PBMC cultures than healthy donors. It should be noted that TGF-β1 was higher in HT patients than healthy donors, irrespective of whether TG, TPO or E. coli LPS was used as the stimulus. The higher baseline concentration of IL-6 and TGF-β1 may support the increased differentiation of Th17 cells in the HT patient group.

The role that TGF-β may play in Th17 differentiation is intriguing. TGF-β is a regulatory cytokine that aids in the differentiation of inducible Tregs (54,315). TGF-β along with IL-6 may have a

more direct role in Th17 differentiation by inducing the expression of RORγt and RORα (49,316). However, TGF-β may also have an indirect role with Th17 differentiation by limiting Th1 differentiation and thereby allowing the differentiation of Th17 cells (48). Moreover, the concentration of TGF-β might also be crucial for Th17 differentiation, where low concentrations (1-10ng/mL) induces Th17 differentiation, and higher concentrations (50ng/mL) inhibits Th17 differentiation and promotes Treg differentiation (95,317).

The imbalance between pro-inflammatory and anti-inflammatory CD4+ T cell subsets, such as Th17, and Treg subsets, respectively, may be one of the pathogenic mechanisms in autoimmune disease (36). This gives credence to our hypothesis that the skewed Th17:Th10 ratio detected in AITD patients may play a role in AITD pathogenesis.

FOXP3 and Th17 plasticity

There is a high degree of plasticity in the Th17/Treg differentiation pathways, but the biological relevance for this is still unclear. Studies have shown that FOXP3+ Tregs are able to produce IL-17 after PMA/ionomycin stimulation, or in an IL-1β-dependent way (101–104). It has also been demonstrated that these IL-17-producing FOXP3+ Tregs were expressing the Th17-specific transcription factor RORγt constitutively alongside the expression of the Treg-specific transcription factor FOXP3 (102,104). If FOXP3+Tregs are able to produce IL-17 or even differentiate into Th17 cells that might help to explain why in some autoimmune diseases a higher percentage of Th17 cells are detected than in healthy individuals. It is still not clear whether these IL-17+FOXP3+ Tregs are able to maintain their suppressive function due to conflicting results (103,104).

In our study, both patient groups had a higher constitutive expression of FOXP3Δ2, but not of total FOXP3, than healthy donors in the unstimulated PBMC cultures. Thus, the patient groups expressed relatively more of the FOXP3Δ2 splice variant than healthy donors. CD4 was chosen as the housekeeping gene because the expression of CD4 does not alter after stimulation (318). Additionally, FOXP3 is expressed by CD4+ T cells and using a house-keeping gene that is specific to the cell population being studied is preferred. Full length FOXP3 functions as suppressors of RORγt (RORC, human ortholog) and RORα, as well as of the NFAT and NF-κB by binding to them and inhibiting their biological activity. Binding of FOXP3 to RORγt and RORα prevents Th17 differentiation, and allows the differentiation of Tregs (94,95,97). In contrast, FOXP3Δ2 is unable to suppress RORγt and RORα, as well as NF-κB because it is unable to bind (93–97,319). Mouse studies have shown that exon 2 is crucial for the binding of FOXP3 to RORγt or RORα or NF-κB (94,97) and is thought to be the vital part of the repressor domain (96). The inability of FOXP3Δ2 to bind to and inhibit RORγt, RORα and NF-κB relies upon the missing exon 2. RORγt is the prevailing transcription factor for Th17 cells, and RORα is upregulated in Th17 cells due to the presence of certain cytokines in the local cytokine environment (49,316). Therefore the inability of FOXP3Δ2 to bind and inhibit RORγt and RORα would allow the differentiation of Th17 cells, and could result in the skewed Th17: Th10 cell ratio observed in paper III.

In summary, our study shows that patients with GD or HT have a higher constitutive expression of FOXP3Δ2 than healthy donors. This elevated expression of FOXP3Δ2 in HT patients may help to explain the preferential differentiation of Th17 cells in HT patients in paper III.

Limitations of the study

Considerations have to be made because all of my experiments in all three papers (I, II and III) were carried out on isolated lymphocytes from the peripheral blood. These may not be functionally or phenotypically similar to the intra-thyroidal lymphocytes driving the disease.

A limitation of our studies was the sparse number of samples per group. For many explorative studies, such as the ones we carried out, a sample size of 10-15 is the norm. In all our papers (I, II and III) we wanted to illustrate qualitative differences in the cytokine production between patients with GD or HT and healthy donors. A previous study by Glick et al, examining Tregs in AITD, had 7 GD patients and 13 HT patients, while Figueroa-Vega et al, studying Th17 cells in AITD, had 8 GD patients and 18 HT patients (41,308). The size of each sample group (paper II: 12 healthy donors, 12 GD patients; 12 HT patients; paper III: 15 healthy donors; 11 GD patients; 10 HT patients) and power calculation was based on previous studies in our group where significant differences were observed with 10-15 subjects in each group (320–322). However, with small sample groups and a low study power, there is a risk of making a type I or a type II error.

Another limitation was that the majority of the GD or HT patients that were recruited for papers II and III were treated with either methimazole or levothyroxine for varying periods of time before blood collection and participation in this PhD study. It should be taken into consideration that the cytokine production for the treated GD or HT patients might be altered due to anti-thyroid drugs, and therefore might be more similar to the cytokine production detected for healthy donors. This might have led us to underestimate the differences in cytokine production between patients and healthy donors. Several reports have linked an association between anti-thyroid medication and cytokine production; Weiss et al reported that anti-thyroid drugs inhibited the proper functioning of lymphocytes (323). Thionamides, a class of anti-thyroid drugs, have been shown to inhibit cytokine production due to the suppression of NF-κB (324). In contrast, Volpe et al reported that the thyroid status, itself, might have an effect on the immune system (325). Severe hyperthyroidism might affect the immune system by altering the numbers and activation status of CD4+ and CD8+ T cells, by increasing the serum concentrations of IL-2, as well as by altering the responsiveness of CD4+ T cells to the thyroid self-antigens (325).

However, it should be noted that all patients with GD or HT had antibody titres well above the normal range for either anti-TSHR or anti-TPO respectively at the time of blood sampling. This was an inclusion criterion for the studies in papers II and III. This indicates that all patients were immunologically active, even though some were receiving anti-thyroid medication or thyroid hormone.

Conclusions

Overall Conclusions of the study

One of the main findings of this PhD study is that the human thyroid self-antigen TG is able to induce antigen-specific production of IL-10 in both CD19+ B cells and CD4+ T cells, in healthy donors as well as in patients with GD or HT. Moreover, TPO, another thyroid specific self-antigen, induced higher frequencies of Th17 cells within the naïve CD4+ T-cell compartment in HT patients than healthy donors. This increased frequency or skewing towards Th17 cells in HT patients could be due to an elevated baseline production of IL-6 and TGF-β1 by PBMC or to an increased expression of mRNA encoding FOXP3Δ2. Notably, the proportions of Th10 cells of GD or HT patients were similar to those in healthy controls.

Furthermore, we demonstrated that patients with GD or HT were not impaired or deficient with regards to inducing an immuno-regulatory response in terms of IL-10 production by CD19+ B cells. IL-10 production by B10 cells was similar between healthy donors and patients with GD or HT, irrespective of the stimulus used. We also showed that B10 cells among healthy donors and patients with GD or HT did not separate into one clearly defined phenotypic subgroup, nor did they express the surface markers that have previously been associated with B10 cells. We did, however, find that B10 cells from HT patients and GD patients had a frequency of B10 cells expressing CD25 and TIM-1.

Thus, these studies have provided insights and extended our understanding of the pathogenesis of GD and HT in terms of the immune system's reaction to thyroid self-antigens.

Perspectives

There are still so many unanswered questions that remain to be answered with regards to the pathogenesis of AITD. It is still not clear as to why thyroid autoimmunity starts to begin with and by what mechanisms autoimmunity is allowed to progress. Some future experiments to aid in our understanding of the pathogenesis of AITD could be:

- To measure the frequency of Th10 and Th17 cells as well as their cytokine products IL-10 and IL-17 in the thyroid tissue of AITD patients.
- To measure the functionality of the IL-10-producing CD4+ T cells or IL-10-producing B cells, in terms of inhibiting cytokine production, proliferation or differentiation of effector CD4+ T cells from AITD patients in the peripheral blood and thyroid tissue.
- To characterize the phenotype of B10 cells in the thyroid tissue of AITD patients.
- To measure other anti-inflammatory cytokines such as TGF-β and/or IL-35. IL-35 has recently been shown to have a regulatory role in autoimmunity (326,327).

From our data HT patients had a higher frequency of Th17 cells and IL-17 production. Inhibiting the Th17/IL-17 axis might be a useful therapeutic treatment. Currently, there are three anti-IL-17A drugs, namely secukinumab, ixekizumab and brodalumab, undergoing clinical trials (328). Secukinumab is a fully human anti-IL-17A monoclonal antibody (mAb) with IgG1 subtype, ixekizumab is a humanized anti-IL-17A mAb, whereas brodalumab is a fully human anti-IL-17RA mAb, which is directed against the IL-17A receptor (328). These therapeutic agents allow the inhibition of IL-17 production as well as the blocking of IL-17A receptor. These therapeutic agents have shown great promise in reducing IL-17 production in various autoimmune diseases such as RA, psoriasis and Crohn's disease (328,329). These therapeutic agents might also have a positive effect in patients with HT by blocking the production of IL-17 or its receptor. Intriguingly, there is also evidence that therapeutic agents can be produced to inhibit the transcription factors ROR-γt and ROR-α, which are central to Th17 differentiation. If these transcription factors can be blocked that would inhibit Th17 differentiation and reduce IL-17 production (330).

Summary

Autoimmune diseases occur due to faulty self-tolerance. Graves' disease (GD) and Hashimoto's thyroiditis (HT) are classic examples of organ-specific autoimmune diseases. GD is an autoantibody-mediated disease where autoantibodies are produced against the thyroid stimulating hormone receptor (TSHR). HT is primarily a T-cell mediated disease, and whether B cells play a pathogenic role in the pathogenesis is still unclear. Both GD and HT are characterized by infiltration of the thyroid gland by self-reactive T cells and B cells.

In the first paper of this thesis, the role of regulatory B cells (Bregs) and regulatory T cells (Tregs) were investigated in the context of GD and HT. First, we studied the role of the thyroid self-antigen, thyroglobulin (TG) in healthy donors. The self-antigen TG, but not the foreign recall antigen tetanus toxoid (TT), was able to induce interleukin 10 (IL-10) secretion by B cells and CD4+ T cells. These IL-10 producing B cells (B10 cells) from healthy donors were enriched with the CD5+ and CD24hi phenotype. In addition, TG was able to induce IL-6 production by B cells. In contrast, TT induced production of Th1-type pro-inflammatory cytokines including interferon-gamma (IFN-γ) and IL-2.

In the second paper, the frequency and phenotype of B10 was investigated in healthy donors and patients with GD or HT. The frequencies of B10 cells were similar in the three groups, irrespective of whether IL-10 was induced by a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin, by CpG oligodeoxynucletodies (ODN) 2006, or by TG. Several phenotypes have been associated with B10 cells such as CD5+, CD25+, TIM-1+, CD24hiCD38hi and CD27+CD43+. We found that larger proportions of B10 cells in patients with GD or HT were CD25+ and TIM-1+ than B10 cells in healthy donors. In healthy donors, B10 cells were CD24hiCD38-, whereas for HT patients these cells were primarily CD24intCD38int. For GD patients, we found lower proportions of B10 cells within the CD27+CD43- and CD27-CD43- fractions than for healthy donors. Our data show that GD and HT are not associated with decreased frequencies of B10 cells. Accordingly, B10 cells may not be confined to one phenotype or subset of B cells.

In the third paper, we studied the balance between IL-17-producing CD4+ T cells (Th17 cells) and IL-10-producing CD4+ T cells (Th10 cells) in healthy donors and patients with GD or HT. In HT patients, we found increased proportions of naïve Th17 cells after stimulation with the thyroid self-antigen thyroid peroxidase (TPO) and the Escherichia coli lipopolysaccharide (E. coli LPS). The proportions of Th10 cells were similar in healthy donors and in HT patients after antigen-specific stimulation. After TG stimulation, an increased Th17:Th10 ratio was found in HT patients within the naïve T cell compartment. Taken together, these data indicate that the thyroid self-antigens TG and TPO induced a skewed Th17:Th10 differentiation in HT patients. IL-6 and TGF-β have been reported to be important for human Th17 differentiation and, accordingly, HT patients showed higher baseline production of IL-6 and TGF-β1 than healthy donors. Moreover, the baseline expression of mRNA encoding the transcription factor Forkhead box protein 3 (FOXP3) was similar in HT patients and healthy donors, but HT patients displayed higher constitutive expression of the splice variant FOXP3Δ2, lacking exon 2, than healthy donors. Full-length FOXP3 has been shown to inhibit Th17 differentiation, while FOXP3Δ2 does not. Thus, increased IL-6 and TGF-β1 in the microenvironment and the increased expression of FOXP3Δ2 may contribute to the skewing of Th17 cells in HT patients.

In conclusion, the human thyroid self-antigen TG is able to induce antigen-specific production of IL-10 in CD19+ B cells and CD4+ T cells among healthy donors and patients with GD or HT. Our data indicates that patients with GD or HT were not impaired in producing IL-10 and thus an immuno-regulatory response. Additionally, TPO, a thyroid self-antigen, induced a higher frequency of Th17 cells in HT. This indicates that Th17 cells may play an important role in HT pathogenesis.

List of Abbreviations

Abbreviation	Meaning
Ab	Antibody
ADCC	Antibody dependent cell mediated cytotoxicity
AITD	Autoimmune thyroid disease
APC	Antigen presenting cell
B10	IL-10-producing B cells
BCR	B cell receptor
Breg	Regulatory B cells
CD	Cluster of Differentiation
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CTL	Cytotoxic T lymphocytes
EAE	Experimental autoimmune encephalomyelitis
EAT	Experimental autoimmune thyroiditis
E. coli LPS	Escherichia coli Lipopolysaccaride
FOXP3	Forkhead box protein 3
GC	Germinal center
GD	Graves' disease
GITR	Glucocorticoid-induced TNFR family related gene
HLA	Human leukocyte antigen
HT	Hashimoto thyroiditis
IFN-γ	Interferon-gamma
IL	Interleukins
iTregs	Inducible regulatory T cells
MHC	Major Histocompatibility complex
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nTregs	Natural regulatory T cells
PBMC	Peripheral blood mononuclear cells
RA	Rheumatoid arthritis
ROR-α	retinoic acid receptor-related orphan receptor – alpha
RORC	retinoic acid receptor-related orphan receptor C
RORγt	retinoic acid receptor-related orphan receptor –gamma t
SLE	Systemic lupus erythematosus
STAT1	signal transducer and transcription activator 1
STAT4	signal transducer and transcription activator 4
TBAb	Thyroid blocking antibody
TCR	T cell receptor
TG	Thyroglobulin
TGF-β	Transforming growth factor-beta
Th	T helper cell
Th0	Naïve CD4+ T cells
TNF-α	Tumor necrosis factor - alpha
TPO	Thyroid peroxidase
TRAb	Thyroid stimulating hormone receptor antibody
Treg	Regulatory T cells
TSH	Thyroid stimulating hormone
TSAb	Thyroid stimulating antibody
TSHR	Thyroid stimulating hormone receptor
TT	Tetanus toxoid

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