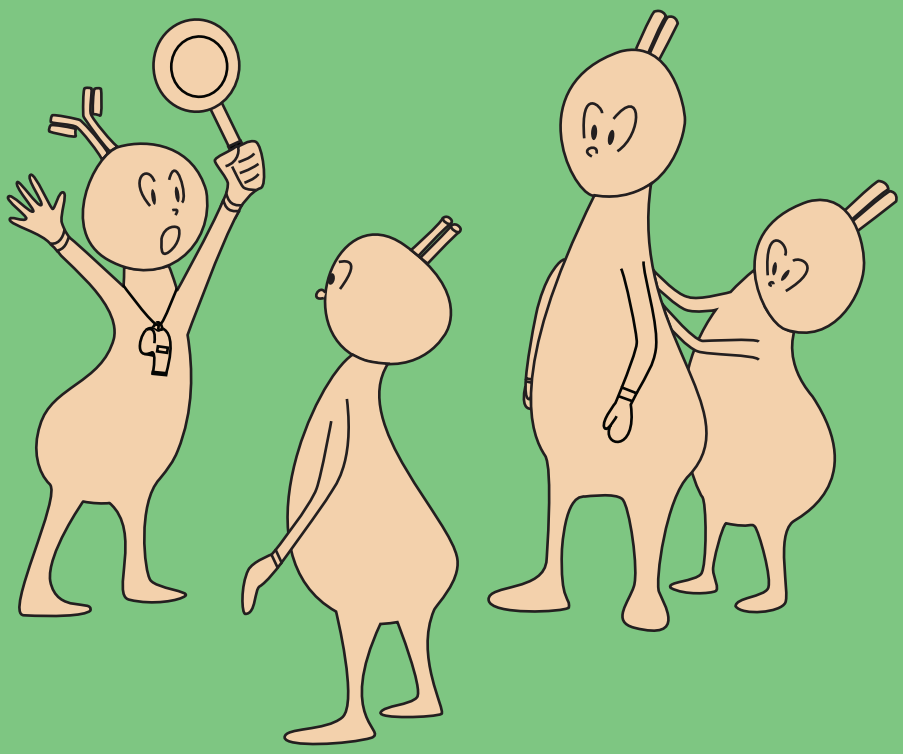
Bidirectional regulation between B cells and T cells



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Bidirectional regulation between B cells and T cells

Bidirectionele regulatie tussen B-cellen en T-cellen

(met een samenvatting in het Nederlands)

Proefschrift

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1

General Introduction

The innate and the adaptive immune system

The vertebrate immune network can be divided into the innate- and the adaptive immune system. The innate immune system provides an early line of defense that reacts to pathogen-associated molecular patterns (PAMPs) via a set of specialized pattern recognition receptors (PRRs), of which the Toll-like receptors (TLRs) are an example. Components of this rapidly acting innate immune system include macrophages and dendritic cells (DCs) that function as antigen-presenting cells (APCs). These APCs phagocytose pathogens and can subsequently activate the adaptive immune system. The adaptive immune system consists of different types of T- and B cells.

Activation of $CD4^+$ T cells, or T helper (T_H) lymphocytes, occurs when a T cell receptor (TCR) on T_H cells recognizes its cognate peptide antigen (Ag) in the context of a major histocompatibility complex (MHC) class II molecules in the form of peptide-MHC class II (pMHC-II) complex displayed by an activated APC. Depending on the various types of PAMPs that are present when an APC (e.g. a DC) encounters an Ag, such as an external pathogen, for the first time, various TLRs and other PPRs are activated. These PRRs provide important information for the DC, since they shape the subsequent immune response and cause it to be tailored towards the detected pathogen. When an activated APC presents Ag to T_H cells, the APC will provide additional information to the T_H cell via membrane-bound signaling-molecules and the secretion of soluble mediators such as cytokines. This series of events is typically followed by proliferation of the T_H cells possessing TCRs matching the specific Ag (a process termed clonal expansion) and the 'skewing' of these T_H cells towards an adequately specialized T_H cell type. Examples of skewed T_H cell subsets include (i) T_H1 cells that are e.g. suited to direct macrophages in the combat against bacteria, (ii) T_H2 cells that can e.g. aid in the provision of immunity towards extracellular pathogens such as parasites, (iii) follicular helper T cells (T_{FH}) that provide crucial help to B cells (discussed below into more detail) and (iv) regulatory T cells that suppress (excessive) immune responses when needed.

B cells have Ag-specific B cell receptors (BCRs) that cause each individual cell to react and clonally expand upon encounter with their cognate Ag. Specialized skewed T_H cells are, as the name indicates, mainly known for helping, regulating and aiding other immune cells to function well. B cells are typically seen as progenitors of 'end-stage effector cells' that secrete antibodies (also named immunoglobulins) that can e.g. directly neutralize toxins, mark pathogens for removal or activate other immune cells.

The roles of B cells in the immune system

Even though the ability to transform into antibody (Ab) secreting cells is one of the most important roles of B lymphocytes in the adaptive immune system, B cells have other functional roles as well. Summarized, B cells possess three interconnected, but separable, roles in the immune network: [1,2]

1. B cells can transform into various types of Ab-secreting cells that play important roles in both the defense against pathogens and the maintenance of immune homeostasis.
2. B cells play an important non-redundant role as APCs when they present peptide Ags to $CD4^+$ helper T cells in MHC-II molecules in the form of pMHC-II complexes.

3. B cells secrete pro- and anti-inflammatory cytokines (including interleukin (IL)-6 and IL-10; [2]), which are important for the regulation and functioning of other immune cells.

Next to that, the roles that B cells play in the organogenesis of secondary lymphoid organs may be considered a fourth role [1,2].

Mouse B cells

Splenic B cell subsets

The mouse spleen contains various B cell subsets. Roughly ~80% of the splenic B cells are Follicular (FO) B cells, about 10-15% are Marginal Zone (MZ) B cells and < 5% are B-1 cells. Strictly spoken, both FO and MZ B cells are B-2 cells, but the term B-2 often refers to FO B cells only [3].

FO B cells are the subset of B cells that typically participate in germinal center reactions, a process that generally yields antibodies with high affinity for a specific Ag. MZ and B-1 cells possess many similarities [3], are collectively referred to as "innate-like" B cells, and perform specialized tasks. Compared to conventional FO B cells, innate-like B cells are relatively strong APCs for $CD4^+$ T cells [4,5], they are more sensitive to TLR-triggering and therefore easier activated by Ags in a non-specific way [3,6], they can quickly release an early wave of antibodies after pathogens are encountered [7], they secrete large amounts of a variety of cytokines [2,6] and they produce 'natural IgM' antibodies (NAbs; discussed below).

Splenic MZ and FO B cells are classically separated on the basis of the expression of CD21 (complement receptor 2), the low affinity Fc epsilon RII (CD23) and a non-classical lipid-Ag presenting MHC protein (CD1d). MZ B cells are $CD21^{hi}$ $CD23^{low}$ $CD1d^{hi}$, whereas FO B cells are $CD21^{low}$ and $CD23^{hi}$ $CD1d^+$ [3].

B-1 cells

B-1 cells differ from B-2 and MZ B cells in their properties, development, phenotype, B cell receptors (BCRs) and anatomical location. They are long-lived, continuously self-renew and do not proliferate after BCR-triggering [3,7-9]. In contrast to FO B cells, B-1 cells can be positively selected for self-reactivity [3,10]. Their exact development remains unclear. B-1's ontogeny has been extensively reviewed [8,11]; B-1 cells can be derived from both the fetal liver and the bone marrow, and evidence points towards the possibility that B-1 cells are both derived from specific progenitors that belong to a separate lineage, and from common B cell precursors that are selected on the basis of their BCR specificity [3]. It is clear that the pool of B-1 cells in adult mice is mainly maintained by self-renewal [3,9], a process in which the spleen plays an important role since splenectomized mice are reported to contain reduced numbers of these cells [12].

B-1 cells exhibit a phenotype that is very different from FO B cells [3,8,9]. Compared to FO B cells, B-1 cells are slightly larger, express slightly higher levels of CD19, but express lower levels of the pan B cell marker B220 (CD45R). They are IgM^{hi} IgD^{low} and $CD23^{lo/-}$ whereas FO B cells are IgM^{low} and IgD^{hi} and $CD23^+$ [3,7-9]. FO B cells do not express CD43, but most of the B-1 cells do [13]. Some B-1 cells in the peritoneal cavity (PerC) are reported to lack this surface molecule and CD43 is furthermore not as highly expressed on B-1 cells as it is on T cells [13]. PerC B-1 cells express intermediate levels of CD11b, whereas B-1 cells in the

spleen are CD11b$^-$, except for the ones that recently migrated from the PerC to the spleen [14]. B-1 cells can be differentiated into B-1a cells that express the pan T cell marker CD5, and B-1b cells that are instead CD5$^-$. The level of CD5 on B-1 cells is about ten times lower than the expression on T cells [3].
The B-1 cell pool expresses a different BCR repertoire compared to B-2 cells; their BCRs and their Abs tend to be polyreactive and therefore react with different Ags, including self-Ags, with low affinity [3,9,15]. B-1 cells are involved in Ag-driven immune responses and can produce Ag-specific antibodies [16-18], but they are also well known for their ability to produce NAbs that are produced without antigenic stimulation.

B cell subsets in the peritoneal cavity

The composition of B cell subsets in the PerC is very different from that in the spleen, and differences between mouse breeds are substantial [19]. The PerC of BALB/c mice contains a relative high percentage of B-1a cells (~70%), a low percentage of B-1b cells (~10%) and about ~20% B-2 cells [[20] and Chapter 3]. The PerC of C57BL/6 mice contains, compared to BALB/c mice, relatively more B-1b cells and less B-1a cells [19].
PerC B-1 cells are attracted towards the PerC due to a specific set of homing receptors. The chemokine CXCL13 (produced by PerC macrophages [21]) attracts them via the receptor CXCR5 [21]. B-1 cells are also attracted to CXCL12 (a ligand for CXCR4) [22]. PerC B-1 cells are thought to enter the PerC from the blood through 'milky spots' (lymphocyte-rich follicular structures) located on the omentum [22]. PerC B cells can exit the PerC through the omentum [22] and via efferent lymphatics through the mediastinal lymph nodes (LNs) [23,24]. TLR-triggering induced down-regulations of integrins (such as CD11b) and CD9 are involved in the exit of B-1 cells from the PerC [7,22].
The PerC of mice contains B-2 cells as well, but they are not identical to splenic B-2 cells. The peritoneal microenvironment has been described to program PerC B-2 cells into B cells with B-1-like properties, including the ability to reenter the PerC after i.v. transfer [21], changes in phenotype (increased expression of CD11b and CD43 [25]) and *in vitro* IgM secretion

Table 1: Selected mouse B cell subsets together with their locations and commonly used phenotypic markers [3,31].

Lineage	B cell subset	Commonly used phenotypic markers	Location
B-2	Follicular (FO) B cells	CD19$^+$ B220$^+$ CD21^{lo} CD23^{hi} CD1d$^+$ IgM^{lo} IgD^{hi} CD43$^-$ CD5$^-$ CD11b$^-$	Spleen (Follicles; 80-85% of splenic B cells), lymph nodes
B-2[1,2]	Marginal zone (MZ) B cells	CD19$^+$ B220$^+$ CD21^{hi} CD23^{lo} CD1d^{hi} IgM^{hi} IgD^{low} CD43$^-$ CD5$^-$ CD11b$^-$	Spleen (Marginal zone; 10-15% of splenic B cells)
B-1[2]	B-1a and B-1b B cells	CD19^{hi} B220^{lo} CD23$^{lo/-}$ CD1d$^+$ IgM^{hi} IgD^{low} CD43$^{+\,(mostly)}$ CD5$^-$ (B-1b) or CD5$^+$ (B-1a) CD11b$^-$ (spleen) or CD11b$^+$ (PerC).	PerC, pleural cavity, spleen (<5%), LN: minimally, if present at al.

[1]: Strictly spoken, both FO and MZ B cells are B-2 cells, but the term B-2 often refers to FO B cells only

[2]: MZ and B-1 cells are collectively referred to as innate-like B cells.

[25]. The relationship between PerC B-1a and splenic B-1a cells is equally unclear: there are differences in BCR repertoires, gene expression patterns and the B-1 cells in the PerC are described to secrete little IgM whereas the ones from the spleen do [26-28]. At least some of these differences are found to be due to influences of the microenvironment, indicating that at least some of these cells might be intrinsically similar, but just differentially activated [28-30].

B cells as progenitors of Ab-secreting cells

A B cell recognizes its cognate Ag when this Ag binds to one of the multiple identical copies of the Ag-specific BCR on the cell membrane. Naive B cells can turn into Ab-secreting cells upon such a cognate interaction. Next to the cognate Ag interaction, B cells may receive important co-stimulatory signals from specialized $CD4^+$ helper T cell subsets when the B cell presents Ag-derived peptides in pMHC-II complexes to the T_H cell.
B cells can turn into Ab-secreting cells via at least three different routes:

1. Some specific subsets of B cells, which include B-1 cells, can turn into Ab-secreting cells without any antigenic stimuli at all (i.e. NAbs).
2. B cells can turn into Ab-secreting cells via extrafollicular (EF) antibody responses; this route may or may not depend on T cell help [32,33].
3. B cells can turn into Ab-secreting cells via a germinal center (GC) reaction; this route is typically highly dependent on T cell-derived help.

The specificity of the Abs formed via any of three routes is derived from the specificity from the BCR present on the B cell that recognized the Ag at first, although it may have been 'fine-tuned' in a process termed affinity maturation. Next to that, the constant fragment (fragment crystallizable region) of the antibody that determines the Ab isotype, and the thereof derived biological effector functions of the antibody, may have been altered in a process named class switch recombination (CSR) or isotype switching.

Germinal Center reactions typically depend on T cell help

B cells that are activated when Ag binds their BCR can enter the extrafollicular or the germinal center route. The choice between these two routes may depend upon the strength of the initial interaction between the BCR and its Ag [34]. The two routes possess certain similarities, but the extrafollicular pathway typically yields Abs that did not underwent as much affinity maturation as compared to Abs derived from the GC pathway [32,33].
B cells that enter the GC pathway will follow a process that typically depends on T cell help. GC B cells express both the activation marker GL7 [35] and the death receptor CD95 (FAS) [35]. GC B cells rapidly proliferate and during this process, mutations in their antibody-encoding DNA are introduced that result in the formation of a variety of B cell clones with slightly different BCRs. This process is termed somatic hypermutation (SHM). Following SHM, the newly formed BCRs will be tested for their ability to obtain Ag from specialized follicular dendritic cells (FDCs) that retain Ag in the GC. Only B cell clones with improved BCRs will obtain sufficient Ag and the necessary help from $CD4^+$ helper T cell when the Ag-derived peptides are presented in a pMHC-II complex [36].
T_H cells directed by the expression of the transcription factor B cell lymphoma 6 (BCL-6)

will specialize themselves to provide T cell help to GC B cells and are named follicular helper T cells (T_{FH}) [33,37,38]. Similarly to mature B cells, T_{FH} cells express high levels of the B cell homing chemokine receptor CXCR5 and low levels of the T cell area homing receptor chemokine receptor CCR7. This combination of homing receptors causes T_{FH} cells to be attracted by the chemokine CXCL13, the ligand of CXCR5, which results in the ability of T_{FH} cells to reach the GC that are positioned in the B cell follicles. T_{FH} cells provide help to B cell that present their cognate Ag in a pMHC-II complex via a combination of direct cell-cell contact and soluble mediators. T_{FH} cells express high levels of inducible T cell costimulator (ICOS; CD278) [39,40] and programmed cell death protein 1 (PD-1; CD279) [39,40]. Together with CD40 Ligand (CD40L), the costimulatory molecules ICOS and PD-1 provide help to GC B cells via CD40, ICOS Ligand (ICOSL; CD275; B7-H2) and PD-1 Ligand 1 (PD-L1; CD274 B7-H1) expressed on the B cells [33,37,38,41] respectively. GC-influencing soluble mediators secreted by T_{FH} cells include the cytokines IL-2, interferon (IFN)-γ, IL-4 [41,42] and especially IL-21 [33,37,38,41,42]. Elevated production of IL-17 by T_{FH} cells is sometimes said to be associated with autoimmunity [41].

The end-results of a GC reaction in which T_{FH} cells have provided help to B cells is multiple: B cells that have maturated their affinity via SHM and likely have switched their isotypes via CSR can eventually downregulate BCL-6, upregulate the transcription factor B-lymphocyte-induced maturation protein 1 (Blimp1) and thereby differentiate into long-lived plasma cells (PCs) that are specialized in secreting large amounts of immunoglobulins. Alternatively, they form memory B cells (MBCs) that can quickly form PCs and/or new GCs when Ag will be encountered for a second time. Both the Ag-specificity and the isotype of the Abs secreted by PCs and the BCRs expressed by MBCs are determined during the GC reaction [33,37,38,41,42]. A third cell-type that can be the result of either the GC reaction or, more typically, an EF B-cell response, is the short-lived plasmablast (PB), an in-between differentiated B cell that secretes large amounts of immunoglobulins but commonly underwent less affinity maturation as compared to PCs and MBCs [43,44].

Antibodies: molecules with mixed functions

The fragment crystallizable region (Fc) of the Ab defines the Ab-isotype, and its biological effector functions. Naive B cells express BCRs of the IgM and IgD isotype. B cells that differentiate into PCs or PBs without CSR will secrete Ab of the IgM isotype. CSR (in mice) can exchange the constant fragment into that of the IgG3, IgG1, IgG2b, IgGa, IgE or IgA isotype. Each isotype has its own specialized effector functions in the immune network.

Broadly summarizing, most isotypes possess strictly either pathogen-neutralizing and/or immune-component stimulating actions. IgM is in this sense an exception since some specific NAbs idiotypes have the ability to down-regulate immune responses and prevent the onset of autoimmunity [45-47]. Protective 'regulatory NAbs' are in fact autoantibodies; they bind to oxidation-associated membrane epitopes of apoptotic cells such as phosphorylcholine [45-47]. This binding protects the host with a variety of mechanisms that include the tolerogenic phagocytic clearance of these apoptotic cells by immature DCs and macrophages [48,49] and suppression of the production of the pro-inflammatory cytokines IL-6 and Tumor Necrosis Factor (TNF)-α [48] by monocytes. Regulatory NAbs were demonstrated to protect mice

against the development of collagen-induced arthritis *in vivo* [48]. However, NAbs can also be involved in the pathogenesis of intestinal ischemia-reperfusion injury [50] and cerebral injury following ischemic stroke [51] and are thus not *per se* protective in all situations. Besides, Ag-induced IgM [16] is virtually impossible to separate from Natural IgM, and may have very different functions, including pathogenic roles in autoimmune diseases such as rheumatoid arthritis (RA) [52]. Most research on regulatory NAbs is performed with B-1 cell-derived NAbs of the prototypic T15 idiotype that recognize phosphorylcholine [22,45-47].

B cells as non-redundant APCs

Early-stage immune response

Since Ag-specific B cells internalize Ag via BCR-receptor-mediated endocytosis, they possess the ability to efficiently pick up very small amounts of Ag, and subsequently present its peptides in a pMHC-II complex to T_H cells [53]. B cells without a BCR specific for a certain Ag do not efficiently do so and are, especially in comparison to DCs, poor APCs [2,54]. At early stages of an immune response there are relatively few B cells that have a BCR specific for a specific Ag [53,55]. Moreover, non-activated naive B cells do not express high levels of the co-stimulatory molecules CD80 and CD86 that are needed to efficiently prime $CD4^+$ T cells [53,56-58]. It has been suggested that non-activated, resting, B cells induce tolerance or forkhead box P3 (FoxP3) expression in $CD4^+$ T cells [59,60]. Because of these reasons, the role of B cells as APCs in the early-stages of an immune response was originally thought to be limited and its contribution is currently still controversial [2,53-55]. Recent research shows that the role of B cells enacting roles as APC at early stages might be more important than previously anticipated. Studies performed with mice that had a MHC-class II-deficiency in B cells only, indicated that primary T cell expansion and differentiation *in vivo* requires Ag presentation by B cells at early time points after immunization [61]. Other studies that made use of fluorescent Ag, have demonstrated the presence of Ag on B cells virtually immediately after *in vivo* administration [62,63]. However, Ag-presentation by B cells alone has been shown insufficient for the full-blown activation of T_H cells [64].

Late-stage immune response

At later stages of an immune response, the number of Ag-specific B cells is much higher due to clonal expansion, and the importance of B cells enacting roles as APCs is well known [2,53-55]. Various research groups have shown that B cells are non-redundant APCs that are important for the functioning of T_H cell subsets *in vivo*, both in protective immune responses and in autoimmunity. B cell deficient mice, e.g., show defects in their development of protective T_H1 cell immunity against Salmonella. This defect is independent of B-cell derived Abs since transgenic mice that have B cells that cannot secrete Abs were well protected against this bacteria [65]. This thus suggests that B cells might either serve as important APCs and/or secrete cytokines important for the development of a T_H1 response [65]. Other studies, performed with mice that possessed a MHC-class II-deficiency in B cells only, more specifically indicated that T cell expansion and differentiation *in vivo* requires Ag presentation by B cells [61]. In addition, experiments performed with the proteoglycan-induced arthritis (PGIA) murine model for RA, have shown that Ag-specific B cells are non-redundant APCs [66].

More specifically, the expression of CD80/86 on B cells was essential for the activation of autoreactive T cells and the development of arthritis [67]. The importance of B cells in T cell functioning is furthermore supported by the fact that B-cell depletion therapy (e.g. anti-CD20 mAb; Rituximab) is well known to influence the induction, maintenance and reactivation of $CD4^+$ T cells and T cell driven autoimmune diseases [2,55].

B cells as cytokine-releasing immune regulators

Pioneering work from the group of Lund et al. [68] has shown that B cells have the ability to regulate T_H cell differentiation through the production of cytokines. This work focused on the differentiation of T_H1 and T_H2 cells via IFN-γ/IL-12-secreting 'B effector 1 cells' and IL-4/-6/-10-secreting 'B effector 2 cells' respectively and it showed that B-cell mediated differentiation and/or amplification of T_H cell subsets can also be mediated via the secretion of B-cell derived cytokines, a mechanism that can be independent of Ag-presentation by B cells [68].

Discovery of IL-10-producing regulatory B cells

Subsequent work on cytokine-releasing and immune regulating B cells was performed in the next decade and mostly focused on the immune dampening role of IL-10 secreting B cells named "regulatory B cells" (B_{REGS}). Reports on B_{REGS} appeared as early as 1974 [69] but have originally been met with skepticism, mainly because IL-10 is a pleiotropic cytokine that was known before to enhance B cell survival, proliferation and antibody production [70]. The work of Fillatreau et al. [71] in 2002 however, clearly showed in an elegant way that B cell-derived IL-10 possesses immunoregulatory roles in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis. Wildtype (WT) mice spontaneously recover from EAE, whereas bone marrow chimeras in which the disability to produce IL-10 is limited to the B cell compartment, do not. The spontaneous recovery was rescued after a transfer of IL-10 capable B cells [71]. Most research groups agree that a *bona fide* B_{REG} can only be identified as such when its immunoregulatory role has been functionally demonstrated. There was however an obvious need to identify the phenotype of the B_{REG} in order to allow e.g. isolation of B cell subsets with putative regulatory functions for subsequent further research.

Phenotypes of regulatory B cells

The work from the groups of Mauri and Tedder, have shown that IL-10-production by autoimmune disease-suppressing activated splenic B cells is concentrated in specific B cell subsets. Mauri identified the transitional type 2-marginal zone precursor B-cell subset (T2-MZP) as a key IL-10-producing B cell subset [72,73] and Tedder termed all IL-10-producing B cells 'B10' cells, and then identified the $CD5^+CD1d^{hi}$ B cell subset as a B cell population that contains a relative large amount of B10 cells [74,75]. Both the T2-MZP and the $CD5^+CD1d^{hi}$ B cell subsets contain functionally well-described disease-suppressing B_{REGS}, although they appear to be different in nature: T2-MZP B cells are immature B cells, whereas the exact lineage or identity of the newly defined B10 $CD5^+CD1d^{hi}$ B cells is not exactly clear [74]. Positivity for CD5 would suggest these cells are derived from the B-1 lineage, although as mentioned earlier, peripheral B-1 cells can never be identified as such based on the expression of one

surface marker only, since multiple other B cells may express similar surface molecules as well. High expression of CD1d would suggest B10 cells to be derived from MZ B cells. Given the fact that the gating strategy for the selection of $CD5^{+}CD1d^{hi}$ cells is more or less arbitrary set, it seems fair to assume that these cells most likely include some B-1 cells, some MZ B cells and possibly some cells with an intermediate phenotype.

B_{REGS}: origin and mechanism of activation

Despite all efforts, both the origin and the exact mechanism of activation of B_{REG} currently remain unclear. The work of Mauri and Tedder indicates that apparently separate B cell subsets possess the common ability to perform regulatory B cell functions [72,74]. This suggests that autoimmune suppressive B_{REGS} might not be derived from a common progenitor, and points towards the possibility that functional B_{REGS} may instead originate from a specific activation-status. Indeed, *in vitro* treatments can induce IL-10 production in a variety of B cells subsets [6], although most IL-10 producing B cells seem (related to) innate-like B cell subsets such as B-1 cells or MZ B cells [6,76].

Stimuli that are known to induce IL-10 secretion by B cells and/or induce or activate functionally active B_{REGS} include (i) microbial products that trigger TLRs such as LPS and CpGs [77-79] (ii) stimulation via the costimulatory molecule CD40 on B cells [79-81] (iii) a combination of TLRs and signals via CD40 [79,82,83] and (iv) cytokines such as IL-21 [82]. Limited evidence indicates that (v) Ag-specific signals via the BCR may be involved in the *in vivo*-activation of B_{REGS} as well [73,75]: *in vivo*-activated B_{REGS} that are activated and suppress in one model do not downregulate inflammation when transferred to a secondary model that is mediated via another Ag [73,75]. Mentioned before, IL-10 secretion on its own does not define a B cell subset as a B_{REG}. Activated B cells often produce multiple cytokines at the same time [6,84] and B cell-derived IL-6, has been shown to play a detrimental role in EAE [85]. Key to the identification of specific B cell subsets as genuine B_{REGS} is the determination of a functional immunosuppressive role, either *in vivo* or *in vitro*.

In summary, B cells were found to have the ability to down-regulatory inflammatory responses via the secretion of IL-10. Research focused on the identification of the exact B cell subset responsible for these actions have yielded different results, which suggests that the origin of functional B_{REGS} does not solely depend on the specific subset, but instead on other factors such as the activation status of the B cell and its specific microenvironment.

T- and B cell communication in host-protection and autoimmune diseases

Under normal homeostatic conditions, the immune system continuously clears pathogens while it remains tolerant to self. The foundation of this separation is based on the selection of T- and B cells early in ontogeny resulting in the removal of T- and B cells that have an Ag-specific receptor with a high potential of attacking self [86,87]. Next to this, regulatory T cells (T_{REGS}) that restrain undesired inflammatory responses are educated during the same selection process and are furthermore continuously being shaped later in life [88]. The latter can take place when steady-state (i.e. non-activated) APCs phagocytose (self-)Ag in the absence of PAMPs and present the thereof derived self-peptides in a pMHC-II complex to T helper cells [88-90]. In the event that an immune reaction against a (self-)Ag is initiated, the

subsequent reciprocal interplay between T- and B cells largely shapes the outcome of the (auto)immune response. The three earlier introduced B cell roles (antibody-production, Ag-presentation and the secretion of cytokines) play an important role in this process. Table 2 provides examples of how B cell-mediated T cell communication and regulation may contribute to both the initiation/aggravation as well as the prevention/amelioration of an autoimmune disease such as arthritis. In general, the in Table 2 presented concepts are not limited to autoimmune conditions but instead applicable to host-protective immune responses as well. Even though B cell depletion therapies have shown to be very effective tools in the treatment of a variety of autoimmune diseases [103,104] the examples in Table 2 show that not all B cell-mediated T cell regulations are detrimental for the development or progression of autoimmune conditions. There is a need for more research in order to determine the exact roles of the different B cell subsets and/or activation statuses during host-protection, autoimmunity and the maintenance of homeostasis.

Outline of this thesis

The overall aim of this thesis is to get a better understanding of the variety of roles B cell subsets play in immune regulation, and how the reciprocal interplay between B– and T cells determines the outcome of an immune response.

In **Chapter 2** we studied what the effects are of targeting a single peptide, which represents an immunodominant T cell epitope of the cartilage proteoglycan, to steady-state DCs on the subsequent induction of arthritis using whole proteoglycan protein. Our studies focused on the effects that alterations in the proteoglycan-specific T cell population had on the subsequent germinal center formation, proteoglycan-specific antibody production and the development of autoimmune arthritis.

In the next two chapters we explored the immunoregulatory potential of the innate-like peritoneal cavity B-1a cells subset on T cells. The immunoregulatory abilities of B cells are becoming clear just now, but the capabilities of cells from the B-1 subset remained largely unexplored so far. In **Chapter 3** we examined the effects peritoneal cavity B-1a cell exert on $CD4^+$ helper T cells when this specific B cell subset functions as Ag-presenting cells *in vitro*. In addition, we used a combination of adoptive B- and T cell transfers to investigate how B-1a cells from the peritoneal cavity can present Ag to T cells in the periphery *in vivo*.

Chapter 4 concentrates on the potential ability of the peritoneal cavity B-1a cell subset to perform immunoregulatory roles as B_{REGS}. This chapter first points out that described B_{REGS} possess multiple similarities with peritoneal cavity B-1a cells. Following this finding, we analyzed the capacity of peritoneal cavity B-1a to perform B_{REG} roles using an *in vitro* suppression assay.

In **Chapter 5** we aimed to elucidate the B cell epitopes on the protein-backbone of the self-Ag mouse proteoglycan-derived G1 domain after induction of arthritis using immunization with proteoglycan. Moreover, we analyzed the difference in B cell response between proteoglycan- and G1 domain-induced arthritis and we explored the presence of anti-G1 domain autoantibodies in the sera and synovial fluid of patients with rheumatoid arthritis.

Chapter 6 summarizes the results described in this thesis and discusses the drawn conclusions together with their implications.

Table 2: The reciprocal interplay between T– and B cells shapes an adaptive immune response.

B cell role	Examples of B cell-mediated T cell regulation *	Examples of reciprocal T cell-mediated B cell regulation
Ab production	(+): Autoantibodies directed against self-Ag (such as cartilage-derived proteoglycan**; chapter 5) aid in the phagocytosis of these Ags and facilitate subsequent pathogenic T cell activation/skewing by various APCs, including B cells. (–): NAbs possess anti-inflammatory properties [45-47] and are shown to possess protective roles in models of RA [48].	T_{FH} cells provide signals that are crucial during affinity maturation, CSR and the development of Ab-secreting PCs [[91,92] and chapter 2].
Ag presentation	(+): Ag presentation by B cells, and in particular B cell-derived signals via CD80/86, are essential for the activation of pathogenic T_H-subsets (possible including T_{FH} cells) in autoimmune arthritis [93,94]. (–): Ag-presentation by B_{REG}s is involved in the described *in vivo* and *in vitro* promotion of IL-10-producing T cells by B_{REG}s associated with induced protection against experimental RA [[95] and chapter 3].	Presumably only Ag-specific B cells are good APCs [2,53,54] signals from T cell regulate the affinity maturation process needed to form these Ag-specific cells [91,92].
Cytokine secretion	(–): IL-10-producing B_{REG}s ameliorate arthritis and/or downregulate the functioning of proinflammatory T cells [73,96-98]. (+): IL-6-production by B cells in EAE is correlated with increased amounts of IL-17-producing $CD4^+$ T cells [85] and IL-6 plays pro-inflammatory roles in (proteoglycan-induced) arthritis as well [99].	CD40-triggering can evoke B cells to secrete cytokines [79-81]. Constitutively activated T cells provide signals to B cells CD40 [100] and this may take place in an Ag non-specific way [101]. Also, IL-10-producing T cells can induce IL-10-producing B cells [102].

* Examples are marked with (+) or (–): (+) demonstrate a pro-inflammatory action correlated to the initiation/aggravation of auto-immunity; whereas (–) indicate regulations related to anti-inflammatory actions correlated with the prevention/amelioration of auto-immunity.

** The reader is kindly referred to the introduction of **chapter 4** for an in-dept introduction on the self-Ag cartilate-derived proteoglycan.

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2

DEC205-targeted tolerogenic vaccination promotes immune tolerance in experimental autoimmune arthritis

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Abstract

Objective: Previous studies have indicated that targeting of (self-)antigen to the endocytic receptor DEC205 on steady-state dendritic cells can be a powerful method to induce antigen-specific immunological tolerance. The aim of the current study is to examine whether DEC205 targeting of a single immunodominant peptide derived from human cartilage proteoglycan (PG) can promote immune protection against experimental arthritis. Complementary experiments aimed to elucidate possible mechanisms of induced protection.

Methods: Mice were treated with recombinant anti-DEC205-PG peptide fusion antibodies before the induction of arthritis. Adoptively transferred PG-specific $CD4^+$ T-cells were used to study the effect of DEC205 targeting on antigen-specific T-cells *in vivo*. Additionally, the generation of germinal centers and pathological autoantibodies were analyzed using flow cytometry and ELISA, respectively.

Results: Targeting of DEC205 efficiently protected against the development of experimental arthritis. Subsequent studies showed that DEC205 targeting induced anergy/deletion of PG-specific $CD4^+$ T-cells, resulting in increased proportions of PG-specific $FoxP3^+$ regulatory T-cells. This coincided with a reduction in the amounts of PG-specific follicular helper T-cells, germinal center B-cells, and titers of PG-reactive antibodies.

Conclusions: Our study supports a model in which DEC205 targeting deletes and anergizes PG-reactive arthritogenic $CD4^+$ T-cells, leading to a lack of necessary germinal center B-cell support by follicular helper T-cells. Reduced germinal center formation results in lower autoantibody titers and compromises arthritis development. This study may indicate the potential of prospective tolerogenic vaccination to interfere with autoimmune diseases, such as rheumatoid arthritis.

Introduction

Many different cell types, including $CD4^+$ T-cells, have been implicated in the pathogenesis of rheumatoid arthritis (RA) [1]. Indeed, the synovial tissue of RA patients is infiltrated with interferon (IFN)-γ- and interleukin (IL)-17-producing $CD4^+$ T-cells, resulting in synovial inflammation and joint cartilage destruction [2]. Blockade of T-cell co-stimulation with a therapeutic agent has proven to be an effective treatment, further corroborating a critical role for T-cells in RA [3].

The cartilage proteoglycan aggrecan (PG) is a key antigen (Ag) targeted by self-reactive T-cells in the joints of RA patients [4-6]. In BALB/c mice, immunization with human PG promotes a form of arthritis (PG-induced arthritis; PGIA) that shows many similarities to human RA [7,8]. PGIA is a T-cell-dependent and antibody/B-cell-mediated autoimmune disease [9-12]. In this model, B-cells play a double role, since they are required as Ag presenting cells (APCs) for the induction of severe RA [11,13,14], and for the production of pathological autoantibodies [9,11,15,16].

Previous studies have indicated that Ag targeting to the endocytic receptor DEC205 in the steady-state may represent a powerful method to induce Ag-specific immunological tolerance to self [17-22]. In this manner, autoimmunity can be prevented by the interference with pathological T-cell responses, both through dominant and recessive tolerance mechanisms. DEC205-targeted dominant tolerance can be achieved by the Ag-specific enhancement of $FoxP3^+$ regulatory T-cell (T_{REG}) activity [19-24], whereas recessive tolerance mechanisms include deletion and/or induction of anergy in pathogenic T-cells [17,18,22,23]. Consistently, DEC205 targeting has been shown to promote immune tolerance in the spontaneous NOD mouse model of autoimmune diabetes [23,25] and peptide-induced mouse models of experimental autoimmune encephalomyelitis [18,22,24]. Compared to peptide-induced autoimmune models, PGIA appears fundamentally different in that autoimmunity is induced by human cartilage-derived whole PG protein, which exhibits a high degree of sequence homology with murine PG and substantial complexity with regard to contained T-cell epitopes. Indeed, systemic immunization with whole PG protein leads to activation of a variety of PG-specific T-cell clones, with the PG peptide 70-84 (PG_{70-84}) representing an immunodominant epitope [26,27]. The overall aim of the current study was to examine whether DEC205-targeted tolerogenic vaccination with a single immunodominant peptide of a cartilage-derived self-Ag can be used to protect against experimental arthritis. In addition, the impact of induced tolerance on eliciting arthritis-inducing T- and B-cell-mediated autoimmune responses by systemic immunization was studied.

Materials & Methods

Mice

Female BALB/c mice (retired breeders) were purchased from Charles River Laboratories. TCR-5/4E8(PG_{70-84})-Tg BALB/c mice [28] were bred and kept under specific pathogen free conditions. CD90.1 BALB/c mice were purchased from Jackson Laboratories and backcrossed to WT BALB/c (Charles River) mice. All mice were kept under standard housing conditions at the Central Animal Laboratory of The Utrecht University. Animal experiments were approved

by the Animal Ethics Committee from The Utrecht University.

Recombinant fusion antibody production

Eukaryotic expression vectors encoding the mouse IgG1 IgH chain cDNA of cloned anti-DEC205 (NLDC-145, αDEC205) and III/10 isotype control, and their respective Ig-κ L chain cDNA were provided by the Nussenzweig laboratory at Rockefeller University [17,18]. The IgH chains contain point mutations that prevent Fc-receptor binding [17]. DNA fragments (**Oligo1-for-PG** 5'-CTA-GCG-ACA-TGG-CCA-AGA-AGG-AGA-CAG-TCT-GGA-GGC-TCG-AGG-AGT-TCG-GTA-GGT-TCA-CAA-ACA-GGG-CC-3', **Oligo1-rev-PG** 5'-TTC-AGT-GGC-CCT-GTT-TGT-GAA-CCT-ACC-GAA-CTC-CTC-GAG-CCT-CCA-GAC-TGT-GTC-CTT-CTT-GGC-CAT-GTC-G-3', **Oligo2-for-PG** 5'-ACT-GAA-GGG-CGC-GTG-CGG-GTC-AAC-AGT-GCC-TAT-CAG-GAC-AAG-TAT-TAT-GAC-GGT-AGG-ACA-TGA-TAG-GC-3', **Oligo2-rev-PG** 5'-GGC-CGC-CTA-TCA-TGT-CCT-ACC-GTC-ATA-ATA-CTT-GTC-CTG-ATA-GGC-ACT-GTT-GAC-CCG-CAC-GCG-CCC-3') encoding the human PG peptide 70-84 (ATEGRVRVNSAYQDK) were added in frame to the C-terminus of anti-DEC205 and III/10, and recombinant antibodies (Abs) were produced, as described before [21,29].

Treatment, arthritis and T-cell transfer

Mice were intraperitoneally (i.p.) treated with αDEC205-PG, as indicated (see figure legends for details on amounts and time points). III/10-PG isotype control Abs and PBS injections were included as controls. Arthritis was induced by i.p. injection of 250 μg PG protein and 2 mg dimethyldioctadecylammonium bromide (DDA; Sigma) on days 0 and 21 as described before [30]. Onset and severity of arthritis was determined using a visual scoring system based on swelling and redness of paws [30]. In T-cell transfer experiments, PG-specific TCR-PG Tg $CD4^+$ T-cells were obtained from the spleen of naïve TCR-5/4E8-Tg mice by negative selection with sheep anti-rat-IgG Dynalbeads (Dynal, Invitrogen) using an excess amount of mAbs to B220 (RA3-6B2), CD11b (M1/70), MHC class II (M5/114), and CD8 (YTS169). Enriched T-cells were routinely ~90-95% pure. BALB/c or congenic BALB/c CD90.1 recipient mice were injected intravenously (i.v.) with indicated amounts of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes)-labeled T cells.

Flow cytometry, intracellular cytokine staining and ELISA

Single cell suspensions were prepared from spleen and draining lymph nodes LN (dLN) of the paws (brachial, axillary, popliteal). For flow cytometry, cells were stained with mAbs to CD5 (53-7,3), CD90.2 (53-2.1) (eBioscience), CD4 (RM4-5) or CD25 (PC61) (BD Biosciences). FoxP3 (FJK 16S) staining was carried out according to manufacturing protocol (eBioscience). Prior to intracellular staining with mAbs to IL-17 (eBio17; eBioscience) and IL-10 (JES5-16; BD Bioscience), T-cells were stimulated with PMA (50 ng/ml) / ionomycin (500 ng/ml) for 5 hours in the presence of brefeldin A (1 μg/ml; all from Sigma Aldrich). Samples were analyzed on a Canto II (BD Biosciences). PG-specific Abs in sera were measured by ELISA as described elsewhere[30]. PG-specific IgG1 and IgG2a Ab levels were compared to a standard of pooled sera from arthritic mice and expressed in relative units.

Statistical analysis

For statistical analysis Prism (GraphPad software Inc.) was used. Two-tailed Student's t-tests and one-way ANOVA (two-tailed) with Bonferroni correction were applied, when applicable. Differences were considered significant at $p < 0.05$.

Results

Efficient presentation of DEC205$^+$ DC-targeted PG$_{70-84}$ peptide to CD4$^+$ T-cells in vitro and in vivo

DEC205 is highly expressed on splenic CD11c^{hi}CD8$^+$ DCs, whereas it is absent or expressed very dimly on various other cell-types ([31] and Supplementary Figure S1A). *In vitro* targeting to DEC205$^+$ DCs by recombinant αDEC205-PG fusion Abs established that the immunodominant MHC class II-restricted PG$_{70-84}$ peptide [26,27] is efficiently processed and presented to TCR transgenic CD4$^+$ T-cells reactive to PG, as compared to the isotype-control Ab III/10-PG (Supplementary Figure S1B). Pre-incubation of DCs with free synthetic PG-peptide resulted in less PG-specific T-cell proliferation (Supplementary Figure S1B). I.p. administration of fluorescently labeled αDEC205 mAb confirmed that splenic CD11c^{hi}CD8$^+$ DCs were effectively targeted *in vivo* as well ([32] and Supplementary Figure S1C). Overall, these data show that i.p. administration of αDEC205-PG can lead to enhanced presentation of PG peptide to PG-specific CD4$^+$ T-cells by CD11c^{hi}CD8$^+$DEC205$^+$ splenic DCs.

DEC205 targeting reduces arthritis and delays disease onset

To study whether DEC205 targeting in the steady-state is protective in PGIA, cohorts of mice were treated with different doses of αDEC205-PG or III/10-PG at different time points before arthritis induction. The results consistently showed that αDEC205-PG pretreatment substantially reduces the development of PGIA (Figure 1A). In line with the observation that high doses of III/10-PG cause PG-specific T-cell proliferation *in vitro* (Supplementary Figure S1B), the *in vivo* treatment of mice with a high dose of III/10-PG isotype-control also promoted protection against PGIA, albeit to a much lower extent as compared to αDEC205-PG (Figure 1A). Indeed, administration of a single dose of small amounts of αDEC205-PG (as low as 50 ng/mouse) at an early time point before disease induction was found to be sufficient to efficiently delay disease onset and reduce symptoms (Figure 1B). In conclusion, DEC205 targeting of PG$_{70-84}$ peptide appears suitable to substantially ameliorate symptoms of whole PG protein induced arthritis over a range of recombinant αDEC205 Ab doses and different time points of administration.

Different mechanisms underlying DEC205$^+$ DC-targeted T-cell tolerance have been proposed in different studies. This includes deletion and induction of an anergic phenotype in residual Ag-specific T-cells, which is accompanied by increased expression levels of CD5 on Ag-specific T-cells [17] and reduced secretion of IL-17 [22], as well as conversion of initially CD4$^+$Foxp3$^-$ T-cells into Foxp3$^+$ T$_{REG}$-cells [19]. In our experiments, amelioration of PGIA appeared to correlate with reduced proportions of dLN-derived IL-17$^+$ T-cells (Figure 1C), whereas expression levels of CD5 and percentages of FoxP3$^+$ T$_{REG}$-cells among CD4$^+$ T-cells in dLNs were not altered (Figure 1D-E). However, the humoral immune response was altered in αDEC205-PG-protected animals, as judged by reduced IgG2a serum levels against PG (Figure

1F). This finding is in agreement with earlier reports on a correlation between the onset and severity of PGIA and PG-specific IgG2a auto-Ab titers [33,34].
In summary, αDEC205-PG-mediated protection against experimental arthritis is accompanied by a reduction in both the percentage of dLN-derived $CD4^+IL\text{-}17^+$ T-cells and serum levels of anti-PG IgG2a Abs.

DEC205 targeting transiently promotes PG-specific $FoxP3^+$ T_{REG}-cells

To address possible changes in FoxP3 expression in PG-specific $CD4^+$ T-cells upon αDEC205-

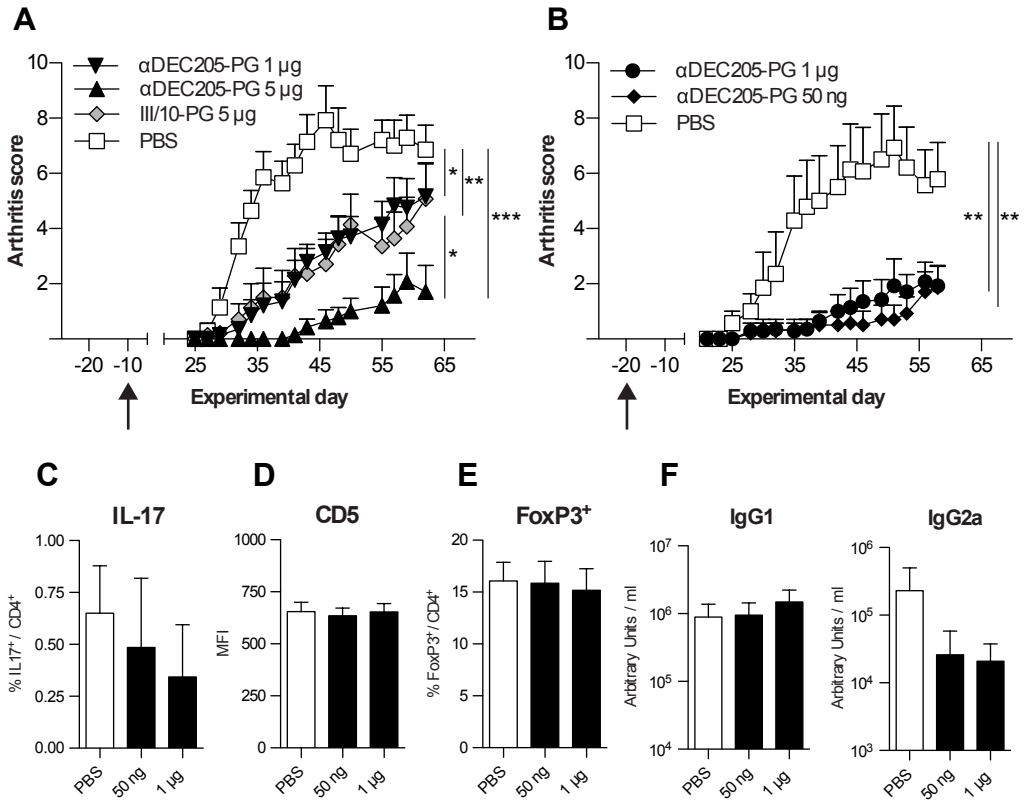

Figure 1: DEC205 targeting ameliorates clinical symptoms and delays onset of PGIA
Arthritis was induced with or without the pre-administration of fusion Abs (αDEC205-PG or III/10-PG) on either day -10 (A) or -20 (B-F). (A and B) Arthritis severity expressed as mean arthritis scores ± SEM of 6 mice per group. The area under the curve was used as measure of disease severity; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ indicate statistically significant differences of indicated vs. PBS-treated control animals (one-way ANOVA) or the 5 µg αDEC205-PG and the 5 µg III/10-PG group (Student's t-test). (C-E) dLNs of the paws were isolated on day 58 and the $CD4^+$ T-cell population was analyzed for the expression of IL-17 (C), CD5 (D) and FoxP3 (E) by flow-cytometry. (F) Serum obtained on day 58 was analyzed by ELISA for the presence of IgG1 and IgG2a Abs against PG. Mean values ± S.D. are shown in C-F.

PG-mediated activation, we analyzed proliferation and FoxP3 expression (Figure 2A) in CD90.2$^+$ PG-specific T-cells that had been transferred into WT and congenic CD90.1 WT recipients. As expected, αDEC205-PG administration induced significantly more proliferation of transferred PG-specific CD4$^+$ T-cells than the isotype-control Ab (Figure 2B). In addition, higher proportions of dividing PG-specific CD4$^+$ T-cells expressed FoxP3 (Figure 2C) and IL-10 (Figure 2D), as compared to the same cells in III/10-PG isotype control-treated animals. Fourteen days after treatment, the proliferation profiles of transferred CD4$^+$ T-cells in αDEC205-PG and isotype control-treated animals were similar to day four (Figure 2B vs. E), but the percentage of FoxP3$^+$ PG-specific CD4$^+$ T-cells did not differ anymore between the αDEC205-PG and III/10-PG treated groups (Figure 2C vs. F).
In summary, αDEC205-PG-treated mice showed increased percentages of PG-specific

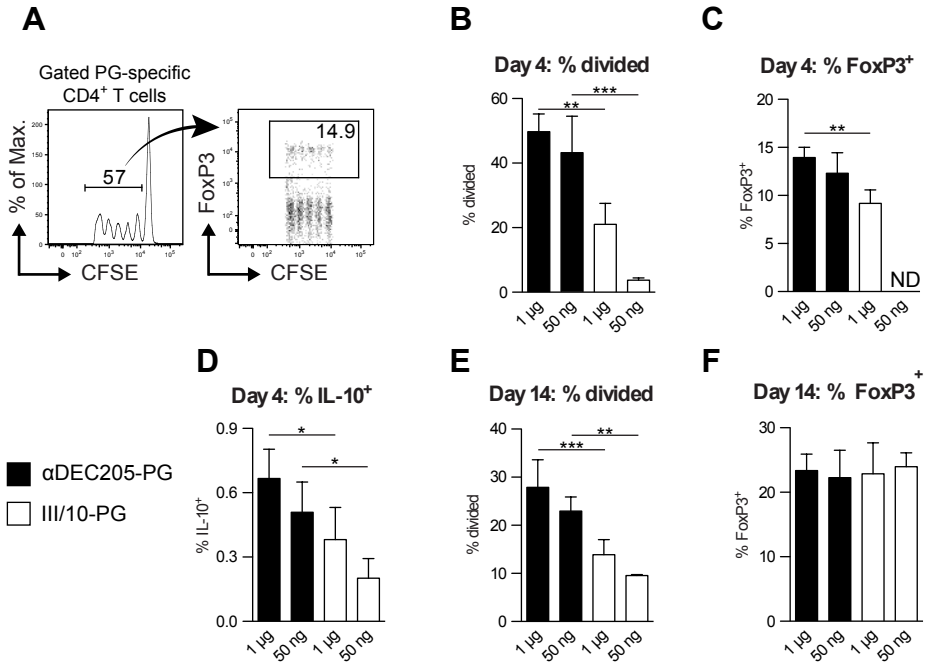

Figure 2: DEC205 targeting enhances promotion of FoxP3 expression in PG-TCR-specific CD4$^+$ T-cells after four, but not after 14 days.

PG-specific CFSE-labeled CD4$^+$CD90.2$^+$ T-cells were i.v transferred into WT (A-D) or CD90.1 congenic WT (E-F) recipient mice. The next day, mice were treated i.p. with 50 ng or 1 µg αDEC205-PG or isotype-control Ab. Spleens of recipient mice were analyzed after 4 (A-D) or 14 days (E-F). (A) Gating strategy to analyze the effect of mAb treatment on cell division (B and E) and percentage of FoxP3$^+$ PG-specific T-cells (C and F). ND = Not determined (amount of proliferated T-cells too low). (D) Percentage of IL-10$^+$ T-cells among the PG-specific CD4$^+$ T-cell population on day 4. (B-D) Mean ± S.D. of 3-5 animals per group. B-C and E-F: Similar data were obtained in an additional experiment. ** p<0.01, and *** p<0.001 indicate statistically significant differences between indicated groups. The Student's t-test was applied and only animals that received identical Ab doses were compared.

CD4⁺Foxp3⁺ T-cells at day four, but not day fourteen, as compared to isotype control-treated mice. This observation raised the possibility that additional tolerance mechanisms such as deletion and/or induction of anergy may contribute to the above observed αDEC205-PG-mediated protection against PGIA.

DEC205 targeting induces deletion/anergy in PG-specific CD4⁺ T-cells

To address the possibility that αDEC205-PG administration induces deletion and/or anergy in PG-specific CD4⁺ T-cells, we next tracked the proliferation and accumulation of CD90.2⁺ PG-specific CD4⁺ T-cell populations in congenic CD90.1 recipients that were treated with αDEC205-PG or control, and subsequently immunized with PG in adjuvant. Even though immunization induced robust proliferation of PG-specific CD4⁺ T-cells (Figure 3A and B),

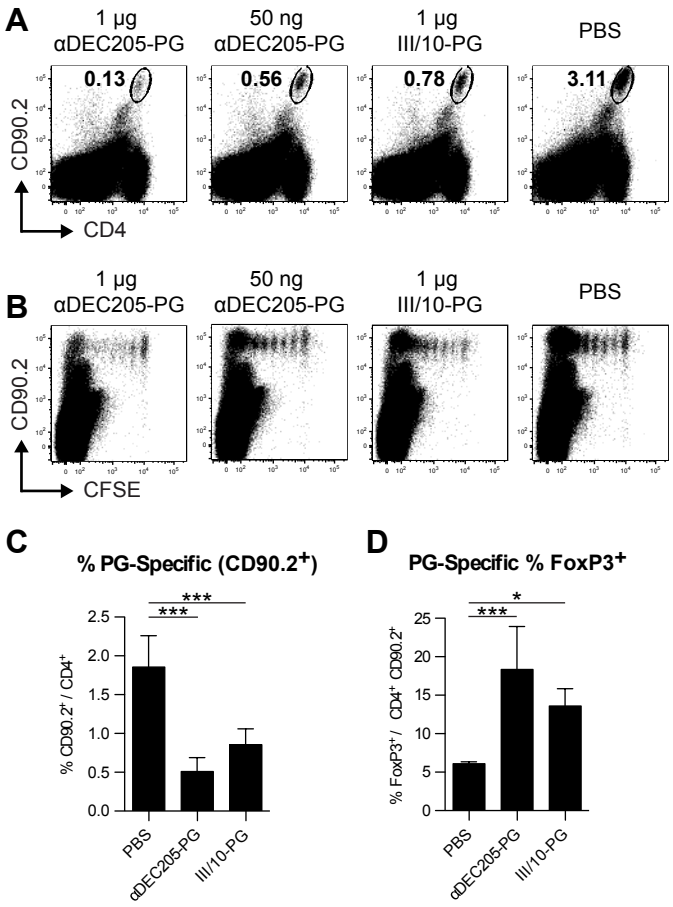

Figure 3: DEC205 targeting induces deletion/anergy in PG-specific CD4⁺ T cells.

PG-specific CFSE-labeled CD4⁺ CD90.2⁺ T-cells were i.v. transferred into CD90.1⁺ recipient mice (day 0) that were treated i.p. with the indicated amount (A and B) or 200 ng (C and D) αDEC205-PG, III/10-PG or PBS on day 1. The mice were subsequently immunized with PG/DDA on day 21, and spleens were analyzed on day 25 (A and B) or 31 (C and D). (A) Representative FACS-plots of CD4⁺CD90.2⁺ PG-specific T-cells within the endogenous CD4⁺ T-cell population. The percentage shown in the plots indicate the relative presence of CD90.2⁺-PG specific T cells among the CD4⁺ T cell population. (B) Representative FACS-plots of the proliferation profile of PG-specific CD4⁺ T-cells as determined by CFSE dilution. (A and B) Representative FACS-plots from 2-3 mice/group. (C) Relative presence of CD90.2⁺ PG-specific CD4⁺ T-cells within the total CD4⁺ population. (D) Relative presence of PG-specific CD4⁺FoxP3⁺ T_{REG}-cells among the transferred CD4⁺CD90.2⁺ PG-specific T-cell population. (C and D) Mean ± S.D. of n = 6 mice/group. * P<0.05, ** p<0.01, and *** p<0.001 indicate statistically significant differences between indicated groups. A one-way ANOVA was applied. B-D: Similar data were obtained from at least three independent experiments.

αDEC205-PG pretreatment resulted in a substantially reduced number of PG-specific $CD4^+$ T-cells in spleen (Figure 3A and C) and LNs (data not shown), as compared to control-treated animals. This indicated that αDEC205-PG treatment induced deletion of PG-specific $CD4^+$ T-cells and/or that PG-specific $CD4^+$ T-cells in the control-treated animals proliferated more vigorously compared to the ones in the αDEC205-PG-treated animals. The latter would imply that αDEC205-PG treatment anergized (part of the) PG-specific $CD4^+$ T-cells.
Importantly, we observed that the small population of residual PG-specific $CD4^+$ T-cells resulting from αDEC205-PG-mediated deletion/anergy induction, contained increased proportions of $FoxP3^+$ T_{REG}-cells (Figure 3D), perhaps due to preferential deletion and/or reduced proliferative expansion of PG-specific $CD4^+Foxp3^-$ T-cells, although proliferative expansion of preformed $Foxp3^+$ T_{REG}-cells or *de novo* generation of T_{REG}-cells cannot be formally excluded.

αDEC205-PG administration compromises germinal center formation

Since αDEC205-PG-treated mice developed lower PG-specific Ab-titers after immunization (Figure 1F), we investigated whether this reduced humoral immune response was caused by T-cell alterations that subsequently negatively affected germinal center (GC) formation. To this end, GC development (as measured by the percentage of $FAS^+GL\text{-}7^+$ GC B-cells) in the spleen and peritoneal cavity-draining mediastinal LNs ([35], MedLN) was analyzed 10 days after immunization with PG. At this time-point, GCs start to develop, whereas clinical disease does not.
We found that GC formation was indeed compromised at day 10 in αDEC205-PG-protected animals. Compared to control mice, lower percentages of B-cells were found in GCs (Figure 4A-B and 4H), and fewer PG-specific T-cells exhibited a follicular helper T-cell (T_{FH}) phenotype (Figure 4C-F). Consistently, PG-specific Ab-titers were reduced (Figure 4G). Since Ag-specific T_{FH}-cells are crucial in normal GC development [36,37] we next investigated whether αDEC205-PG-mediated reduction of GCs resulted from dominant tolerance mechanisms (as suggested by elevated levels of $FoxP3^+$ T_{REG} cells; Figure 3D) or from the reduced availability of PG-specific T-cells (Figure 3C) that develop into GC-supporting T_{FH}-cells. Therefore we adoptively transferred either PG-specific $FoxP3^+$ T_{REG}-cell enriched ($CD4^+CD25^+$) T-cells or 'effector T' (T_{EFF})-cell enriched ($CD4^+CD25^-$) T-cell populations (Figure 5A) into mice that were subsequently treated with αDEC205-PG or PBS and immunized with whole PG to induce GC formation.
αDEC205-PG treatment reduced GC development compared to PBS-treated control mice in both recipients of T_{REG} and T_{EFF}-cells (Figure 5B). However, reduced GC formation did not correlate with the number of $FoxP3^+$ T_{REG}-cells (Figure 5D, right) in αDEC205-PG-treated or PBS-treated T_{REG}-cell recipients. This observation suggests that PG-specific $FoxP3^+$ T_{REG}-cells were not likely responsible for the differences in GC development, indicating that the deletion of PG-specific T_{EFF}-cells (derived from both recipients and the small T_{EFF}-cell population within transferred enriched T_{REG}-cells) instead may impinge on GC development in this experimental system. This notion is further supported by the finding that the reduced availability of PG-specific T-cells in αDEC205-PG-treated T_{EFF}-cell recipients (Figure 5C) correlated with smaller numbers of PG-specific T_{FH}-cells (Figure 5E) and smaller GCs (Figure

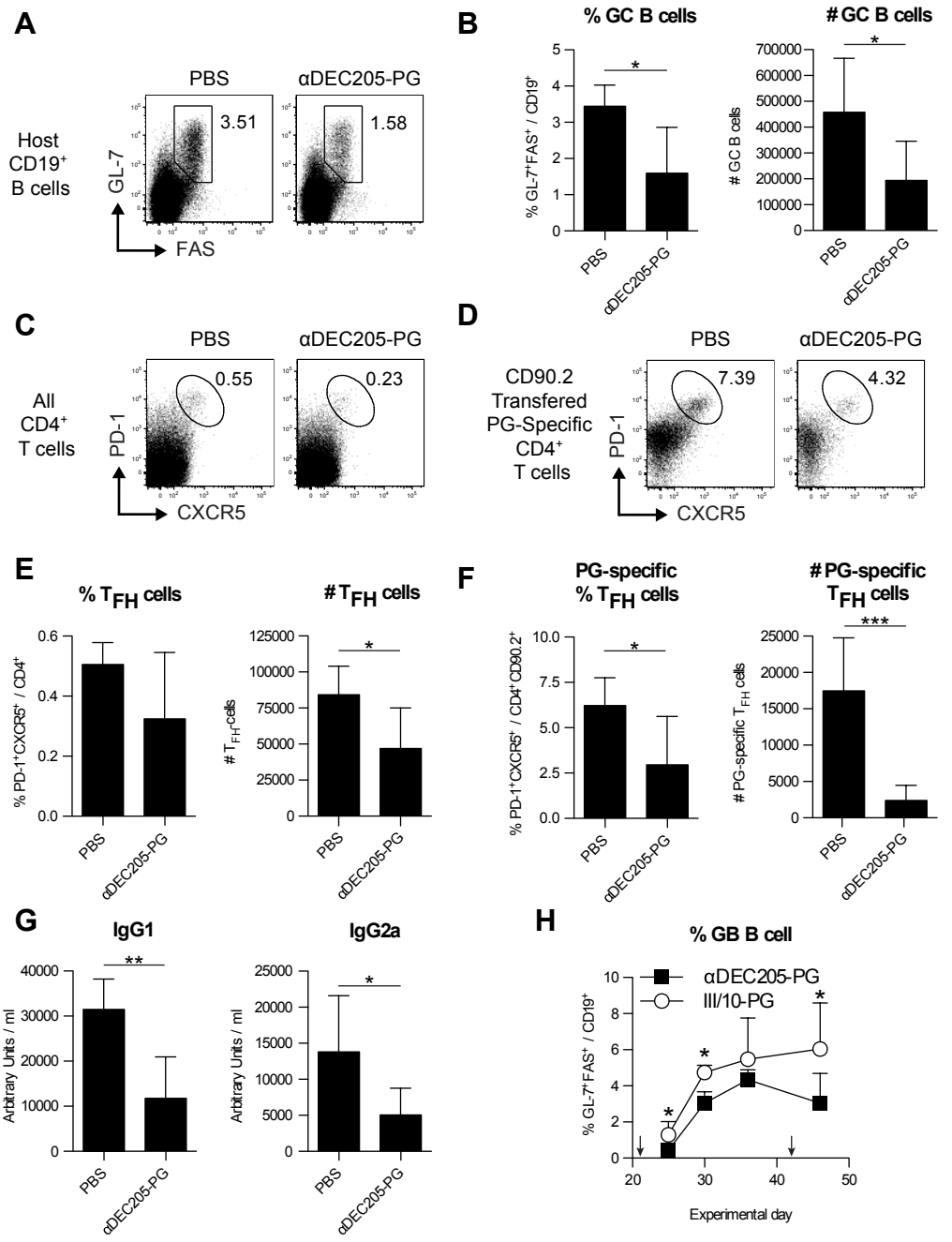

Figure 4: αDEC205-PG administration compromises GC formation.

PG-specific CD4+CD90.2+ T-cells were i.v. transferred into CD90.1+ congenic recipient mice (day 0) that were treated (day 1) with αDEC205-PG or PBS. The mice were immunized (day 21) with PG/DDA and sacrificed (day 31 for A-F, or at indicated time points in H) in order to analyze PD-1+CXCR-5+ T_{FH}-cells and GL-7+FAS+ B-cells within GCs (GC B-cells) in spleen (A-F) and MedLN (H). (A) Representative FACS-plots for the identification of GC

5B) in a system where overall very low levels of PG-specific FoxP3$^+$ T_{REG}-cells were present (Figure 5D).

Discussion

Recombinant αDEC205 antibodies fused to disease-relevant auto-antigens have been developed and used successfully for tolerogenic protection in autoimmune disease models, including autoimmune diabetes [23,25] and encephalomyelitis [18,22,24]. In this study, we demonstrated that administration of a single dose recombinant αDEC205 antibody fused to the cartilage-derived immunodominant PG_{70-84} efficiently ameliorated the severity of PG-induced arthritis. PGIA is an experimental arthritis model substantially different from previously employed models in that disease is induced by immunization with a complex whole protein and the immune effector cells contribute to autoimmune destruction.

Based on the results reported in previous studies, potential mechanisms of αDEC205-PG_{70-84}-induced suppression of PGIA were explored in more detail. Earlier it was described that DEC205 recombinant fusion Abs could promote autoimmune-protection via deletion of Ag-specific T-cells [17,38], CD5-mediated T-cell unresponsiveness [18], induction of T-cell anergy and reduced IL17 secretion [22], or FoxP3$^+$ T_{REG}-cell induction as reported for pancreatic β-cell-specific T-cells [21], and non-self Ags [19,20,39,40]. Our findings demonstrated that αDEC205-PG treatment altered the PG-specific CD4$^+$ T-cell population in a complex way: PG-specific CD4$^+$ T-cells proliferated extensively early (i.e. 4 days) after αDEC205-PG administration and the percentage of FoxP3$^+$ T_{REG}-cells was significantly enhanced compared to controls at this time point. However, this difference was not observed at a later time point, possibly because the proliferating T-cells became anergic and/or were deleted. Immunization of αDEC205-PG-treated animals with PG in adjuvant supported this notion, since αDEC205-PG-treated animals contained lower numbers of PG-specific CD4$^+$ T-cells after the challenge compared to control animals. Importantly, the small amount of CD4$^+$ T-cells detected after immunization was greatly enriched in FoxP3$^+$ T_{REG}-cells, which suggests that the FoxP3$^-$ cells were deleted more efficiently. Together, these data clearly indicate that DEC205 targeting induced T-cell deletion and/or anergy, while the relative amounts of FoxP3$^+$ T_{REG}-cells were increased at the same time. However, the increase in Foxp3$^+$ T_{REG}-cells appeared only transient, suggesting a minor role in protection. Future studies are warranted to elucidate the relative contributions of recessive and dominant tolerance mechanisms in DEC205-mediated protection from PGIA.

B-cells, and (B; left) percentages and (B; right) absolute numbers of GC B-cells. (C-D) Representative FACS-plots for the identification of (C) T_{FH}-cells, or (D) transferred PG-specific CD4$^+$CD90.2$^+$ T-cells, and (E) percentage (left) and absolute numbers (right) of T_{FH}-cells. (F) Percentages (left) and absolute numbers (right) of PG-specific CD4$^+$CD90.2$^+$ T_{FH}-cells. (G) Anti-PG serum titers of both the IgG1 (left) and IgG2a (right) isotype on day 31. (H) Development of GCs (as determined by the relative percentage of GC B-cells) in the MedLN of αDEC205-PG and III/10-isotype control-treated mice. The animals were immunized on day 21 and 42 (indicated by arrows). (A-F) n=6 mice per group. A-G: Similar data were obtained from at least 2 (1 for G) independent experiments. * P<0.05, ** p<0.01, and *** p<0.001 indicate statistically significant differences between indicated groups. The Student's t-test was applied.

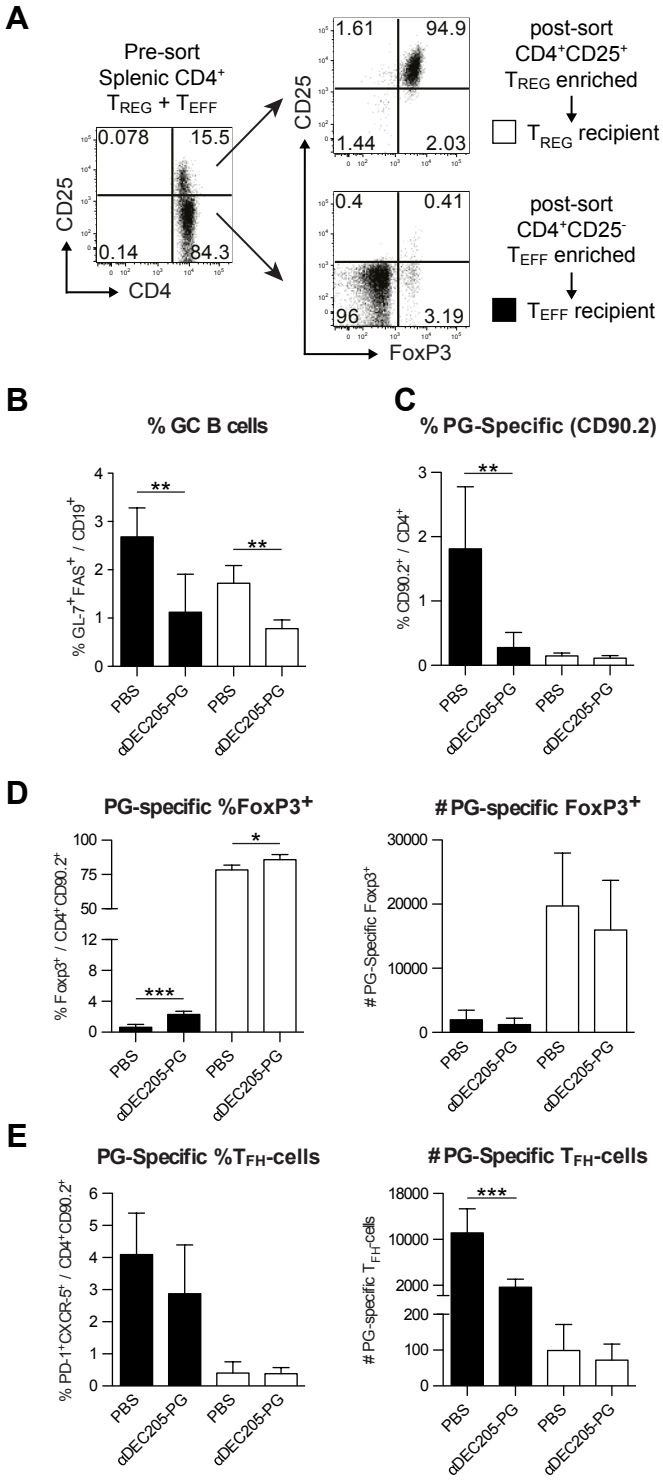

Figure 5: αDEC205-PG treatment affects GC formation by deletion/ anergy of T_{EFF}-cells.

PG-specific CD90.2⁺-enriched CD4⁺ CD25⁻ T-cells ('effector T-cells'; T_{eff}; 1.7×10^6; black bars) or CD4⁺CD25⁺ T-cells ('Treg'; 0.3×10^6; white bars) were i.v. transferred into CD90.1⁺ congenic WT recipient mice (day 0) that were treated (day 1) with αDEC205-PG or PBS. The cohorts of mice were immunized (day 21) with PG/DDA and spleens were analyzed at day 32. (A) FACS-plots of intracellular FoxP3 staining of enriched and FACS-sorted T_{REG} (top) and T_{EFF} (bottom) cell populations used for adoptive transfer. (B) GC formation and (C) percentage of transferred CD90.2⁺ cells within the total CD4⁺ T-cell population. (D and E) Percentage (left) and absolute numbers (right) of FoxP3⁺ T_{REG}-cells (D) and CD4⁺ T-cells with the T_{FH}-cell phenotype (E) among the transferred PG-specific CD4⁺CD90.2⁺ T-cell population. (B-E) Mean ± S.D. values of n=6 (T_{EFF}-cells) or n=5 (T_{REG}-cells) mice per group. * $P<0.05$, ** $p<0.01$, and *** $p<0.001$ indicate statistically significant differences between indicated groups. The Student's t-test was applied.

Subsequent studies showed that the αDEC205-PG-mediated changes in transferred PG-specific $CD4^+$ T-cells compromised the formation of GCs and PG-specific Abs important for the induction of PGIA. Smaller GCs and lower Ab-titers were also observed when animals were treated without the transfer of $CD4^+$ T-cells (data not shown). Experiments in which PG-specific T_{REG}-cells or T_{EFF}-cells were transferred showed that this effect was mainly caused by a reduction in potentially pathogenic PG-specific $CD4^+$ T-cells as compared to control-treated animals, rather than by increased numbers of PG-specific T_{REG}-cells. However, we cannot formally exclude the possibility that T_{REG}-cells play a role in disease reduction in αDEC205-PG-treated animals.

Our data support a model in which PG_{70-84}-specific $CD4^+$ T-cells are anergized/deleted and therefore, cannot provide the necessary support to GC B-cells as differentiated T_{FH}-cells. In line with this interpretation, Murad et al. who had molecularly manipulated the immunodominant T-cell epitopes present in PG found that immunization without PG_{70-84} significantly diminished the subsequent immune response compared to WT Ag [26], further corroborating that the absence of a single immunodominant T-cell specificity has the potential to compromise the entire immune response to a protein Ag. The fact that the human PG used for disease induction is highly homologous to the murine PG may be an important aspect in this, since it presumably limits the number of high affinity T-cell epitopes present in human PG.

A significant number of RA patients reacts to PG-derived T-cell epitopes [41-44], but the relative contribution of these T-cell epitopes to the overall progression of disease is unknown. Detailed knowledge on relevant self-Ags will be critical for future human translation of tolerogenic vaccination against RA. Therapies that combine an immunomodulatory treatment with a DEC205-targeted self-Ag would bring such tolerogenic vaccination within reach [23,45].

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Competing Interests

The authors declare no conflict of interest.

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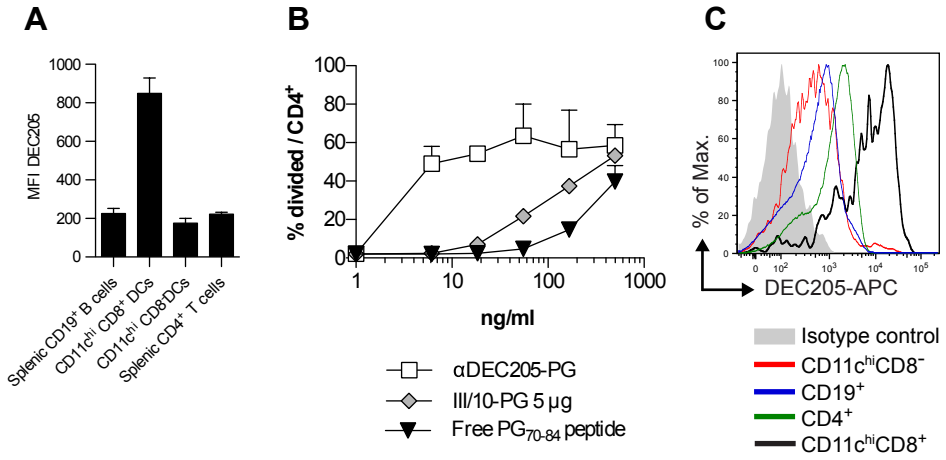
Supplementary Figure S1:



Supplementary Figure S1: αDEC205 mAb binds to splenic CD8⁺ DCs *in vitro* and *in vivo* and Ag delivered via DEC205 fusion Abs is efficiently presented to CD4⁺ T-cells.

(A) Presence of DEC205 on various splenocytes as determined by surface staining and flow-cytometric analysis. The average mean fluorescence intensity (MFI) ± S.D. of three naïve BALB/c mice is shown. (B) Single cell suspensions were obtained from spleen using Collagenase D-treatment (Roche). CD11c⁺ dendritic cells (DCs) were subsequently isolated by FACS-sorting. After incubation with indicated concentrations of αDEC205-PG, III/10-PG isotype control or an equivalent amount of PG-peptide (500 ng/ml recombinant Ab corresponds to ~10 ng/ml free peptide) for three hours (37°C), DCs were washed extensively and cultured with 1 x 10^5 FACS-purified CFSE-labeled PG-specific CD4⁺ T-cells for four days. Average ± S.D. of technical duplicates. Similar data were obtained from two independent experiments. (C) 5 µg of APC-conjugated anti-DEC205 (clone NLDC-145) or respective isotype control Ab was administered i.p. in 200 µl PBS. Spleen cells were isolated after 2½h, and stained for CD19, CD4, CD11c and CD8 to identify the indicated cell-types. Representative data of three anti-DEC205-APC and three isotype control Ab-treated mice.

3

Peritoneal cavity B-1a cells promote peripheral CD4$^+$ T-cell activation

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Summary

Innate-like murine B-1a cells are well known for their ability to secrete natural IgM. Their non-antibody mediated functions, including antigen presentation to $CD4^+$ T cells, are less well explored. Using combined adoptive transfer experiments with peptide-pulsed peritoneal cavity-derived B-1a cells and CFSE-labeled T cells, we show that B-1a cells present antigen to $CD4^+$ T cells from the periphery *in vivo*. *In vitro* characterization, using co-cultures in which B-1a- or splenic B cells presented whole OVA protein to OVA-specific Tg T cells, shows that B-1a cells differentially promote intracellular cytokine-expressing T cells. Peritoneal cavity-derived B-1a cells increase the percentage of IL-10-producing T cells along with IL-4- and IFN-γ-producing $CD4^+$ T cells. These data suggests that B cells in the peritoneal cavity have the potential to influence peripheral immune responses without the necessity to migrate out of this location. This, to our knowledge previously undescribed, immunological pathway potentially plays a role in the presentation of gut microbiota-derived antigens to peripheral T cells.

Introduction

B cells are traditionally known as precursors of antibody-producing cells. Nevertheless, it is well accepted that their non-antibody mediated functions, which include Ag-presentation to $CD4^+$ T cells and the secretion of cytokines, play important roles during both active immune responses and maintenance of immunological homeostasis [1-4].
Unstimulated, resting B cells are reported to preferentially expand $Foxp3^+$ regulatory T cells [5,6]. Likewise, specific subsets of IL-10-producing regulatory B cells are described to be involved in the maintenance or induction of IL-10-producing T_R1 regulatory T cells [7,8]. However, there are few reports on possible specialized roles that other B-cell subsets might exert when acting as APCs.
Murine B-1 cells are innate-like B cells that differ from B-2 cells (follicular B cells and marginal zone B cells) as reviewed by Baumgarth [9], Suzuki et al. [10] and Sindhava et al. [11]. Briefly, B-1 cells are well known for their ability to produce natural antibodies, i.e. polyreactive IgM present in pre-immune mice involved in both the early defense against pathogens and in the maintenance of homeostasis. B-1 cells can furthermore be distinguished from B-2 cells by their abundant presence in the peritoneal cavity (PerC) and the differential expression of cytokines. Especially the predominant secretion of IL-10 is well known [12].
B-1 cells are found in relative large amounts in the PerC where they are characterized by a specific combination of surface markers including CD11b. Most of the B-1 cells in the PerC of BALB/c mice are B-1a cells characterized by the expression of CD5, while a smaller population of $CD5^-$ cells are termed B-1b cells. The absolute number of B-1 cells in the spleen and the peritoneal cavity are thought to be comparable, however the splenic B-1 population is much harder to distinguish from B-2 cells due to the lack of CD11b expression. Other B-1 specific surface markers such as low expression of B220, intermediate expression of CD5 or expression of CD43 can also be markers for B-cell activation, anergy or of plasma blasts [9,13-15], which makes the identification of splenic B-1a cells challenging. The relationship between B-1a cells from the PerC and peripheral (splenic) B-1a cells is not completely clear. A large part of the splenic B-1a-cell population possesses significant differences in B-cell receptor repertoires, gene expression patterns and IgM-secreting properties compared with that of PerC B-1a cells [16-18]. On the other hand, at least some of these differences are shown to be due to influences of the microenvironment in these organs, indicating that differences between these B-1a cell subsets might be due to a different activation status and not due to intrinsic cell or origin differences [18-20]. On top of that, it has been shown that PerC B-1a cells are able to migrate to the spleen after LPS-triggering [18,21-23] and that the spleen is an important organ for the generation or maintenance of PerC B-1a cells as shown by reduced numbers of this B-cell subset in splenectomized mice [24].
Here we compare the Ag presentation of traditional splenic B cells with PerC B-1a cells *in vitro.* Analysis shows that PerC B-1a cells, compared with splenic B cells, increase the percentage of IL-10-, IFN-γ- and IL-4-producing T cells, as determined via intracellular cytokine staining. Even though our data show that PerC B-1a cells only migrate in small numbers from the PerC towards the periphery, we demonstrate that PerC B-1a cells interact with $CD4^+$ T cells from the periphery since peripheral T cells visit the PerC and may engage with PerC B-1a cells at this location. To our knowledge, this pathway has not been described before.

Results

PerC B cells increase IL-10-producing CD4⁺ T cells in vitro

The PerC contains a unique B-cell population that contains a high percentage of B-1 cells. The exact ratios between the various B(-1) cell subsets in this organ, however, varies between mice breeds [25]. Around 60-70% of the PerC CD19⁺ B lymphocytes in BALB/c mice possess the B220^{low} CD11b⁺ CD5⁺ CD23⁻ CD43⁺ IgM^{hi} IgD^{low} phenotype characteristic for B-1a cells (Figure 1A/B). B cells in the spleen can be subdivided into follicular B cells and marginal zone B cells based on their expression of CD21/35 and CD23 while B-1 cells, that are included in the B220^{low} CD5⁺ population, constitute only a minor fraction (1~5%) of the splenic B-cell population (Figure 1C).

To determine if the above described PerC B-cell population (Figure 1A) differentially increases CD4⁺ T-cell subsets compared with the splenic B-cell population, we first analyzed both the Foxp3- and IL-10-expression of T cells when both B-cell populations were used as APCs. To that matter, CFSE-labeled OVA-specific T cells were activated with either naïve PerC or splenic B cells in the presence OVA protein.

The relative expression of Foxp3 in the T cells that had been primed by B cells from the PerC tended to be lower compared with that of T cells primed by splenic B cells (Figure 2A/B). However, when PerC B cells were used as APC, a significantly higher percentage of T cells produced IL-10 compared with cultures in which splenic B cells had been used as APC (Figure 2C /2D). In order to determine whether this effect was due to the presence of B-cell-secreted IL-10, we selectively neutralized this cytokine using a neutralizing monoclonal antibody against

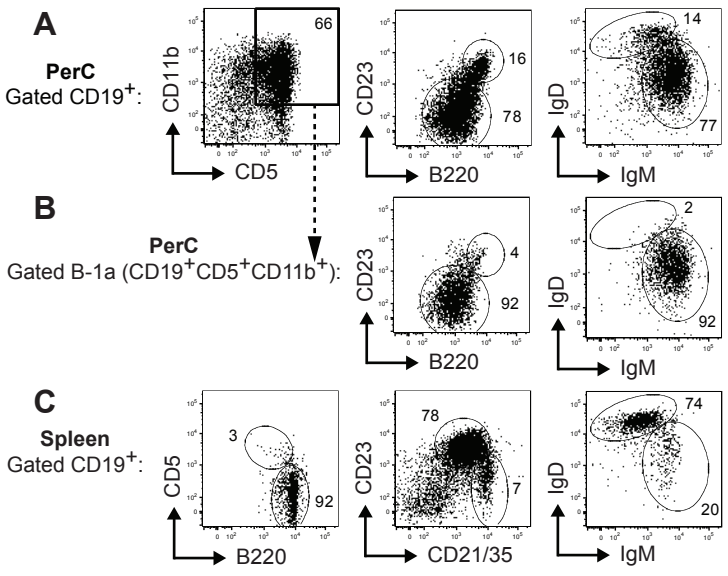

Figure 1, The PerC of BALB/c mice contains a high percentage of B-1a cells. (A) Peritoneal cavity washout cells were stained for CD19 and subsequently for CD11b and CD5, CD23 and B220 or IgD and IgM. Shown are the various B-cell populations that can be differentiated after gating on CD19⁺ cells. (B) Peritoneal cavity washout cells were stained as in (A), and the expression of CD23 and B220 or IgD and IgM is shown after gating on CD19⁺ CD5⁺ CD11b⁺ cells. (C) Splenic B cells were stained with CD19 and subsequently with B220 and CD5, CD23 and CD21/35 or IgD and IgM. Values next to gates indicate the percentage of cells within gates. Flow cytometry plots are from one experiment representative of at least three independent experiments performed.

IL-10. A reduction in the amount of T cells positive for intracellular IL-10 was found, but the selective enrichment of the cytokine-production cells was not completely blocked (Figure 2E).

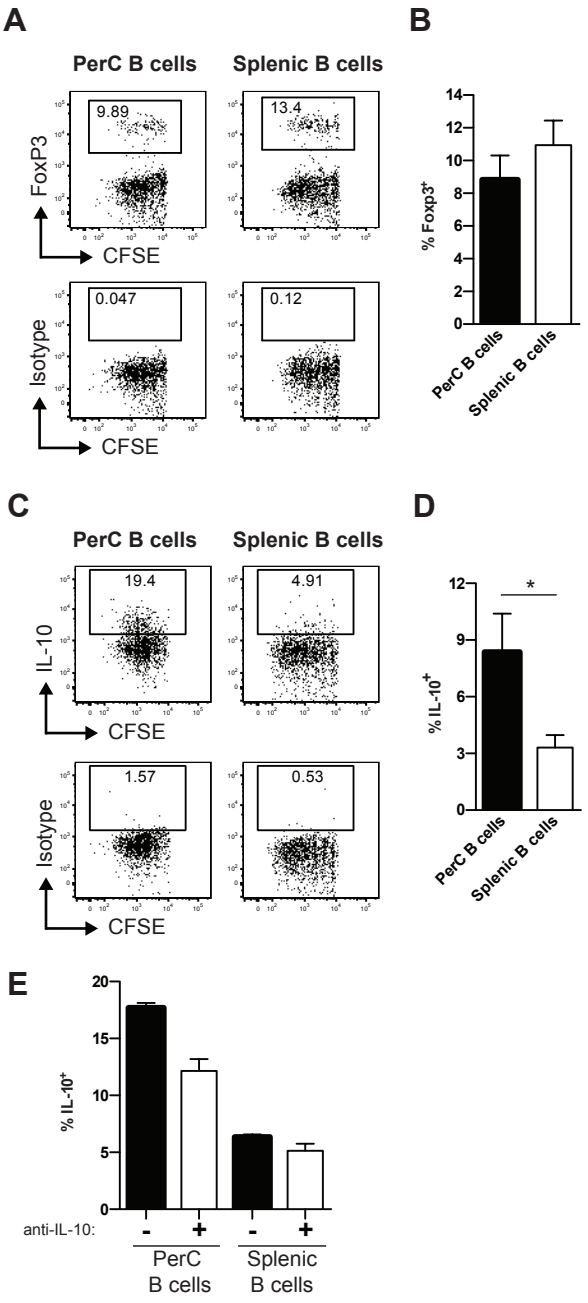

Figure 2. PerC B cells enrich for IL-10-producing CD4+ T cells.

B cells were isolated from the peritoneal cavity and the spleen using CD19-MicroBeads, and co-cultured for 96 h with CFSE-labeled CD4+ OVA-specific T cells in the presence of whole OVA protein. Intracellular Foxp3 in the proliferated T cells was stained after co-culture and IL-10 was stained intracellularly after stimulation. (A) Flow cytometry plots showing Foxp3-stainings are from one experiment representative of three independent experiments performed. (B) The percentage of Foxp3-expressing T cells (gating as in (A)) after co-culture with both B-cell subsets is compared. Data are shown as mean + SEM of 3 samples pooled from three independent experiments. (C) Flow cytometry plots showing IL-10-stainings are from one experiment representative for six independent experiments performed. (D) The percentage of IL-10-expressing T cells (gating as in (C)) after co-culture with both B-cell subsets is compared. Data are shown as mean + SEM of 6 samples from six independent experiments. *p<0.05, Student's t-test. (E) The percentage of IL-10-expressing T cells (gating as in (C)) in co-cultures with or without the presence of neutralizing mAb against IL-10. Data are shown as mean ± SD of 2 samples and are from one experiment representative of two independent experiments performed.

PerC B-1a cells differentially increase CD4$^+$ T-cell subsets in vitro compared with splenic B cells

We sought to determine if the B-1a cells present in the PerC B-cell population were responsible for the enrichment of IL-10-producing T cells demonstrated in Figure 2. CD19$^+$ CD11b$^+$ CD5$^+$ B-1a cells were FACS-sorted from the PerC B-cell population and these cells were used as APCs in co-cultures in a similar way as described above. To further characterize T-cell subsets by their cytokine-profile, we analyzed IL-10, IFN-γ, IL-17 and IL-4 production by T cells. When FACS-sorted B-1a cells were used as APCs, the enrichment of IL-10-producing T cells was similar compared with that of cultures where the whole PerC B-cell population were used as APC (Figure 3A and 3B). This indicates that indeed PerC B-1a cells have the potential to increase the percentage of IL-10-producing T cells. Furthermore, more IL-4-producing cells were observed, coinciding with a small increase in the percentage of IFN-γ-producing cells (Figure 3A and 3B). No differences in the amount of T cells capable of expressing IL-17 were found, mainly because almost none of the T cells were producing this cytokine under these conditions.

Since B-1a cells are present in both the PerC and the spleen, we performed co-cultures using splenic B-1a cells to investigate if this B-cell subset enriches T-cell subsets in a similar way as PerC B-1a cells. The splenic CD19$^+$ B220^{low} CD5$^+$ B cell population that contains splenic B-1a cells, was FACS-sorted and splenic CD19$^+$ B220^{hi} CD5$^-$ B cells served as a control. The data indicate that the B-1a-enriched splenic B-cell fraction tended to enrich IL-10-producing T cells as well, although the increase was not to the same extend as seen by PerC B-1a cells. Splenic B-1a cells furthermore seemed to enrich IL-17-producing T cells (Figure 3C).

In summary, our data show that PerC B-1a cells, compared with splenic B cells, increase the percentage of IL-10-, IFN-γ- and IL-4-producing T cells, as determined by intracellular cytokine staining. Especially the percentage of IL-10 and IL-4 positive cells was increased (~3.2-fold and ~5.3-fold respectively vs. ~1.6 for IFN-γ). Interestingly, when splenic B-1a cells are used, IL-17-producing T cells are enriched.

PerC B-1a cells create different cytokine environments after T cell-B cell communication

The cytokines that are secreted into the supernatant after T cell–B cell communication were measured and are compared in Figure 4. Significant higher amounts of IL-10 were found when PerC B cells or FACS-sorted B-1a cells were compared with splenic B cell-cultures. Compared with splenic B cells, IL-6 levels were significantly higher in PerC B (and PerC B-1a) cell cultures. IL-2 levels were significantly higher when whole PerC B cells were used, whereas they were only slightly enriched when FACS-sorted B-1a cultures were compared with splenic B cell cultures. IFN-γ and IL-17 levels were roughly equal in all co-cultures performed.

PerC B cells or splenic B cells in the presence of OVA protein (without T cells), produced 6 fold lower levels of IL-10 while IFN-γ, IL-17a and IL-2 became undetectable. IL-6, on the other hand, was still present (Figure 4). This finding indicates that most of the cytokines detected in the supernatant of the co-cultures resulted from communication between T and B cells.

In summary, we detected increased levels of IL-6 and IL-10 when FACS-sorted PerC B-1a cells presented Ag to Ag-specific T cells whereas Ag-presentation by splenic B cells resulted in a cytokine-environment that is characterized by the virtual absence of these cytokines.

PerC- B-1a cells are not exclusively restricted to the PerC

The above presented data show that PerC B-1a cells can differentially activate CD4+ T cells. However, physiologically, the PerC contains relatively few CD4+ T cells ([26] and data not shown). We therefore addressed whether PerC B-1a cells can migrate towards the periphery to communicate with peripheral T cells. For this purpose, we FACS-sorted PerC B-1a cells, labeled them with the red fluorescent dye PKH-26 and adoptively transferred them i.p. to acceptor mice that were s.c. immunized the next day. Four days later, the mice were analyzed for the presence of the transferred PKH-26+ cells. Flow cytometric analysis in combination with viable cells counts of the draining lymph nodes, as well peripheral lymph nodes, the spleen and the blood showed that most of the transferred B cells remained in the PerC (Figure 5B). However, a small population of transferred cells was consistently detected in the periphery

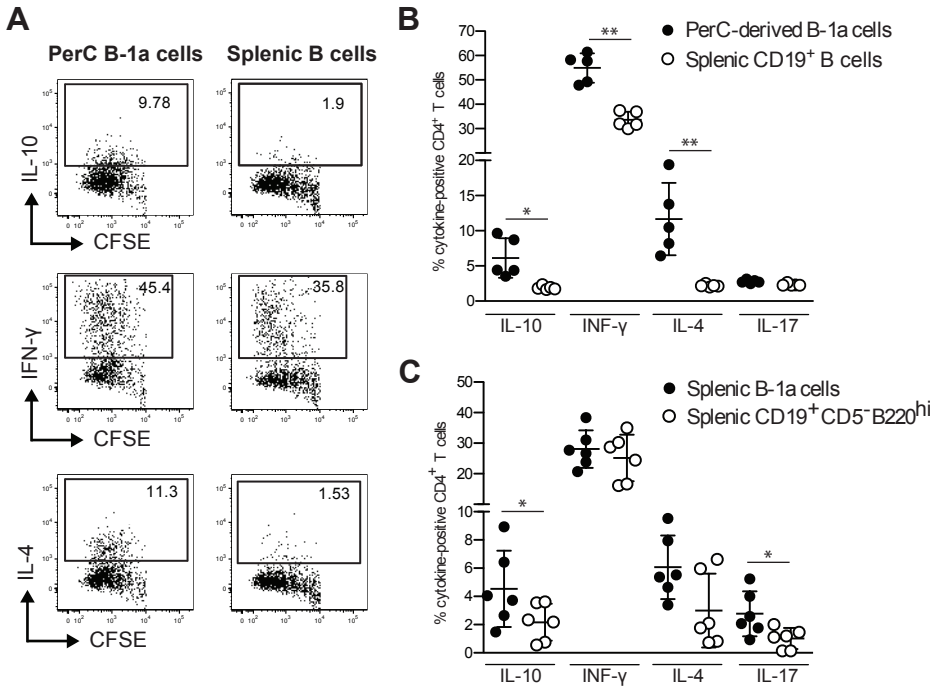

Figure 3. Compared with splenic B cells, PerC B-1a cells differentially enrich T-cell subsets.

CD19+ CD5+ CD11b+ B-1a cells were FACS-sorted from the PerC and compared with splenic B cells in a co-culture that was performed in a similar way as described in the legend of figure 2. Cytokines in the proliferated T cells were stained intracellularly after restimulation. (A) Flow cytometry plots showing gating strategies are representative of two independent experiments. (B) The percentage of cytokine-expressing T cells after co-culture with B-cell subsets from the PerC and the spleen is compared. Gating was performed as in (A). Data are shown as mean ± SD and are pooled from two independent experiments with technical duplicate or triplicate measurements. (C) The percentage of cytokine-expressing T cells after co-culture with the indicated splenic B-cell subsets is compared. Data are shown as mean ± SD and are pooled from three independent experiments with duplicate measurements. *p <0.05; **p<0.01; Student's t-test).

(Figure 5B). Migration of PerC transferred B-1a cells towards the periphery was associated with the loss of CD11b (Figure 5C), which is in agreement with LPS-induced B-1a migration experiments performed by others [18,22]. The expression of CD5 was stable after migration (data not shown).
In conclusion we found that B-1a cells preferentially remain in the PerC, but that they are not exclusively restricted to this location, since small amounts of PerC B-1a cells were shown to migrate to the periphery under our experimental conditions.

PerC B-1a cells present Ag to CD4⁺ T cells from the periphery in vivo

The finding that PerC B-1a cells migrated towards the periphery in small numbers only, questioned the likelihood that these cells migrate to the periphery to subsequently present Ag to T cells. Therefore, the capacity of PerC B-1a cells to present Ag to peripheral T cells *in vivo* was further studied. Isolated PerC B-1a cells were *ex vivo* pulsed with $OVA_{323-339}$ peptide and transferred to the PerC of acceptor-animals via the i.p. route. CFSE-labeled OVA-specific (KJ1-26⁺) CD4⁺ T cells were transferred to the periphery via the i.v. route on the same day. The animals were sacrificed 4 days later and analyzed for the presence of proliferating transferred T cells in various organs. Proliferating KJ1-26⁺ T cells were detected mainly in the PerC, in very small amounts in the mediastinal LNs, but not in the spleen or in other peripheral LNs (Figure 6A). This finding strongly suggests that i.v.-transferred CD4⁺ T cells from the periphery entered the PerC to engage with Ag-presenting PerC B-1a cells. In a follow-up experiment we transferred T cells via the i.p. route to the PerC as well, to allow further analysis of the *in vivo*-activated T cells that were otherwise present in small amounts. I.p.-transferred T cells that were *in vivo*-activated by PerC B-1a cells were not restricted to the PerC but instead are were located in the spleen (6B upper panel), mediastinal lymph nodes (6B lower panel) and other peripheral lymph nodes including the brachial, axillar, mesenteric and lumbar lymph nodes (data not shown). Using this model, we were not able to detect differences in T-cell

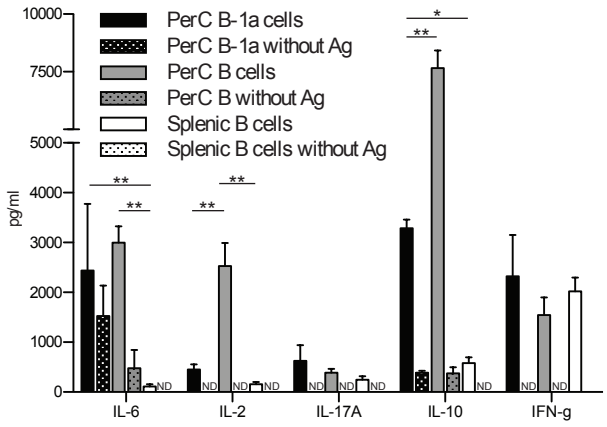

Figure 4. PerC B cells, B-1a cells and splenic B cells create differential cytokine environments after T cell–B cell communication. PerC CD5⁺ CD11b⁺ B-1a cells and total PerC B cells were compared with splenic B cells in co-cultures with T cells (as described in figure 2). The concentrations of the indicated cytokines were determined using a multiplex assay. The values are presented as the mean + SEM of 6 (PerC B-1a) - 20 (PerC and splenic B cells) samples pooled from 2 - 6 separate experiments. Statistical significant was calculated using a One-way ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$, **$p < 0.01$. ND: not detected.

cytokine profiles after *ex vivo* stimulation (Figure 6C) in a similar way as we showed earlier in figure 3. Nevertheless, PerC B-1a-activated T cells found in the spleen proliferated more vigorously compared to the ones activated by splenic control B cells (Figure 6D).

Discussion

Ag-presentation by B cells has long been disregarded, but several papers unambiguously demonstrated the important contribution of Ag-presentation by B cells for effective immune-responses [2,4,27].

However, few papers describe the Ag presenting capacity of specialized B-cell subsets. In the current study we describe how B-1a cells from the PerC enrich IL-10, IL-4 and IFN-γ-producing T cells when acting as APC *in vitro*. In a similar study, Zhong et al. [28] compared the APC abilities of PerC B-1a and splenic B cells as well and focused on Foxp3+ regulatory T-cell differentiation. They found that cultures in which PerC B-1a cells were used as APCs contained fewer Foxp3+ regulatory T cells than cultures where splenic B-2 cells were used. This was also seen in our experiments (Figure 2A/B).

Interestingly, the same paper describes that B-1a preferentially promoted T_H1 and T_H17 cell differentiation (measured via intracellular cytokine-staining of IFN-γ and IL-17 respectively) [28]. Although we did find an increase in the numbers of IFN-γ-producing T cells in our

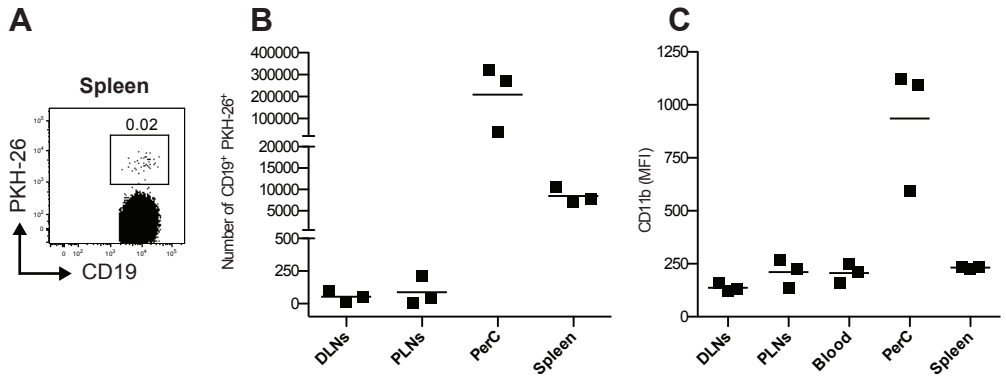

Figure 5. PerC B-1a cells are not exclusively restricted to the PerC.

CD19+ CD5+ CD11b+ B-1a cells were FACS-sorted from the PerC, labeled with the fluorescent dye PKH-26 and transferred to the PerC of recipient mice that were subsequently locally s.c. immunized with OVA and the adjuvant dimethyldioctadecylammonium bromide. After 96 h, the mice were sacrificed and the presence of transferred B-1a cells in the indicated organs was analyzed by flow cytometry. (A) Flow cytometry plot of detected transferred B-1a cells in the spleen of recipient mice. Plot is representative of two individual experiments performed. (B) Number of viable i.p. transferred B-1a cells in indicated organs. DLNs: draining (axillar) lymph nodes, PLNs: peripheral (inguinal) lymph nodes. The relative amount of viable PKH-26+ B-1a cells was ~0.02% out of the total splenic CD19+ population and ~10% in the PerC. Each symbol represents one individual mouse and data shown are pooled from 2 experiments each performed with 1 or 2 recipient mice. The bar represents the mean. (C) MFI of CD11b on transferred cells in indicated organs. Each symbol represents one individual mouse and data shown are pooled from 2 experiments each performed with 1 or 2 recipient mice.

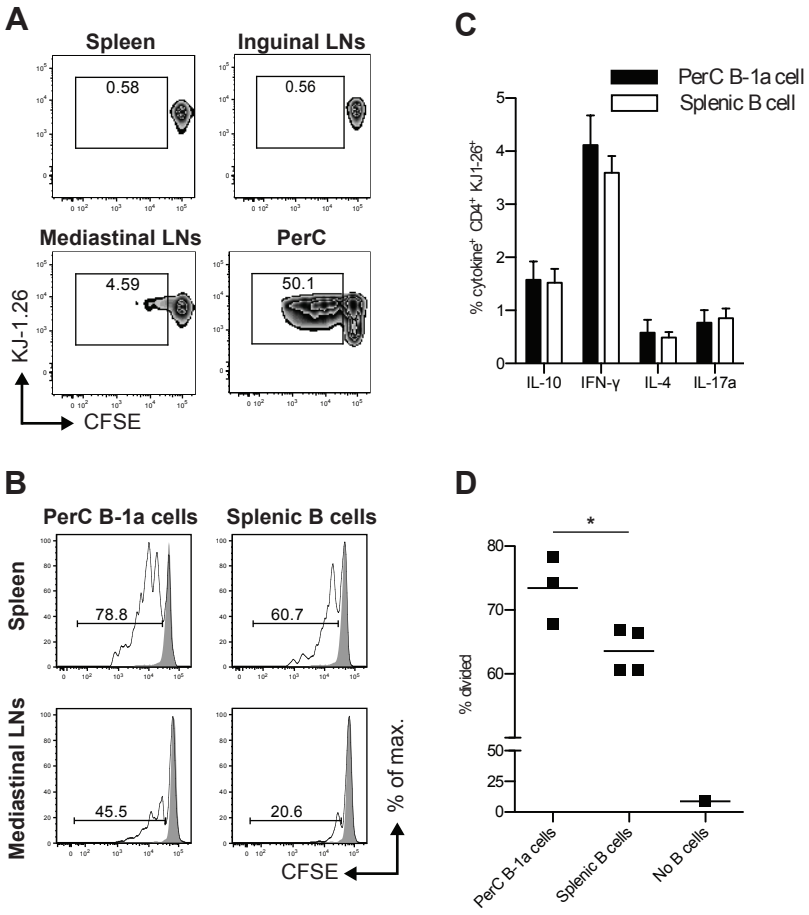

Figure 6. PerC B-1a cells present antigen to peripheral T cells, which are differentially activated.
(A) FACS-Sorted CD19$^+$ CD5$^+$ CD11b$^+$ B-1a cells were *ex vivo* pulsed with OVA peptide and subsequently i.p. transferred to the peritoneal cavity of recipient mice. CFSE-labeled CD4$^+$ OVA-specific (KJ1-26$^+$) T cells were transferred via the i.v. route to the same animal. Shown are gated KJ1-26$^+$ T cells in the indicated organs of recipient mice 96 h after transfer. Flow cytometry plot is from one experiment representative of two independent experiments. (B) B cells were transferred as in (A) whereas CFSE-labeled T cells were now transferred via the i.p. route. Shown are gated KJ1-26$^+$ T cells in the spleen and mediastinal lymph nodes of recipient mice that received T cells in combination with indicated peptide-pulsed B-cell subsets. Shaded areas indicate T cells from a control animal that did not receive peptide-pulsed B cells. (C) The percentages of indicated-cytokine positive cells out of the KJ1-26$^+$ T-cell population in the spleen of recipient mice (treated as in (B)), after *ex vivo* stimulation with PMA/ionomycin, followed by intracellular cytokine staining. Data are shown as mean + SEM of 6 (PerC B-1a) - 8 (splenic B cells) samples pooled from three individual experiments performed. (D) The percentage of divided KJ1-26$^+$ T cells (gated as in (B)) in the spleen. Data shown are from one representative experiment out of three experiments performed. *p <0.05, Student's t-test).

experimental setting, we found this increase relatively modest compared with the increase in the number of IL-10- and IL-4-producing cells (ratio PerC B-1a to splenic B-cell cultures ~1.6-fold for IFN-γ, ~3.2-fold for IL-10 and ~5.3-fold for IL-4; see Figure 3B). No differences in the relative number of T cells expressing IL-17 were detected.

Interestingly, IL-17-producing T cells did seem to be enriched when splenic $CD5^{+}B220^{low}$ B cells were used in co-cultures. It is difficult to assign this finding specifically to splenic B-1a cells due to the fact that the $CD5^{+}B220^{low}$ fraction possibly contained other cells than B-1 cells. B-1 cells are notoriously hard to identify in any organ other than the body cavities due to the lack of CD11b expression and the presence of large amounts of other cells with similar surface markers [9]. CD5 is a marker for anergic B cells as well [29], and activated B cells can downregulate their expression of B220 [13,14]. The splenic $CD5^{-}$ $B220^{hi}$ B cell fraction consists mainly of follicular B cells, but it should be noted that it contains smaller amounts of multiple other B cell subsets, including marginal zone B cells. Although it is technically challenging to obtain sufficient amounts of cells, it would be of interest to see a side-by-side comparison of the multiple splenic- and PerC B cell subsets enacting roles as APCs in a future study.

Analysis of the supernatants from our co-cultures showed that communication between PerC B(-1a) cells and T cells resulted in a cytokine environment with increased levels of IL-10, IL-6 and IL-2 while the levels of IL-17a and IFN-γ were not significantly altered. The latter observation supports the notion that no apparent differentiation of T_H17 cells took place in co-cultures where PerC B(-1a) cells were used as APCs. This finding suggests that some of the cytokines in the PerC B-cell subset were possibly derived from other sources, such as $CD5^{-}$ B-1b cells or PerC B-2 B cells.

Discrepancies between cytokine levels detected in the supernatant and the cytokines that were detected intracellularly can occur due to the fact that the T cells were restimulated with PMA/ionomycin in order to facilitate intracellular detection. The supernatant furthermore contains cytokines that are produced not only by the T cells, but by the B cells as well.

Data from our migration- and *in vivo* experiments with pulsed B cells demonstrated that B-1a cells from the PerC communicate with T cells from the periphery. This finding is important since the apparent physical separation of the B-1 cells in the PerC and the bulk of the T cells in the periphery might suggest otherwise. Migration from PerC B-1a cells towards the periphery would facilitate PerC B-1a–T cell contact, but our data show that this does not happen in abundant quantities. Data from parabiosis experiments show that B-1a cells are continuously circulating from the PerC through the omentum, towards the blood [30]. While other reports indicate that in situ labeled PerC B-1a cells showed only minimal migration towards peripheral lymphoid tissues other than the pleural cavity [31]. In general, both these reports are in agreement with our observations, since, in our model, most PerC B-1a remained inside the PerC and only relatively small amounts ended up in the periphery. After TLR-triggering [21-23] or due to influenza virus infection [32] B-1a cells do migrate towards the periphery (spleen or lungs and local lymph nodes respectively) in abundant amounts.

Our *in vivo* study with peptide-pulsed B cells suggests that it is not necessary for B-1a cells to migrate out of the PerC in large amounts in order to present Ag to T cells from the periphery since peripheral T cells visit the PerC and engage with B cells in this location (Figure 6A). Lábadi et al. indeed showed that amongst in situ labeled PerC lymphocytes, T cells have the fastest migration rates [31]. Two recent studies indicated that the PerC T lymphocyte population

is highly enriched in T cells with an Ag-experienced/memory phenotype [26,33], but not with Foxp3$^+$ regulatory T cells [26]. Earlier, it was already shown that Ag-experienced T cells present in the PerC include cells that were primed locally in the lung as well, supporting the presupposition that peripheral T cells migrate to the PerC and come into contact with PerC B cells [34]. Our experiments with i.p. transferred B and T cells furthermore demonstrate that T cells activated in the PerC do not stay in this location, but instead migrate towards the periphery (Figure 6B).

Differences in cytokine profiles of T cells activated by B-1a cells *in vitro* (Figure 2 and 3) or *in vivo* (Figure 6D) can be the result of differences in microenvironments. *In vitro* co-cultures are efficiently simulating specific microenvironments that are important for the enrichment of specific T-cell subsets. We showed that enrichment of IL-10-producing T cells is dependent on a high concentration of soluble IL-10 (Figure 2C), and we indeed demonstrated that high amounts of IL-10 were present in the supernatants of our co-cultures with PerC (B-1a) cells (Figure 4). *In vivo,* splenic B cells transferred to the PerC might have been influenced by the microenvironment of the PerC, programming them into cells with B-1 like properties. The influence of the PerC microenvironment on splenic B cells has been described before [35,36]. Alternatively, *ex vivo* T-cell stimulation might not have been sensitive enough to detect differences in *in vivo* T-cell activation by the different B-cell subsets.

The finding that peripheral T cells migrate to and from the PerC, where they interact with B-1a cells, raises the question of what the contribution of this interaction on the T-cell pool might be. Evidence suggests that a large proportion of the peripheral memory T-cell pool is in fact specific for commensal microbes from the gut [37]. It is tempting to speculate that PerC B-1a cells play an unexplored role in sensing gut microbiota-derived Ags [21] or whole bacteria [38,39], which they subsequently present to peripheral (memory) T cells. Alterations in the PerC B-1 cell population from germ-free and SPF mice support the hypothesis that PerC B-1 cells sense luminal Ags [21,40].

In summary, our data shows that PerC B-1a cells communicate with CD4$^+$ T cells from the periphery *in vivo*, and that PerC B-1a cells differentially enrich IL-10-, IL-4- and IFN-γ-producing T-cell subsets *in vitro*. To our knowledge, this immunological pathway had not been described before. Given the fact that B-cell depletion therapies are currently being exploited for usage in a variety of T-cell-mediated autoimmune diseases [4], it is of utmost importance to understand how various B-cell subsets influence peripheral T-cell populations.

Materials and Methods

Mice

BALB/c mice (8-12 weeks) were purchased from Charles River Laboratories (Maastricht, the Netherlands) and kept under standard housing conditions at the Central Animal Laboratory (GDL) of the Utrecht University. DO11.10 (OVA-TCR Tg) were bred and kept at the GDL under specific pathogen free conditions. In one experiment, mice were immunized with 100 µl containing 1 mg dimethyldioctadecylammonium bromide and 100 µg whole chicken ovalbumine grade VII (OVA; Sigma-Aldrich Chemie B.V.). All animal experiments were approved by the Animal Ethics Committee (DEC) from the Utrecht University.

Cell isolation, labelling and cell-transfers

$OVA_{323-339}$-TCR Tg $CD4^+$ T cells were obtained from the spleen of naïve DO11.10 mice by negative selection with sheep-anti-rat IgG Dynalbeads (Dynal, Invitrogen) using an excess amount of anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC class II (M5/114), anti-CD8 (YTS169) monoclonal Abs. Enriched T cells were routinely pure between 85 and 95%. Labelling of cells with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) was performed as previously described [41].
B cells were isolated from naïve BALB/c mice. Splenic B cells were isolated by positive selection using CD19-MicroBeads (MACS Miltenyi) and splenic B-cell subsets were isolated by FACS-sorting (BD INFLUX). B cells were routinely pure >95% based on their expression of CD19. PerC wash-out cells were obtained by an i.p. injection of 10 ml of PBS supplemented with 2% FCS and 2 mM EDTA (Gibco) that was subsequently collected using a Pasteur pipette. Whole PerC B cells were isolated by positive selection using CD19 MicroBeads and PerC B-1a cells were isolated by FACS-sorting (based on the expression of $CD19^+CD5^+CD11b^+$). Supporting Information Figure 1 shows the reanalysis of purified cell populations used. In some experiments, PerC B-1a cells were labeled with PKH-26 (Sigma) according to manufacturer's instructions. Viable cell counts were determined by Tryptan blue exclusion.
In experiments were cells were transferred, 2×10^6 B cells (Figure 5 and 6) and 1×10^7 OVA-TCR Tg CFSE-labeled $CD4^+$ T cells (Figure 6) were transferred via the i.p. and/or i.v.-route. $OVA_{323-339}$ peptide pulsed B cells (15 μg/ml; 2 hours; 37 °C; 5% CO_2) were washed three times before transfer.

Flow cytometry and Intracellular cytokine staining

Single-cell suspensions were washed and subsequently stained in the presence of Fc block (2.4G2) for CD11b (M1/70), CD19 (1D3), CD21/35 (7E9), CD23 (B3B4), CD4 (RM4-5), CD43 (S7), CD45R/B220 (RA3-6B), CD5 (53-7.3, biotin conjugate), DO11.10 TCR (KJ1-26), IgD (11-26c.2a) and IgM (II/41) from BD, eBiosciences or Biolegend. After washing, the samples were analyzed on a FACS Canto II (BD Biosciences) and analyzed with FlowJo software (Tree Star). For intracellular cytokine staining, total cells from co-cultures were stimulated for 5 hours in the presence of 50 ng/ml PMA, 500 ng/ml ionomycin, 1 μg/ml LPS and 1 μg/ml Brefeldin A (all from Sigma). Cells were then washed, stained extracellularly as indicated earlier, permeabilized using Cytofix/Cytoperm-solution (BD) according to manufacturer's instructions and subsequently stained intracellularly for cytokines in the presence of Fc block (2.4G2) using antibodies against IFN-γ (XMG1.2), IL-10 (JES5-16E3), IL-17 (eBio17B7) and IL-4 (11B11) from BD or eBiosciences.

Co-culture

Indicated B cells (3×10^5) were co-cultured with OVA-TCR Tg $CFSE^+$ $CD4^+$ T-cells (1×10^5) in the presence of 100 μg/ml OVA in IMDM supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml) (all from Gibco) at 37 °C and 5 % CO_2. In some experiments, IL-10 secreted into the supernatant was neutralized using 20 μg/ml rat anti-mouse IL-10 mAb (JES-2A5).

Luminex

Cytokines secreted during the cultures were determined by Luminex. Briefly, fluoresceinated microbeads coated with capture antibodies for simultaneous detection of IFN-γ (AN18), IL-2 (JES6-1A12), IL-10 (JES5-2A5), IL-17a (TC11-18H10) and IL-6 (MP5-20F3) were added to 50 µl of culture supernatant. Cytokines were detected by biotinylated antibodies against IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-10 (SXC-1), IL-17a (TC11-8H4.1) and IL-6 (MP5-32C11) followed by PE-labeled streptavidin (All from BD Biosciences Pharmingen). Fluorescence was measured using a Luminex model 100 XYP (Luminex, Austin, TX, USA) and the cytokine concentrations were quantified using a calibration line from recombinant cytokines.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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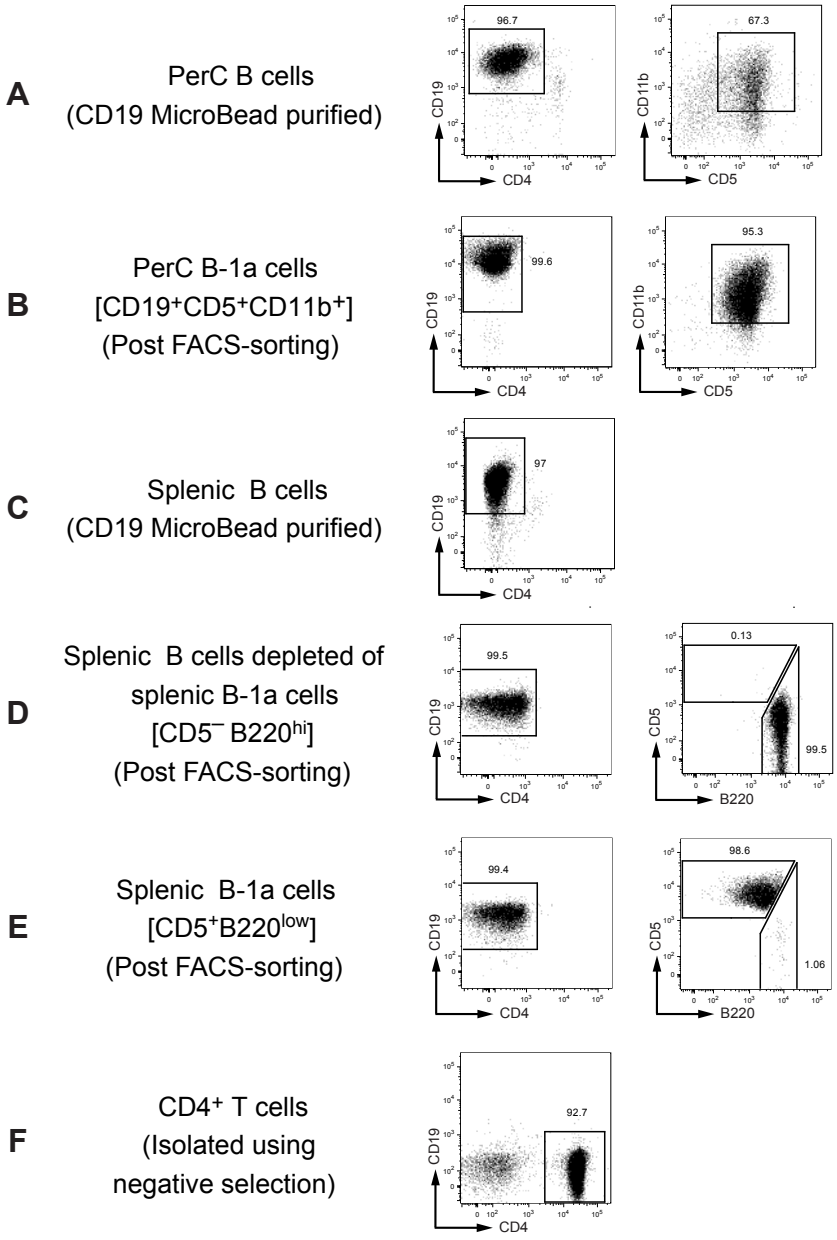
Supporting Information: Figure 1



Supporting Information Figure 1. Representative flow cytometry plots of reanalyzed purified cell populations. Flow cytometry plots of reanalyzed purified cell populations. (A) PerC B cells were used in figure 2 and 4; (B) Perc B-1a cells were used in figure 3A, 3B, 4, 5 and 6; (C) Splenic B cells were used in figure 2, 3A, 4 and 6; (D) Splenic B cells depleted of splenic B-1a cells and (E) the splenic B-1a cell population were used in figure 3C. (F) CD4+ T cells were used in figure 2, 3, 4 and 6.

4

Activated peritoneal cavity B-1a cells possess regulatory B cell properties

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Abstract

Previous studies have suggested that murine peritoneal cavity-derived B-1a cells possess similarities with described regulatory B cell subsets. The aim of the current study was to examine the potential immunoregulatory function of peritoneal cavity-derived B(-1a) cells. *In vitro* activation of peritoneal cavity-derived B- and B-1a cells shows that activation of these B cells with anti-CD40 and LPS induces these cells to secrete more IL-10, IL-6 and IgM as compared to splenic B cells. In a suppression assay, CD40/TLR4-activated peritoneal cavity B cells possess regulatory B cell functions as they inhibit the capacity of $CD4^+$ T cells to produce both tumor necrosis factor-α and interferon-γ. Splenic B cells did not show this, whereas non-activated peritoneal cavity B cells augmented the capacity of $CD4^+$ T cells to produce tumor necrosis factor-α, while the ability to produce interferon-γ was not altered. The current paper compares splenic B cells to peritoneal cavity B(-1a) cells in an *in vitro* activation- and an suppression-assay and concludes that peritoneal cavity B(-1a) cells possess properties that appear similar to splenic autoimmune-suppressive regulatory B cell subsets described in the literature.

Introduction

Research in the past decade has convincingly shown that certain B cell subsets, nowadays referred to as "regulatory B cells" (B_{REG}s), possess the capacity to down-regulate immune-responses via the secretion of interleukin (IL)-10. There is no definite surface marker or master-transcription factor to identify B_{REG}s, and they are functionally defined by their immune-suppressive action, either *in vitro* or *in vivo* [1-3]. The best described B_{REG}s, with well confirmed regulatory functions, include the splenic $CD21^{hi}CD23^{hi}CD1d^{hi}$ transitional 2 marginal zone precursor B cells described by the group of Mauri [1,4] and the IL-10-producing B cells termed 'B10' cells characterized by the group of Tedder. The latter are mainly found within the $CD1d^{hi}CD5^{+}$ splenic B cell subset [3,5].

B-1a cells are innate-like B cells that have the ability to produce polyreactive natural antibodies, secrete large amounts of cytokines (among others IL-10), and are known for their expression of CD5 [6-8]. There are multiple similarities between B-1a cells and specific B_{REG} subsets (particularly B_{REG}s with the $CD5^{+}$ $CD1d^{hi}$ B10 phenotype), but it is still unclear how these subsets are related, or if they are possibly even the exact same cells [1,3,6].

B_{REG}s need to be activated in order to exert their suppressive functions, and activation of B_{REG}s presumably takes place *in vivo* in the context of inflammation. Although the exact mechanism is incompletely understood, both the groups of Mauri and Tedder have shown that *in vivo* activated B_{REG}s are more potent suppressors of autoimmunity than their non-activated counterparts [4,9]. There is evidence that this activation is antigen-specific, since B_{REG}s that are *in vivo* activated by one antigen (Ag), do not protect in inflammatory models induced by another Ag [4,5]. *In vitro*, B_{REG}s can be activated in an antigen-nonspecific way to secrete IL-10 and suppress by a variety of stimuli that include activation through Toll-like receptor (TLR)-ligands [10-13], CD40 [12,14,15], a combination of these two [9,12,16], or the cytokine IL-21 [9].

B_{REG}-mediated amelioration of autoimmune diseases in animal models (*e.g.* antigen-induced arthritis, collagen-induced arthritis and experimental autoimmune encephalomyelitis) is typically dependent upon IL-10 but besides that relatively little is known about the mechanism of action. Most reports indicate that B_{REG}s influence T cell activation. Protection induced via the adoptive transfer of B_{REG}s often correlates with a reduction in interferon (IFN)-γ-, IL-17- and/or tumor necrosis factor (TNF)-α-positive T cells [4,9,17,18] and sometimes increased levels of $Foxp3^{+}$ regulatory T cell (T_{REG}s) [19] or IL-10-producing T cells [20]. Furthermore, B cell depleted mice or studies using $IL10^{-/-}$ B cells show that B cell-derived IL-10 is needed *in vivo* to maintain the levels of IL-10-producing T cells [18,21] and Foxp3-positive T_{REG}s [18,22] found in wild type mice.

In vitro B_{REG} suppression assays are sometimes used to decipher immunosuppressive mechanisms. Although, B_{REG}s are reported to limit T cell proliferation *in vivo* [4,9] in a similar way as T_{REG}s do, this is only occasionally seen in *in vitro* B_{REG} suppression assays [23] and most reports do not detect this type of inhibition [10,17]. Instead, B_{REG} suppression assays show that B_{REG}-derived IL-10 inhibits the promotion of proinflammatory cytokine (IFN-γ and TNF-α) positive $CD4^{+}$ T cells [15,17] the production of TNF-α by monocytes [11,24] or T cell activation by dendritic cells [10,17]. Human B_{REG}s are reported to possess identical functions *in vitro* [25-27].

The capacity to produce IL-10 is a *sine qua non* for a B_{REG}, but the capacity of a B-1 cell to produce this cytokine does not automatically define B-1 cells as B_{REG}s. IL-10 is a pleiotropic cytokine with a variety of functions [28], and the exact function exerted may depend upon several micro environmental factors *e.g.* other cytokines secreted by the same B cell. Furthermore it has been demonstrated that B cells with IL-10-secreting capabilities often possess the ability to secrete IL-6 as well, and B-cell derived IL-6 plays a prominent role in the pathogenesis of autoimmune diseases [29]. Numerous other studies aimed to elucidate the exact phenotype of B_{REG}s, and found that their phenotype partially overlaps with (splenic) B-1a cells [3,5]. This signifies that populations deemed B_{REG}s (and isolated as such) contain B-1a cells as well, either as an 'irrelevant contaminant' or possibly as the actually functional immunosuppressive cell. In the current paper, we examined whether the well-defined B-1a cell containing peritoneal cavity B cell population possessed an immunoregulatory function.

Materials and Methods

Mice and Ethic Statement

Female BALB/c mice (10-12 weeks old) were purchased from Charles River Laboratories (Maastricht, the Netherlands) and kept under standard housing conditions at the Central Animal Laboratory of the Utrecht University. All animal experiments were approved by the Animal Ethics Committee from the Utrecht University (DEC Numbers: 2011.II.05.90, 012. II.08.108 and 2012.II.11.157). All efforts were made to minimize animal discomfort.

Cell isolation

Peritoneal cavity (PerC) washout cells were obtained by an i.p. injection of 2 ml air and 8 ml of phosphate buffered saline supplemented with 2% fetal calf serum and 2 mM ethylenediaminetetraacetic acid (Gibco) that was subsequently collected using a transfer pipette. The washout cells were passed through a 27G needle to obtain a single cell suspension. Splenic $CD4^+$ T cells and $CD19^+$ B cells from spleen and PerC were isolated from naïve mice using positive selection via magnetic-activated cell sorting using CD4- and CD19-MicroBeads (Miltenyi) respectively, and routinely between 95-99% pure based on the expression of CD4 and CD19. PerC B-1a cells were isolated by Fluorescence activated cell sorting (FACS) using a BD Influx Cell Sorter (BD Biosciences) based on the expression of CD19, CD5 and CD11b.

Flow cytometry and intracellular cytokine staining

Single-cell suspensions were washed and subsequently stained in the presence of Fc block (2.4G2) for CD11b (M1/70), CD19 (1D3), CD4 (RM4-5), CD5 (53-7.3, biotin conjugate), CD80 (16-10A1), CD86 (GL-1), CD40 (3/23) and major histocompatibility complex (MHC) class II (M5/114.15.2) using monoclonal antibodies (mAbs) from BD or eBiosciences. After washing, the samples were analyzed on a BD FACSCanto II Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). For intracellular cytokine staining, total cells from co-cultures were stimulated for 5 hours in the presence of 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 500 ng/ml ionomycin, 1 μg/ml lipopolysaccharide (LPS; Escherichia coli 0127:B8) and 1 μg/ml Brefeldin A (BFA) (Sigma). Cells were then washed,

stained extracellularly as indicated earlier, permeabilized using Cytofix/Cytoperm-solution (BD) according to manufacturer's instructions and subsequently stained intracellularly for cytokines in the presence of Fc block (2.4G2) using mAbs against IL-10 (JES5-16E3), IFN-γ (XMG1.2) and TNF-α (MP6-XT22) from BD or eBiosciences.

In vitro B cell activation

B cells were *in vitro* activated using LPS (10 μg/ml), an agonistic mAb against CD40 (αCD40) (10 μg/ml; clone FGK45), a combination of these two (10 μg/ml LPS and 10 μg/ml αCD40), IL-21 (100 ng/ml recombinant protein; eBiosciences) or by α-CD40 followed by LPS (10 μg/ml) during the final 5 hours of an 48 hours culture as described in literature [9,12,16]. The cells were washed extensively before being used in other assays. All cultures took place in IMDM supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin (500 units/ml) and streptomycin (50 μg/ml; all from Gibco) at 37 °C and 5 % CO_2 in either 96-well plates (1×10^5/well) or 24-well plates (2×10^6/well).

B_{REG} – T cell suppression assay

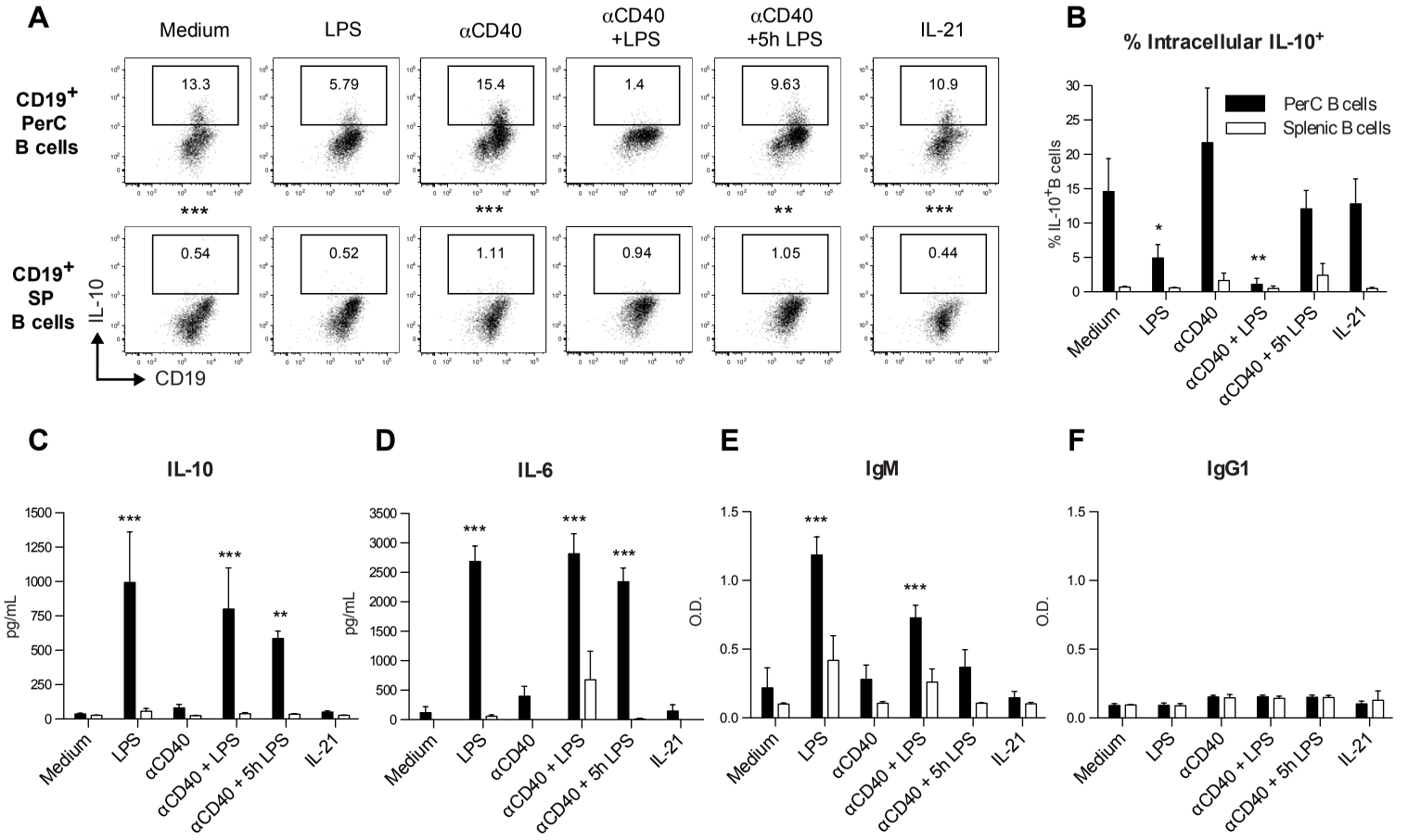
The suppression assay was performed as described [15,17], briefly: indicated B cells (2×10^5) were 1:1 co-cultured with $CD4^+$ T cells for 72h in the presence of anti-CD3 ('soluble'; 1 μg/ml; clone 145-2C11), while PMA, ionomycin, LPS and BFA were added during the final 5 hours of culture to facilitate intracellular cytokine staining (see above). TNF-α, IFN-γ and IL-10 positive T cells were determined intracellularly as described above.

Luminex

Cytokines secreted during the cultures were determined by Luminex before the stimulation with PMA, ionomycin, LPS and BFA took place. Briefly, fluoresceinated microbeads coated with capture antibodies for simultaneous detection of IFN-γ (AN18), IL-2 (JES6-1A12), IL-10 (JES5-2A5), IL-17a (TC11-18H10) and IL-6 (MP5-20F3) were added to 50 μl of culture supernatant. Cytokines were detected by biotinylated antibodies against IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-10 (SXC-1), IL-17a (TC11-8H4.1) and IL-6 (MP5-32C11) followed by phycoerythrin-labeled streptavidin (All from BD Biosciences Pharmingen). Fluorescence was measured using a Luminex model 100 XYP (Luminex, Austin, TX, USA) and the cytokine concentrations were quantified using a calibration line from recombinant cytokines.

ELISA

The presence of IgG1 and IgM in the supernatant of indicated stimulated B cells was determined by enzyme-linked immunosorbent assay (ELISA). 96-well ELISA plates (NUNC Maxisorp) were coated with polyclonal goat anti-mouse immunoglobulins (Dako Z0420). Free binding sites were blocked with a blocking buffer containing 4% horse serum (Gibco) and 1% Tween-80 (Sigma). Sera were diluted in a buffer containing 4% horse serum, 1% Tween-80 and 0.5 M NaCl, and mouse immunoglobulins were determined using horseradish peroxidase-conjugated goat anti-mouse IgG1 (Cat. 1070-05) or IgM (Cat. 1020-05, both from Southern Biotech Association).


A
Medium
LPS
αCD40
αCD40 +LPS
αCD40 +5h LPS
IL-21
CD19+ PerC B cells
13.3
5.79
15.4
1.4
9.63
10.9
***
***
**
***
CD19+ SP B cells
0.54
0.52
1.11
0.94
1.05
0.44
IL-10
CD19
B
% Intracellular IL-10+
PerC B cells
Splenic B cells
% IL-10+B cells
C
IL-10
pg/mL
D
IL-6
pg/mL
E
IgM
O.D.
F
IgG1
O.D.
Medium
LPS
αCD40
αCD40 + LPS
αCD40 + 5h LPS
IL-21

Statistical analysis

Statistical analysis was performed as indicated in the figure legends using GraphPad Prism (GraphPad Software Inc.).

Results

B-1a cells share multiple characteristics with B_{REG}s, including the production of IL-10

Previous studies have indicated that the similarities between B-1a cells and described B_{REG}s are not limited to their (partial) overlap in phenotype. Transgenic mice with in- or decreased amounts of certain regulatory B cell subsets often contained parallel alterations in B-1a levels, suggesting that these cells are either the same cells and/or possess similar development pathways. Similarities in reactions to certain activating triggers further supported the notion that these cells are not only phenotypically similar, but also functionally (Table S1).

To investigate if the B-1a cell containing PerC B cell population produced IL-10, PerC B cells and splenic B cells were stimulated for 48h with LPS, αCD40 or a combination of these stimuli. At the end of the 48h activation period, the cells were analyzed for their potential capacity to produce IL-10 (as determined by intracellular cytokine staining after restimulation) and the supernatant of the cells was analyzed for secreted cytokines and immunoglobulins. Figures 1A and 1B show that a significantly higher percentage of the PerC B cells possess the potential capacity to produce IL-10 after a 48h-culture compared to Splenic B cells, either without stimulation (medium control) or after stimulation with αCD40, αCD40 in combination with a brief 5h LPS stimulation or IL-21. None of these stimuli significantly increased the number of IL-10-producing PerC B cells compared to the medium control (Figure 1B), but treatment with LPS, αCD40+LPS or αCD40+5hr LPS did significantly alter the produced quantities of IL-10 (Figure 1C). Analysis of IL-6 (Figure 1D) secretion into the supernatant showed that PerC B cells produced IL-6 together with IL-10. LPS and αCD40+LPS furthermore also significantly increased IgM (Figure 1E), but not IgG1 (Figure 1F) secretion by PerC B cells compared to the medium control.

Figure 1: *In vitro* activated PerC B and PerC B-1a cells produce IL-10 and IL-6.

Splenic B cells and PerC B cells were cultured for 48h in the presence of medium only or indicated stimuli (see materials and methods). Intracellular IL-10 staining was performed after additional stimulation with PMA/ionomycin/LPS and both representative flow cytometric plots (A) and the percentage of IL-10^+ B cells (B) are shown, whereas the levels of IL-10 (C), IL-6 (D), IgM (E) and IgG1 (F) were determined directly from the supernatant (before restimulation took place) using Luminex (IL-10 and IL-6) and ELISA (IgM and IgG1). Shown in B-F are the mean ± S.D. of pooled results from two identical replicate experiments (with 2–5 technical replicates each) that produced similar data. Statistically significant differences between PerC- and splenic B cells after stimulation were calculated using a two-way ANOVA with Bonferroni post-hoc test (A), whereas statistically significant difference between the various stimuli and the medium control of PerC B cells (B-F) were calculated using a one-way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

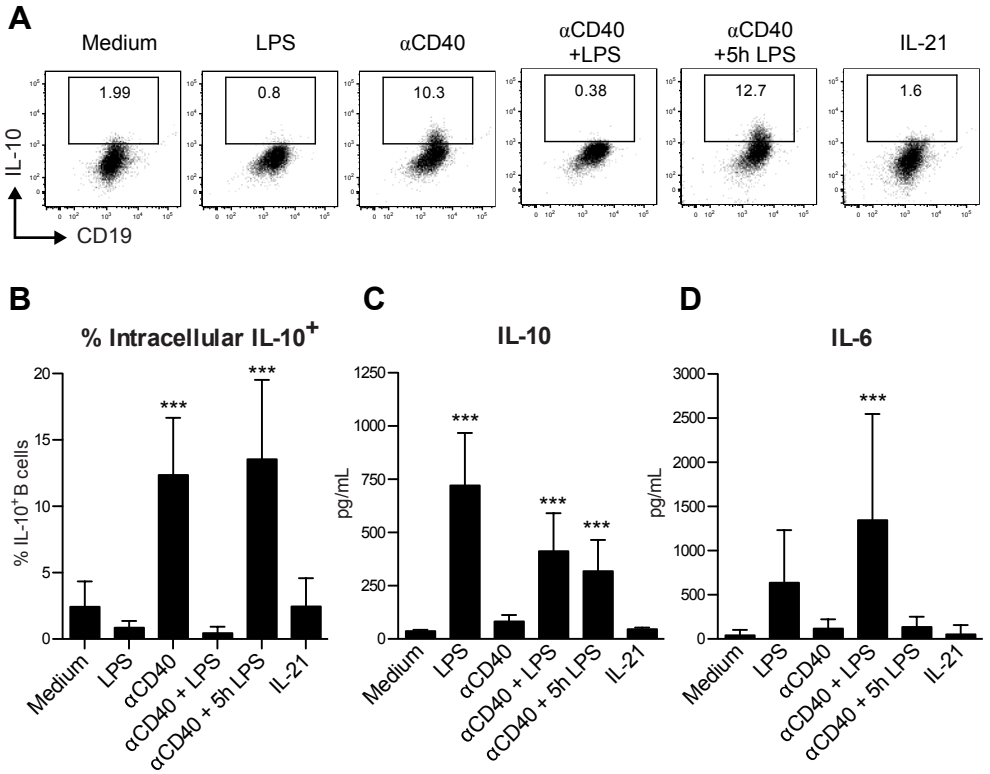


Figure 2: PerC B-1a cells secrete IL-10 and IL-6 similarly to the undifferentiated PerC B cell population

FACS-purified CD19$^+$ CD11b$^+$ CD5$^+$ PerC B-1a cells were cultured for 48h in the presence of medium or indicated stimuli. Intracellular IL-10 staining was performed after additional stimulation with PMA/ionomycin/LPS and both representative flow cytometric plots (A) and the percentage of IL-10$^+$ B cells (B) are shown, whereas the levels of IL-10 (C) and IL-6 (D) were determined directly from the supernatant (before restimulation took place) using Luminex. Shown in B-D are the mean ± S.D. of pooled results from two identical replicate experiments (with 2–5 technical replicates each) that produced similar data. Statistically significant differences between the various stimuli and the medium control (B-D) of PerC B-1a cells were calculated using a one-way ANOVA with Bonferroni post-hoc test. ** $p < 0.01$, *** $p < 0.001$.

PerC B-1a cells secrete IL-10 and IL-6 similarly to the undifferentiated PerC B cell population

The PerC B cell population from BALB/c mice consist for about 65-70% out of B-1a cells [30], which suggested that the high percentage of B cells capable of IL-10-producion (Figure 1A) and the cytokines detected in the supernatant (Figure 1B-D) were at least partially derived from B-1a cells. To investigate how purified PerC B-1a cells reacted to CD40 and TLR signaling, cells were sorted prior to *in vitro* stimulation. Figure 2A and 2B indicate that the percentage of PerC B-1a cells with the potential capacity to produce IL-10 increased after a 48h activation period with both αCD40 and αCD40+5hr LPS. The amount of IL-10 secreted into the supernatant (Figure 2C) was significantly increased compared to the medium control after stimulation with LPS, αCD40+LPS and αCD40+5h LPS, whereas the amount of IL-6 (Figure

2D), was only significantly different after αCD40+LPS treatment. This data supports a model in which αCD40 increases the percentage of B cells that is capable to produce IL-10, whereas LPS induces actual IL-10 secretion. This notion was described before by Lampropoulou et al. [10]. Since αCD40+5h LPS stimulated the PerC B(-1a) cells in such as way that (i) a high percentage of the B cells possessed and maintained the capacity to produce IL-10 (Figure 1A, 1B, 2A and 2B) while (ii) high amounts of IL-10 were secreted into the supernatant (Figure 1C and 2C), the subsequent experiments were performed with this stimulation-method only.

PerC– and splenic B cells possess different surface markers after activation

B cells may influence T cells not solely via the secretion of cytokines but as well via direct cell-cell contact. To determine the potential differences in co-stimulative abilities between the activated and non-activated PerC- and splenic B cells, we analyzed the expression of CD80,

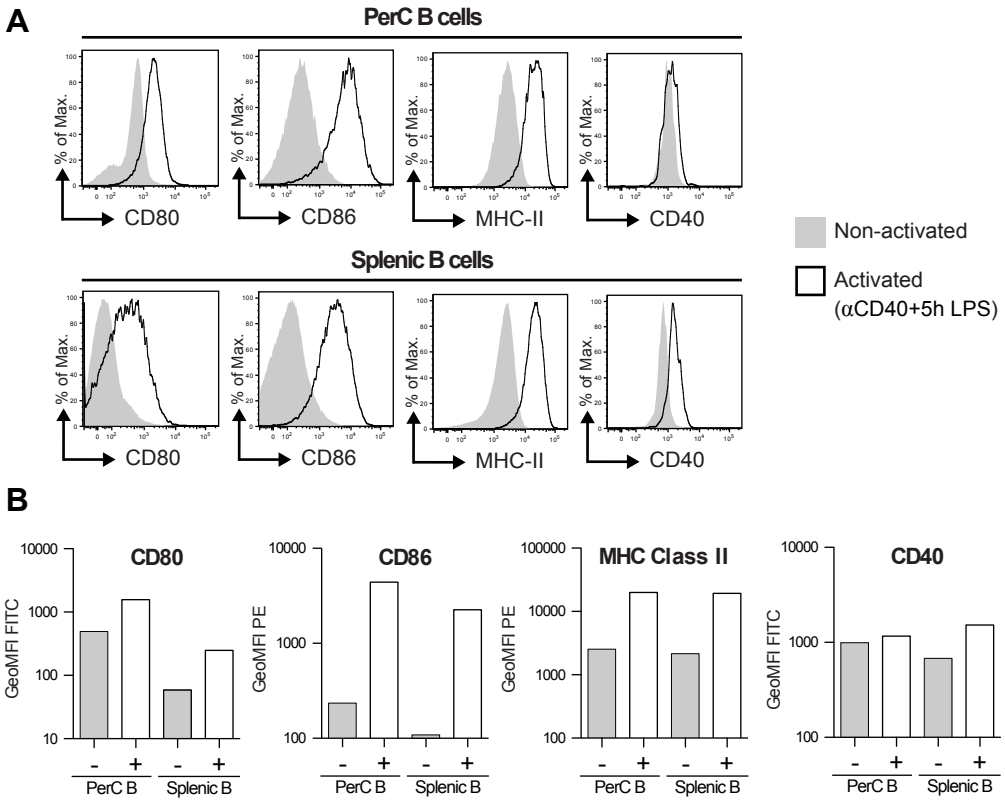


Figure 3: PerC– and Splenic B cells possess different surface markers after activation

Indicated B cell populations were stained extracellular with mAbs against CD19 and indicated markers after both a 48h period of stimulation using αCD40+5hr LPS (+ : activated; see materials and methods) and after being isolated freshly (– : non-activated). Shown in A are expression levels of indicated markers on $CD19^+$ B cells (representative plots of two identical replicate experiments), in B the average geometric mean fluorescence intensity (GeoMFI) levels from two identical replicate experiments.

CD86, MHC class II and CD40 of these populations. Figure 3 shows that PerC B cells express higher levels of CD80, CD86 and MHC class II compared to splenic B cells. After activation with αCD40+5hr LPS, both subsets upregulated CD80, CD86 and MHC class II and the differences in expression between PerC B cells and splenic B cells became, although still present, much smaller. Activation upregulated CD40 expression on splenic B cells only (Figure 3).

Activated PerC B cells suppress pro-inflammatory cytokine production by CD4⁺ T cells

Since B_{REG}s have been described to inhibit the capacity of polyclonally activated CD4⁺ T cells to produce TNF-α and IFN-γ [15,17] we examined the capacity of PerC B cells to do the same. The splenic B cells, which served as a control, and the PerC B cells were activated using αCD40+5hr LPS, and subsequently added to CD4⁺ T cells that were activated with anti-CD3 mAb. The PerC B cell population was found to possess B_{REG} functions since the added PerC B cells reduced the capacity of CD4⁺ T cells to produce both TNF-α (Figure 4A and 4B) and IFN-γ (Figure 4C and 4D), whereas splenic B cells did not show any effect.

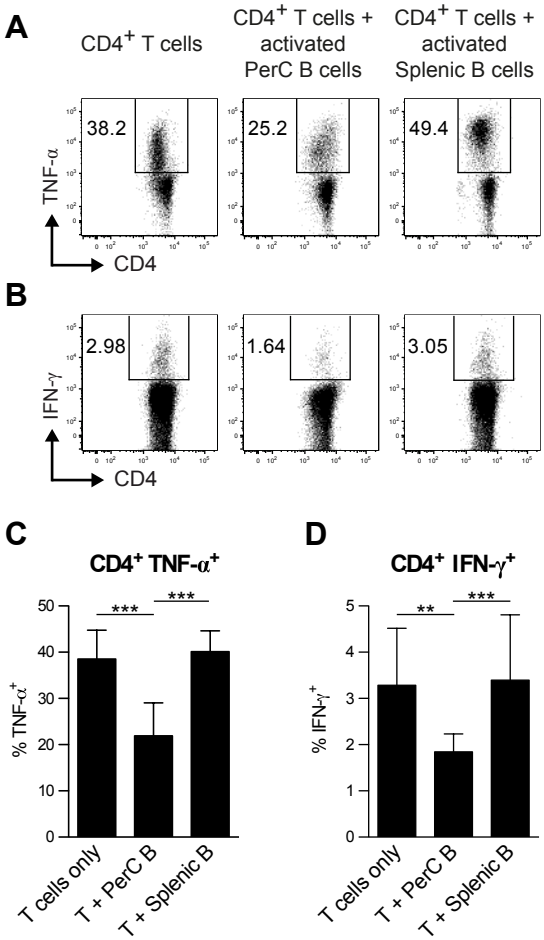


Figure 4: Activated PerC B cells suppress TNF-α- an IFN-γ-production by CD4⁺ T cells

Splenic- and PerC B cells were activated *in vitro* for 48h using αCD40+5hr LPS (see materials and methods), and co-cultured with splenic CD4⁺ T cells that were stimulated polyclonally with anti-CD3 for 72h. The cells were additionally stimulated with PMA/ionomycin/LPS during the last 5 hours of the co-culture in order to facilitate intracellular TNF-α and IFN-γ staining. Shown are representative plots of TNF–α (A) and IFN-γ (C) production by CD4⁺ T cells, and the percentages of T cells producing TNF-α (B) and IFN-γ (D). E and F additionally show the data for indicated non-activated B cell subsets. Shown in B, D and E are the mean ± S.D. of pooled results from two identical replicate experiments (with at least triplicate technical replicates each) that produced similar data. Statistically significant differences between the various culture conditions (B and D) were calculated using a one-way ANOVA with Bonferroni post-hoc test. ** $p < 0.01$, *** $p < 0.001$.

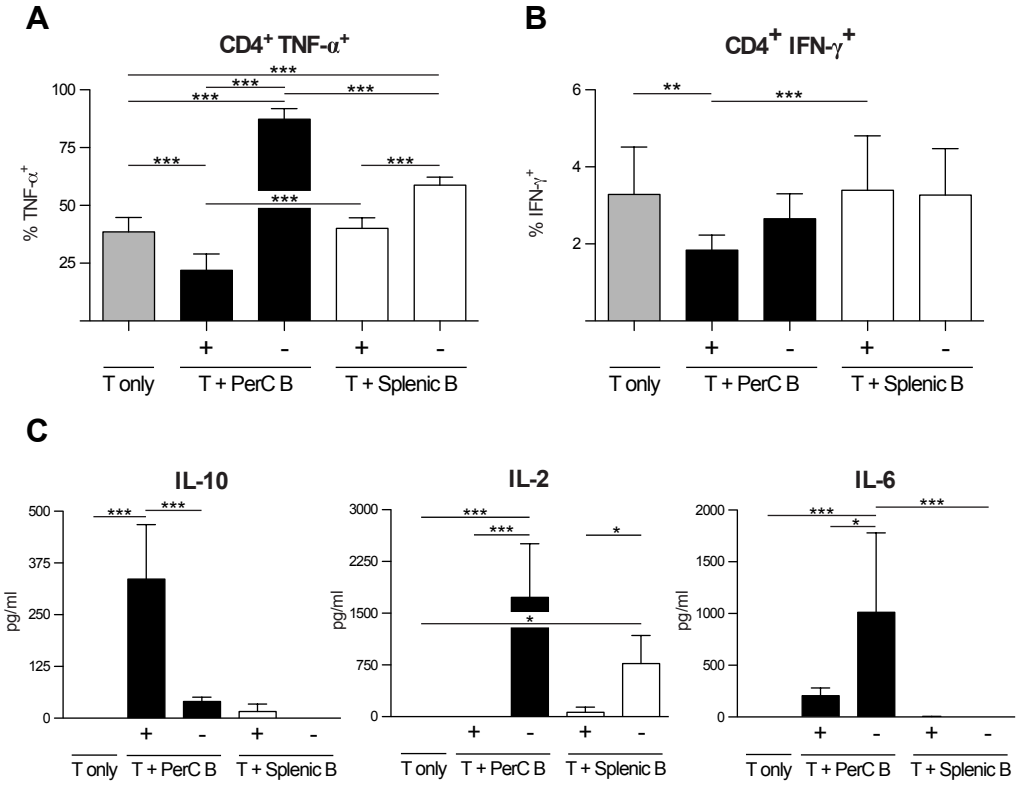


Figure 5: Activation status of B cells is critical for the effects exerted on T cells

B- and T cell cultures and subsequent intracellular staining were performed as explained in the legend of Figure 4. The percentages of T cells producing TNF-α (A) and IFN-γ (B) after co-culture with indicated activated (+) or non-activated (–) B cell subsets is shown. In A and B the mean ± S.D. are depicted of pooled results from two identical replicate experiments (with at least triplicate technical replicates each) that produced similar data. The cytokine levels of indicated B- and T cell cultures (C) were determined from the supernatant using Luminex. Levels shown in C are the mean ± S.D. of pooled results from two identical replicate experiments (with at least triplicate technical replicates each). Statistically significant differences between the various culture conditions were calculated using a one-way ANOVA with Bonferroni post-hoc test. *p< 0.05, ** p< 0.01, *** p < 0.001.

Activation status of B cells is critical for the effects exerted on T cells

Interestingly, the effect the B cells populations exerted on the activated T cells depended greatly upon the activation status of the B cells. Freshly isolated, PerC- and splenic B cells did not possess regulatory B cell roles. Instead non-activated B cells even augmented the capacity of T cells to produce TNF-α (Figure 5A) whereas the capacity to produce IFN-γ was not altered (Figure 5B).

Evaluation of the cytokine levels in the B-T cell co-cultures, indicated that co-cultures in which activated PerC B cells were used, resulted in a cytokine environment with relatively

high levels of IL-10 and low levels of IL-2 and IL-6 (Figure 5C). Co-cultures in which non-activated PerC B cells were used, resulted in a cytokine environment that was characterized by the virtual absence of IL-10, but increased levels of IL-2 and IL-6 (Figure 5C). Co-cultures with non-activated splenic B cells induced IL-2 production only. No cytokines were detected when T cells were cultured alone (Figure 5C).

Discussion

The aim of the current study was to examine the potential immunoregulatory functions of peritoneal cavity-derived B(-1a) cells. Our investigations show that peritoneal cavity-derived B cells can fulfill regulatory B cell roles *in vitro*, when activated as described [9,12,16]. Activation of PerC B- and B-1a cells via CD40 triggering and short term TLR4 activation induced prolonged IL-10 production and IgM secretion. Reports in the literature describe that IgM can possess regulatory functions in specific situations [31], but the complex-analysis needed to verify this possible activity is beyond the scope of the current paper.

These observations indicated that PerC B cells behave identically to functional B_{REG}s described in the literature following a variety of stimuli [12], except for stimulation with IL-21. In our study, IL-21 did not activate PerC B(-1a) cells similarly to B10 B_{REG}s, as this cytokine induces direct IL-10 production by the latter cells without the necessity of restimulation [9].

The co-stimulatory molecules CD80, CD86 and MHC Class II were upregulated after stimulation on both PerC- and splenic B cells, but the upregulation was greater in PerC B cells as compared to splenic B cells (Figure 3). Of note, unstimulated PerC B cells possessed significant levels of CD80, whereas splenic B cells did not. These data suggest that $CD4^+$ T cells might preferentially form more and/or longer interactions with activated PerC B cells as compared to their non-activated counter parts or splenic B cells. These differences are also reflected in the powerful capabilities of innate-like B cells (e.g., B-1 cells) to serve as antigen presenting cells [32,33].

The activation status of PerC B cells appeared to be decisive for the capacity of PerC B cells to exert regulatory B cell roles *in vitro*. Non-activated PerC B cells that were co-cultured with $CD4^+$ T cells induced a predominantly pro-inflammatory environment with increased levels of B-cell derived proinflammatory IL-6 [29] and an augmented amount of TNF-α-competent T cells. Activated PerC B cells on the other hand, produce relatively more IL-10 and induced an anti-inflammatory environment with increased levels of IL-10, decreased levels of IL-6 and less TNF-α and IFN-γ-competent T cells. Future studies should point out the exact working mechanism of this PerC B cell regulatory role. Such studies may want to focus on, e.g., the IL-10-, IL-10 receptor-, dose- and contact-dependency of the described effect.

It is important to realize that the PerC B cell population contains distinct B cells; 60-70% of the PerC B cells in our BALB/c mice are B-1a cells [30]. Experiments performed with purified B-1a cells suggest that these cells are (at least partially) responsible for the IL-10 production observed when the PerC B cell subset was stimulated.

Preliminary data from experiments in which we transferred *in vitro* activated PerC- and splenic B cells to mice before the induction of proteoglycan-induced arthritis, did not demonstrate an apparent disease-dampening effect for PerC B cells whereas activated splenic B cells seemed to worsen disease (unpublished observations). CD80/86-mediated autoreactive T cell activation by B cells plays an important role in the induction of severe arthritis in this

model [34]. This can explain the effect of activated splenic B cells that possess upregulated levels of CD80/86 (Figure 3). It is possible that insufficient PerC B cell numbers reached the locations necessary to suppress diseases efficiently, possibly (partly) due to interference caused by the immunization used to induce disease. However, a recent report indicates that PerC B cells can function regulatory B cell roles *in vivo* in a murine model for colitis [35].
In conventional autoimmune situations, when no cells are transferred, endogenously present PerC B cells may become stimulated *in vivo* in a similar way as splenic B_{REG}s [4,9]. *In vivo* stimuli for PerC B cells and splenic B_{REG}s may include *e.g.* constitutively activated T cells that provide signals via CD40 in chronic inflammatory disease [36], a process that can take place in an antigen non-specific way [37]. The localization of B cells in the PerC does not exclude these cells from activation since PerC B cells can migrate from the PerC to the spleen [38]. Furthermore it has been suggested that PerC B cells are activated locally in the PerC after administration of distant local inflammatory mediators [39]. Difference mouse breeds might be useful model systems to analyze the contribution of PerC B-1a cells in autoimmune conditions since differences in the quantities and IL-10-producing capabilities of PerC B-1a cells between e.g. BALB/c and C57BL/6 mice have been described before [40].
B-1a cells are found in the spleen as well; splenic B-1a cells are $CD11b^{-}$, whereas they are $CD11b^{+}$ in the PerC, but the exact relationship between splenic- and PerC B-1a cells is not completely clear [6]. When PerC B-1a cells migrate to the spleen (e.g. after systemic stimulation with LPS), these 'recent splenic immigrants' are still positive for CD11b for several days [38]. Since part of the splenic B10 B_{REG}s that are upregulated after autoimmune inflammation seem to express heightened levels of CD11b as well [5] it is tempting to speculate this population also contains activated PerC-derived B-1a cells.
PerC B-1a cells were earlier described to possess suppressive properties in a contact hypersensitivity model [41] and in a colitis model [35,42]. We now have shown that described splenic autoimmune-suppressive B_{REG} subsets and PerC B-1a cells might be stronger related to each other as thought before since (i) both cells reacted equally on activation-stimuli and (ii) both cells behaved similarly in a B_{REG} suppression assay.

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Supplementary data: Table S1

Table S1: Similarities and equalities between regulatory B cells and B-1a cells

Property	IL-10-producing B10 cells / BREGs with regulatory properties	Splenic- or PerC-derived B-1a cells
$CD5^+$ phenotype	B10 cells are enriched within the splenic $CD5^+CD1d^{hi}$ B cell subset [1]	B-1a cells are defined by their positivity of CD5 [2]
IL-10 production after stimulation	B10 cells are functionally defined by their ability to produce IL-10 [1]	B-1 cells are known to be able to produce IL-10 [3]
$CD19^{-/-}$ mouse[1]	B10 cells are reduced by ~75% in CD19-deficient mice [1,4]	CD19-deficient lack B-1a cells [5,6]
hCD19Tg mouse[1]	B10 cells are expanded in mice that overexpress (human) CD19 [1,4]	hCD19Tg mice have an large increase in their numbers of B-1a cells [5,6]
Chronic Lymphocytic Leukemia (CLL)	CCL cells share phenotypic and functional properties with B10 cells; including their immunosuppressive function [7]	Many suggest that CCL cells are often derived from the B-1 lineage (reviewed by Nordgren et al. [8]).
$CD22^{-/-}$ mouse[2]	B10 cells are expanded in mice that lack CD22 [4,9]	B-1a cells are expanded in $CD22^{-/-}$ mice, although they seem to be functionally impaired in at least some situations [10]
CD40 ligation	B10 cells are expanded in mice with an ectopic expression of CD154 [4,9]	PerC cells vigorously proliferate *in vitro* following CD40 ligation with an agonistic antibody (unpublished observations).
1~3% of $CD19^+$ cells in spleen	About 1~3% of the splenic B cells are functionally defined B10 cells [1]	About 1~3% of the splenic B cells are B-1a cells [2]
Development dependent upon BCR diversity	B10 cells are reduced by ~90% in Tg MD4 mice with a fixed BCR specific for HEL [4]	The B-1 subset does not develop normally in MD4 mice since the PerC B cell maintain a follicular (B-2) cell phenotype [11].

Activation by the protozoa *Leishmania major*	Infection induces IL-10-producing B cells with regulatory properties and a B10 phenotype [12]	Infection causes expansion of B-1a cells *in vivo*, and B-1 cells activated *in vitro* (with leishmanial extracts) produce IL-10 [13]
Activation by the helminth *Schistosoma mansoni*	Infection induces IL-10-producing B cells [3] with regulatory properties [14] / [15]	Infection causes expansion of B-1a cell *in vivo*, and B-1 cells activated *in vitro* (with *Schistosoma mansoni* soluble egg Ag) produce IL-10 [16]
In vitro treatment with BAFF	*In vitro* treatment of splenic B cells with BAFF induces IL-10-producing regulatory B cells that suppress arthritis *in vivo* [17] [4]	*In vitro* treatment of (TLR stimulated) B-1a cells with BAFF augments IL-10 production [18]
48h *in vitro* treatment agonistic CD40 mAb and 5hr LPS	*In vitro* treatment of splenic B cells with these stimuli induces and activates IL-10-producing B10 cells to both become IL-10-competent and secrete IL-10 [4,9,19]	*In vitro* treatment of PerC B(-1a) cells with these stimuli activates these B cells in a similar way (this paper)
Co-culture of the B cells with $CD4^+$ T cells in a regulatory B cell suppression assay	Various regulatory B cells suppress TNF-α- and IFN-γ-competent $CD4^+$ T cells in a suppression assay [20,21]	PerC B(-1a) cells suppress TNF-α-and IFN-γ-competent $CD4^+$ T cells in a similar way (this paper)

1. CD19 is a positive BCR regulator; BCR signalling is decreased in $CD19^{-/-}$ mice, while it is vice versa increased in hCD19Tg that overexpress (human) CD19. hCD19Tg mice have increased amounts of B-1a cells, but decreased amounts of B-1b cells. $CD19^{-/-}$ mice vice versa, possess increased amounts of B-1b cells, but lack B-1a cells [5,6].
2. CD22 is a negative BCR regulator; and it's absence results in increased BCR signalling [22].
3. The regulatory B cells in these articles possess a phenotype other than the $CD5^+$ $CD1d^{hi}$ B10 cells; there are instead: MZ ($CD1d^{hi}$) B_{REG}s [14] / $CD19^+$ IL-10^+ $CD1d^{hi}$ $CD5^+$ $CD21^{hi}$ $CD23^+$ IgD^+ IgM^{hi} [15].
4. This article demonstrates that MZ B cells (and not FO B cells) can gain regulatory B cell roles after BAFF treatment. Splenic B-1a cells were not investigated.

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5

B cell epitopes of the G1 domain of cartilage proteoglycan aggrecan in rheumatoid arthritis and two corresponding mouse models

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Abstract

Introduction: Systemic immunization with human cartilage proteoglycan, or the thereof derived G1-domain, induces autoimmune progressive polyarthritis in susceptible mouse strains that closely resembles certain aspects of human rheumatoid arthritis. Proteoglycan and G1-induced arthritis are T cell-dependent and antibody/B cell-mediated autoimmune diseases in which autoantibodies are directed against the self-antigen mouse proteoglycan. The aim of the current study is to elucidate the B cell epitopes on the protein-backbone of the G1 domain of mouse proteoglycan after immunization with human proteoglycan, and to analyse the difference in B cell response between proteoglycan- and G1 domain-induced arthritis. Moreover, the presence of human G1-derived peptide binding antibodies in the sera and synovial fluid from patients with rheumatoid arthritis was determined.

Methods: The B cell epitopes in mouse G1 domain were mapped using a peptide array by which the binding of antibodies in the sera of arthritic mice was compared to that of naïve mice. The peptide array covered the entire G1 domain by 20-mer peptides, which overlapped by 19 residues. Subsequent analysis of selected epitope-binding autoantibodies was performed using biotinylated-peptide epitopes and conventional enzyme-linked immunosorbent assay.

Results: Multiple B cell epitopes were detected in arthritic mice immunized with either full-length proteoglycan or G1 domain. Autoantibodies directed against G1 peptides in proteoglycan-immunized mice were mainly of the IgM isotype, whereas G1-immunized mice possessed IgG autoantibodies to the same and additional epitopes. Preliminary results show that human rheumatoid arthritis, but not osteoarthritis, patients possess autoantibodies locally in the synovial fluid that bind to proteoglycan G1-derived epitopes. These autoantibodies were only incidentally detected in sera of patients with rheumatoid arthritis.

Conclusions: Both human proteoglycan- and G1 domain-immunized mice develop arthritis and possess autoantibodies to several B cell epitopes of mouse PG that are predominantly contained within four immunodominant regions. Arthritic patients were found to possess autoantibodies against similar targets in the synovial fluid, suggesting that antibodies against the B cell epitopes of the G1 domain play a role in the pathogenesis of human rheumatoid arthritis.

Introduction

Systemic immunization of BALB/c mice with human cartilage proteoglycan (PG) aggrecan induces progressive polyarthritis (PG-induced arthritis, PGIA) that closely resembles certain aspects of human rheumatoid arthritis (RA) as characterized by the clinical features and histopathology [1-3], the presence of anti-citrullinated protein antibodies (Abs) and rheumatoid factor [4] and its high prevalence in aged females [5,6]. PG, one of the most abundant components of cartilage, is a relevant autoantigen in RA since both T [7-10] and B cells [11] respond to PG protein in a significant number of RA patients.

Immunization of mice with the purified recombinant human G1 (rhG1) domain, the arthritogenic part of the PG molecule, induces a similar form of arthritis (G1 domain-induced arthritis, GIA) [4,12]. Differences between PGIA and GIA include that immunization with G1 exhibits a more uniform clinical presentation of disease and a stronger T_H1/T_H17 polarization accompanied with a higher IgG2a/IgG1 ratio of the Abs directed against mouse PG [4,12].

PGIA/GIA are T cell-dependent and antibody/B cell-mediated autoimmune diseases [4,13-18], and either form of arthritis cannot be adoptively transferred into SCID mice using only one of these cell-types [14,16]. PG-specific T_H1 polarized T cells are indispensable for the induction of disease [4,12,19-21], whereas B cells are required for the induction of severe PGIA both as antigen-presenting cells [15,22,23] and for the production of autoantibodies (autoAbs) [13,15,17,18]. The $CD4^+$ T cell response has been relatively well characterized and three immunodominant arthritogenic T cell epitopes are localized in the G1 domain of PG [12,19,24].

Earlier studies have shown that autoAbs directed against mouse PG are good biomarkers for the development of disease since their presence correlates well with both the incidence and the severity of PGIA/GIA [4,12,14-16,25], but like B cells from arthritic mice, these PG specific autoAbs alone are insufficient to transfer disease [14,23,26].

AutoAbs elicited against mouse PG consist of various immunoglobulin isotypes, prevailingly the IgG1 and the IgG2a isotypes. Abs of the IgG2a isotype correlate well with the onset and severity of arthritis [25,27], which may suggest a pathological role for this particular isotype. Other studies that used mice deficient in Fc receptors have shown however, that the development of PGIA is dependent on FcγRIII, but not on FcγRI [13]. FcγRI is a high affinity receptor for IgG2a, whereas FcγRIII is a low-affinity receptor for both IgG1 and IgG2a [13,28]. These reports thus suggest that Abs of both the IgG1 and the IgG2a isotypes are important in the development of disease.

Although the role of PG-specific isotypes in these arthritis models have been relatively well studied, the precise specificities of the pathogenic autoAbs have not been elucidated yet. PGIA is induced by systemic immunization with human PG, and a set of the polyclonal anti-PG Abs cross-react with the self-antigen mouse PG. However, the exact B cell epitopes are unknown. Immunization with synthetic peptides representing T-cell epitopes (either as a single peptide or as pooled peptides) has never induced arthritis [4], but a relatively short protein fragment (G1 domain, ~340 amino acids of the PG core protein) did. This suggests that indispensable arthritogenic B cell epitopes are additionally present in this T-cell epitope-containing region. The rhG1 domain used for GIA induction contains N- and O-linked oligosaccharides as well as keratan sulfate [4], but not a single chondroitin sulfate chain is present within the G1

domain [29,30]. This suggests that anti-chondroitin sulfate Abs are not likely involved in the induction or pathogenesis of GIA. Dayer et al. [31], showed that a monoclonal Ab directed against the protein-moiety of the G1-domain could deplete PG from articular cartilage *in vivo* (the loss of PG was confirmed by toluidine blue PG staining), whereas an anti-keratan sulfate monoclonal Ab did not. This suggests that Abs directed against the protein-backbone of the G1 domain might be involved in the development and pathology of autoimmune arthritis. PG also contains a domain termed G2 that consists of B- and B'-loops (Figure 2) highly similar to those of the G1 domain [32]. This indicates that B-loop specific Abs possibly can bind to the B-loops in both the G1 and the G2 domains of cartilage PG.
In the present study, the autoAbs in arthritic mice directed against the protein-backbone of the self-antigen mouse G1 were characterized and the differences in B cell epitope repertoires between PGIA and GIA were investigated. Immunodominant B cell epitopes in PGIA were detected, to which surprisingly mainly IgM Abs bound, whereas the level of IgG1 Abs was just above the background level. In GIA, the humoral immune response was different because high levels of IgG autoAbs were detected that bound either to the same epitopes as in PGIA, or to other B cell epitopes that are detected only in GIA. Analysis of synovial fluid samples of arthritic patients showed that Abs directed against G1 derived epitopes are present in the synovial fluid of RA patients as well, suggesting a possible pathogenic significance of G1 specific autoAbs in RA.

Methods

Mice

Female BALB/c mice (retired breeders) were purchased from Charles River Laboratories (Maastricht, the Netherlands) and kept under standard housing conditions at the Central Animal Laboratory of the Utrecht University. All animal experiments were approved by the Animal Ethics Committee from the Utrecht University.

Sera and synovial fluids from patients

Sera and synovial fluid samples were obtained from the University Medical Center Utrecht, the Netherlands. These samples included sera from RA patients with a high erythrocyte sedimentation rate (n=10, ESR; Age: 60.6 ± 14.1 mean ±S.D.; Female: 8/10; ESR: 55.1 ± 21.6 (mean ±S.D; mm/1st hr.), from RA patients (n=10) with a low ESR (Age: 52.4 ± 9.7 S.D.; Female: 7/10; ESR: 4.8 ± 1.9 mean ±S.D.), and from healthy subjects without a history of arthritis that were used as controls (n=7, Age: 27.3 ± 1.9 S.D.). In addition, synovial fluid samples were collected from RA patients (n=10, Age: 55.5 ± 17.7 S.D.; Female: 5/10), and osteoarthritic patients (Age: 66.3 ± 5.9 S.D.; Female: 4/6,). Collection of patient material was approved by the medical ethical committee (METC) of the University Medical Center Utrecht and written informed consent was obtained.

Immunizations and induction of PGIA and GIA

PG was isolated from human articular cartilage, deglycosylated with endo-β-galactosidase (EC 3.2.1.103; E-XBG01, QA-Bio) and subsequently depleted of chondroitin sulfate side chains with

testicular hyaluronidase as described [33]. rhG1-mFc2a (rhG1) fusion protein was prepared as described elsewhere [4]. PGIA was induced by two intraperitoneal administrations of 250 µg PG protein and 2 mg dimethyldioctadecylammonium bromide (DDA, Sigma) emulsified in 200 µl phosphate-buffered saline (PBS) on day 0 and day 21. GIA was induced by three intraperitoneal administrations of 100 µg rhG1 fusion protein and 2 mg DDA emulsified in 200 µl PBS on day 0, 21 and day 42. In both models, arthritis scores were determined using a visual scoring system to score paw inflammation on a scale of 0-4 per paw (foot plus ankle) as follows: 0: no signs of inflammation; 1: (a) starting inflammation characterized by light erythema and swelling of the paw or (b) signs of erythema/swelling in one/two digits; 2: (a) progression of inflammation in the paw characterized by an increase in erythema/swelling without contribution of the digits or (b) severe erythema/swelling of three/four digits; 3: (a) combined inflammation characterized by severe erythema/swelling of the paw with a maximum of three inflamed digits or (b) severe inflammation on three to four digits in combination with moderate swelling/redness of the paw; 4: severe inflammation of the entire paw characterized by tension of the paw's skin and signs of inflammation in all digits. Incidence of arthritis denotes the percentage of mice that develop an arthritis score of at least one. A cumulative score ranging from 0-16 is based on individual paw scores of 0-4.
Sera from PGIA mice (n=10) used for the B-cell epitope mapping studies and sera from PGIA mice (n=19) used for the peptide-specific enzyme-linked immunosorbent assays (ELISA) were collected from previous experiments. The animals used in the side-by-side comparison of PGIA (6/6 became arthritic; mean maximum arthritis severity score ± S.D.: 8.9 ± 1.0) and GIA (5/6 became arthritic; mean maximum arthritis severity score ± S.D: 8.8 ± 3.6) were sacrificed at day 70.

Epitope mapping

The G1 domain of mouse PG [GenBank: AAC37670] was synthesized as both linear and cyclic 20-mer peptide arrays [34]. Adjacent peptides were shifted by one amino acid residue so that 19 out of the 20 residues overlapped with their neighboring peptides (Additional File 2). Conformational loops were created by connecting the cysteine residues with a T3 CLIPS™ scaffold [35]. The identification of G1-domain binding sites was performed according to previously published protocols [34,35]. In short: The binding of antibody to each peptide was tested in a PEPSCAN-based ELISA. After pre-incubation with blocking solution (overnight at 4°C) and subsequent washing, the peptide arrays were incubated with sera from mice with PGIA (n=10) and sera from non-immunized naive control mice (n=6), at 1:1000 and 1:500 dilutions, respectively, in blocking solution (4% horse serum, 5% ovalbumin, 1% Tween-80 in PBS). After washing, the peptides were incubated with a 1000-fold dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse-immunoglobulin antibody (Dako) for one hour at 25° C. After washing, the peroxidase substrate solution (0.5 mg/ml 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 0.006% H_2O_2 in 0.05 M citrate buffer, pH 4) was added and after one hour incubation at room temperature the color development was measured. The color development was quantified with a charge-coupled device (CCD)-camera and an image processing system.

Peptide synthesis

The identified antibody binding sites in the G1-domain of mouse G1 domain were reconstructed as peptide mimics and synthesized by standard Fmoc chemistry [36]. CLIPS™ chemistry [37] was used to create stabile cyclic constructs of structured peptides. The peptides were synthesized with an amidated C-terminus and a N-terminal biotin moiety (for coating purposes in ELISA assays) with a 6-aminohexanoic acid spacer to create distance between the peptide sequence and biotin. The purity and identity of the peptides were analyzed by RP-HPLC (C18 column, Waters Corporate, Milford, MA, USA) and electrospray ionization mass spectrometry detection (Aqcuity UPLC-MS, Waters Corporate, Milford, MA, USA).

ELISA

Antibody responses against human PG, mouse PG and the peptides listed in Table 1 were determined by ELISA. Human and mouse cartilage PG aggrecan was purified by CsCl gradient centrifugation as described earlier [1]. Human- (0.5 µg / 100µl / well) and mouse PG (0.75 µg / 50µl / well) in bicarbonate buffer (pH 9.6) were directly coated onto 96-well ELISA plates (NUNC Maxisorp), and for anti-peptide antibody measurements, Avidin (0.4 µg / 100µl / well; Pierce) in bicarbonate buffer (pH 9.6) was coated on ELISA plates (NUNC Maxisorp) followed by incubation with the biotinylated peptides (0.05 µg / 100 µl/ well). Plates were blocked with a buffer containing 4% horse serum (Gibco) and 1% Tween-80 (Sigma). Sera were diluted (1:100 for the detection of peptide-binding Abs and 1:500 for the detection of isotype specific anti-PG Abs) in a phosphate-buffer containing 4% horse serum, 1% Tween-80 and 0.5 M NaCl. The PG/peptide specific Abs were determined using HRP-conjugated polyclonal rabbit anti-mouse immunoglobulins (P0260 from Dako), HRP-conjugated goat anti-mouse IgG1 (Cat. 1070-05), IgG2a (1080-05), IgG2b (1090-05), IgG3 (1100-05) or IgM (1020-05, all from Southern Biotech Association).
Abs in synovial fluid or sera from RA- and osteoarthritis patients (diluted 1:300) was determined in a similar matter, using HRP-conjugated polyclonal goat anti-human Ig (IgM+IgG+IgA, H+L; Cat. 2010-05 from Southern Biotech Association) and expressed as an optical density (O.D.) after subtraction of the background wells with avidin coated only ($O.D. = O.D._{\text{avidin + indicated peptide}} - O.D._{\text{avidin only}}$).

ELISpot

The number of antibody-secreting cells (ASCs) against either G1-derived peptides or PG-protein was enumerated. MultiScreen IP Filter Plate plates (Millipore) were activated with 70% ethanol, coated with avidin (0.4 µg / 100µl / well; Pierce) or human PG-protein (see above) and subsequently blocked with 250 µl/well IMDM culture medium (Gibco) containing 5% fetal calf serum (Gibco). For the enumeration of anti-G1 peptide ASCs, a pool of G1-derived peptides (Hu A, B1, B2, B3, Hu B'1, B'2 and Hu B'3; see Table 1; each at ~7 ng; total 0.05 µg / 100 µl / well) was incubated for 2 hours with avidin-coated wells in a subsequent step. After washing, serially diluted total splenocytes and bone marrow cells were incubated for 24h. ASCs were detected using an alkaline phosphatase-conjugated goat anti-mouse IgG detection-antibody (Cat. 1030-04 from Southern Biotech Association) and the spots were visualized using NBT/BCIP tablets (Roche) according to manufacturer's instructions. Enumeration of

ASCs was done with an Automated ELISpot Assay Video Analysis System (A.EL.VIS GmbH).

Three-dimensional (3D) modelling of the G1 domain

Theoretical structures for the mouse G1 domain were assembled via protein homology modeling using SWISS-MODEL Workspace (swissmodel.expasy.org). Molecule coordinate files of A-loop, B-loop and B'-loop were created based on SWISS-MODEL repository templates 1qz1A, 1o7bT and 1pozA respectively [38,39]. Protein 3D structures were visualized and epitopes were highlighted with RasMol (rasmol.org) software [40].

Statistical analysis

Statistical analysis was carried out using Prism 4.0 (GraphPad software Inc.). Two-tailed Student's t-tests and Mann-Whitney U tests were applied where indicated. Differences were considered significant at $p < 0.05$.

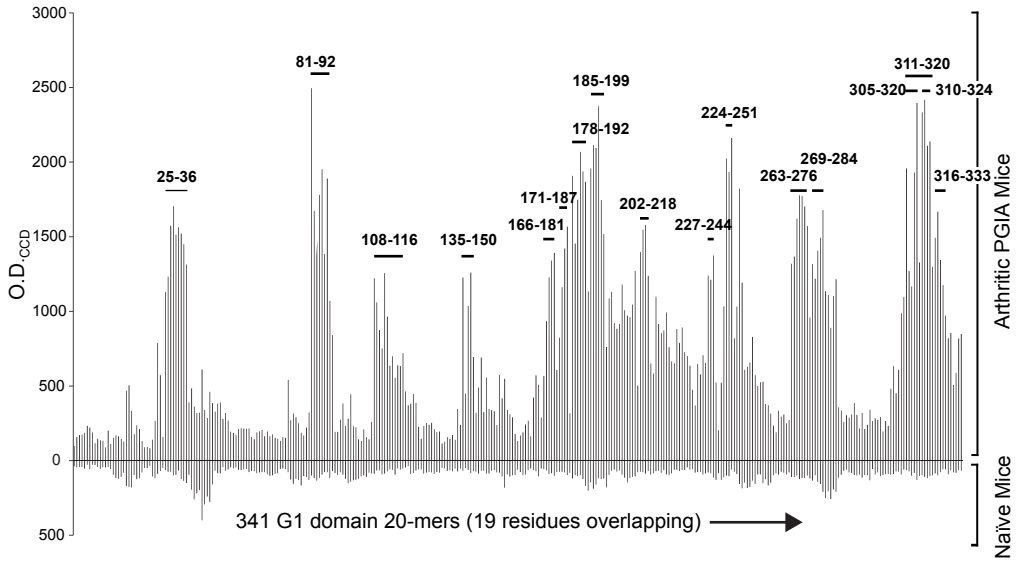


Figure 1: B cell epitope mapping of the G1-domain of mouse PG aggrecan using sera from arthritic PGIA mice. A total of 341 peptides covering the entire G1 domain of mouse PG were synthesized as 20-mer peptides (overlapping by 19 residues). The B-cell epitopes on G1 domain were subsequently mapped by comparing the binding of sera obtained from arthritic PGIA mice (n=10; average on positive y-axis) with that from naive control mice (n=6; average on negative y-axis) to all 341 peptides. The binding intensity of Abs (y-axis; O.D.CCD: determined by CCD camera) to the 341 linear 20-mer peptides (x-axis) is shown. The horizontal bars above the binding intensities indicate the sequence regions from which the peptides used for subsequent analyses were derived of (Additional File 2 shows a representative example). The numbers above the bars are the position of the first and last amino acids of these peptide-epitopes (see Table 1 for details).

Results

Mapping-study of the auto-antigen PG G1 domain reveals its B-cell epitopes

A total of 341 peptides covering the entire G1 domain of mouse PG (including its leader sequence) were synthesized as 20-mer peptides, either as linear-fragments or as small cyclic loops. The B-cell epitopes on G1 were subsequently mapped by comparing the binding of sera obtained from mice with PGIA to that from naive control mice to the 341 peptides. The cyclic peptides were created by the introduction of extra N- and C-terminal cysteine residues that were connected by a CLIPS™ moiety. Antibody binding to both linear (Figure 1) and cyclic peptides (Additional File 1) was determined and the binding of Abs with the 20-mer linear-fragments was highly similar to the binding to the corresponding 20-mer cyclic peptides.

Selection of B-cell epitopes for detailed examination

A selection of 17 immunodominant linear peptides was made based on the B-cell mapping data presented in Figure 1. The selected peptides were typically derived from the minimally required core sequence present in a set of overlapping peptides that were still recognized by Abs (see Additional File 2 for a representative example). Biotinylated versions of the selected peptides were synthesized to allow further analysis of the binding Abs. The selected peptides and their location in the G1 domain are schematically depicted in Figure 2 and listed in Table 1. The selected peptides include 17 linear peptides, six cyclic peptides (representing loops from the B- and B'-domains) and one discontinuous peptide (consisting of sequences around the two cysteine residues in the A-loop of the G1 domain).

Humoral immune response and autoAbs in PGIA and GIA

Immunization with either whole PG or G1 induces arthritis in mice. We compared the humoral immune response in GIA and PGIA arthritic animals by ELISpot and found significantly more ASCs directed against pooled peptides derived from G1 in the spleen (Figure 3A) and bone marrow (Figure 3B) collected from mice with GIA than from mice with PGIA. The number of ASCs directed against whole human PG where equal in PGIA and GIA mice in both locations (Figures 3C and 3D). The various antibody isotype specific responses against human (Figure

Table 1: Synthesized peptides of selected putative B cell epitopes

A total of 17 linear mouse peptides were synthesized based on the results obtained in the B cell epitope mapping (see Figure 1). Ten of the 17 sequences were identical with the human peptides, and the human counterparts for the seven remaining mouse peptides were synthesized as well. A total of eight cyclic epitope were synthesized: two peptides were mouse specific, two peptides represent the human sequence analogues of these and four cyclic peptides were identically between mouse and human. One discontinues mouse epitope (A in Figure 2) and its human counterpart is included as well. Two glycine residues (***GG***, printed in bold italics) were used to link the individual sequences of the discontinuous epitopes. The positions of the peptides in the G1 domain are based upon the sequence of mouse PG aggrecan with subtraction of the first 19-amino acid long leader sequence. The differences between the human and the mouse sequences are underlined and printed in bold. Ahx = 6-aminohexanoic acid spacer; Bio = Biotin. L: linear peptide; D: discontinuous peptide; C: cyclic peptide.

Name	Sequence	Mouse / Human / Identical	Linear / Discontinuous / Cyclic	G1 Loop
25-36	Bio-Ahx-^{25}GSSLTIPCYFID$_{36}$	M	L	A
Hu 25-36	Bio-Ahx-^{25}GS**T**LTIPCYFID$_{36}$	H	L	A
81-92	Bio-Ahx-^{81}YQDKVSLPNYPA$_{92}$	I	L	A
108-116	Bio-Ahx-^{108}DSGIYRCEV$_{116}$	M	L	A
Hu 108-116	Bio-Ahx-^{108}DSG**V**YRCEV$_{116}$	H	L	A
135-150	Bio-Ahx-^{135}VFHYRAISTRYTLDFD$_{150}$	I	L	B
166-181	Bio-Ahx-^{166}PEQLQAAYEDGFHQCD$_{181}$	I	L	B
171-187	Bio-Ahx-^{171}AAYEDGFHQCDAGWLAD$_{187}$	I	L	B
178-192	Bio-Ahx-^{178}HQCDAGWLADQTVRY$_{192}$	I	L	B
185-199	Bio-Ahx-^{185}LADQTVRYPIHTPRE$_{199}$	I	L	B
202-218	Bio-Ahx-^{202}YGDKDEFPGVRTYGIRD$_{218}$	I	L	B
227-244	Bio-Ahx-^{227}CFAEEMEGEVFYATSPEK$_{244}$	I	L	B'
234-251	Bio-Ahx-^{234}GEVFYATSPEKFTFQEAA$_{251}$	I	L	B'
263-276	Bio-Ahx-^{263}TTGQLYLAWQGGMD$_{276}$	M	L	B'
Hu 263-276	Bio-Ahx-^{263}TTGQLYLAWQ**A**GMD$_{276}$	H	L	B'
269-284	Bio-Ahx-^{269}LAWQGGMDMCSAGWLA$_{284}$	M	L	B'
Hu 269-284	Bio-Ahx-^{269}LAWQ**A**GMDMCSAGWLA$_{284}$	H	L	B'
305-220	Bio-Ahx-^{305}GVRTVYLHANQTGYPD$_{220}$	M	L	B'
Hu 305-220	Bio-Ahx-^{305}GVRTVY**V**HANQTGYPD$_{220}$	H	L	B'
310-324	Bio-Ahx-^{310}YLHANQTGYPDPSSR$_{324}$	M	L	B'
Hu 310-324	Bio-Ahx-^{310}Y**V**HANQTGYPDPSSR$_{324}$	H	L	B'
311-320	Bio-Ahx-^{311}LHANQTGYPD$_{320}$	M	L	B'
Hu 311-320	Bio-Ahx-^{311}**V**HANQTGYPD$_{320}$	H	L	B'
316-331	Bio-Ahx-^{316}TGYPDPSSRYDAICYT$_{331}$	I	L	B'
A	Bio-Ahx-^{24}LGSSLTIPCYFIDP$_{37}$***GG***$_{104}$LRSNDSGIYRCEV$_{116}$	M	D	A
Hu A	Bio-Ahx-^{24}LGSSLTIPCYFIDP$_{37}$***GG***^{104}LRSNDSG**V**YRCEV$_{116}$	H	D	A
B1	Bio-Ahx-^{156}CLQNSAIIATPEQLQAAYEDGFHQC$_{180}$	I	C	B
B2	Bio-Ahx-^{180}CDAGWLADQTVRYPIHTPREGC$_{201}$	I	C	B
B3	Bio-Ahx-^{201}CYGDKDEFPGVRTYGIRDTNETYDVYC$_{227}$	I	C	B
B'1	Bio-Ahx-^{254}CRRLGARLATTGQLYLAWQGGMDMC$_{278}$	M	C	B'
Hu B'1	Bio-Ahx-^{254}CRRLGARLATTGQLYLAWQ**A**GMDMC$_{278}$	H	C	B'
B'2	Bio-Ahx-^{278}CSAGWLADRSVRYPISKARPNC$_{301}$	I	C	B'
B'3	Bio-Ahx-^{301}CGGNLLGVRTVYLHANQTGYPDPSSRYDAIC$_{329}$	M	C	B'
Hu B'3	Bio-Ahx-^{301}CGGNLLGVRTVY**V**HANQTGYvPDPSSRYDAIC$_{329}$	H	C	B'

3E) and mouse PG (Figure 3F) were determined by ELISA. The PG-specific isotype profiles were overall quite similar in the two arthritis models, except that arthritic PGIA animals had a significantly higher IgM-response against these antigens than mice in which GIA was induced (Figure 3E and 3F).

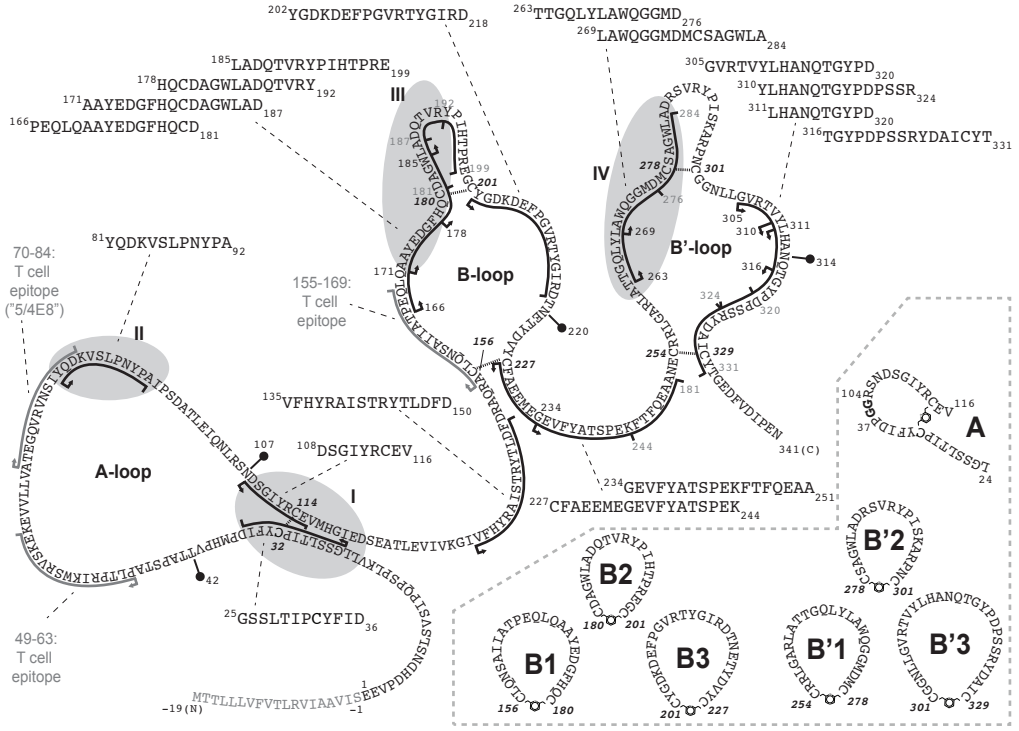


Figure 2: Schematic presentation of the G1 domain of mouse PG aggrecan and the peptide-epitopes that were selected for further analysis by ELISA.

The mouse G1 domain is shown together with the position of the 17 linear, the 6 cyclic (named B1, B2, B3, B'1, B'2 and B'3) and the discontinuous peptide (named A) that were selected and subsequently synthesized based on the results of the B cell epitope scan presented in Figure 1. The positions of the epitopes are based upon the mature protein, i.e., the sequence of mouse aggrecan after deletion of the first 19 amino acid long leader sequence (indicated in gray). The sequences that form the discontinuous peptide A are linked by two glycine residues (bold). Three known arthritogenic T cell epitopes are also indicated in the schematic drawing (gray lines and descriptive text) [12,19,24]. The N- and O-linked glycosylation consensus sites, as described in the literature [29] for the bovine G1 domain, are indicated with black circles: O-linked at position 42 and N-linked at position 107, 220 and 314. Keratan sulfate side chains are present on position 42, 220 and 314 whereas a high mannose oligosaccharide is found on position 107 (all for bovine PG). The oval gray areas numbered with roman numerals indicate the immunodominant regions/epitopes described in the discussion.

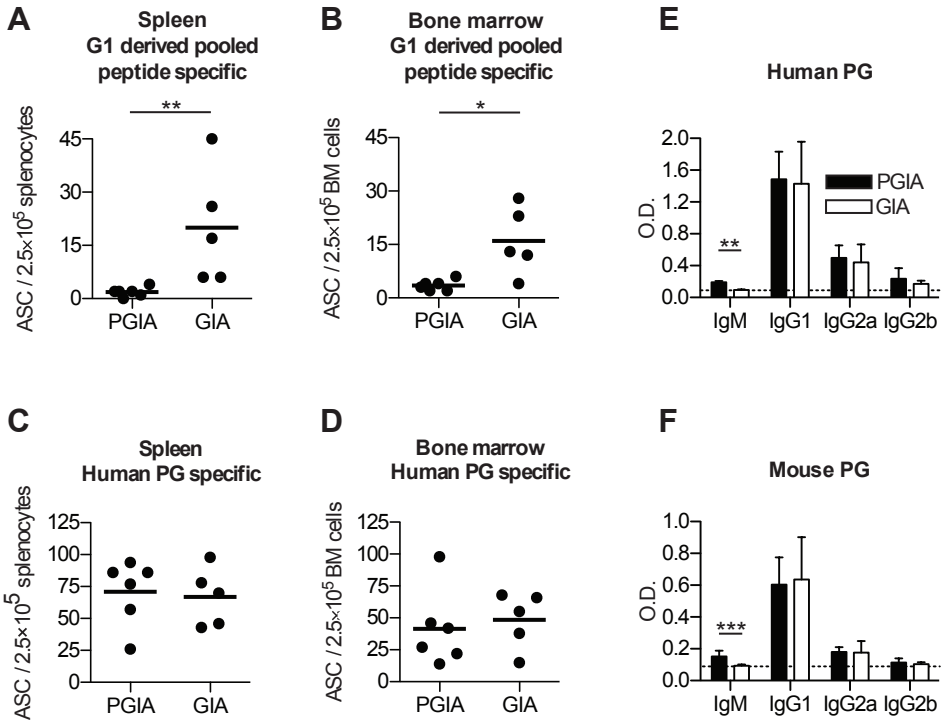


Figure 3: G1 domain specific humoral immune response in PGIA and GIA mice.

ASCs against a pool (see Materials) of G1 derived peptides (A and B) and full-length PGs (C and D) are enumerated in both the spleen (A and C) and the bone marrow (B and D) of mice with PGIA or GIA. ELISA results of sera derived from PGIA and GIA animals against human (E) and mouse PGs (F) are shown. Data represent 6 PGIA (black bars) and 5 GIA (white bars) mice. The dashed line (E and F) indicates the background (no sera) O.D. Statistical significant was calculated using the Mann Whitney U test (A-D) or the student t-test (E and F). *: $p< 0.05$; **: $p<0.01$; ***: $p<0.01$.

Antibody binding to selected G1 peptides from sera of mice with PGIA or GIA

Immunoglobulin binding (sera from arthritic PGIA mice) to the peptides presented in Table 1 was determined by ELISA. Several of the tested G1 peptides showed binding above background level (defined as binding to an irrelevant OVA-peptide ^{323}ISQAVHAAHAEINEAGR$_{339}$). Among these peptides, some 'immunodominant' epitopes (defined by a relative high O.D. in ELISA as compared with OVA-peptide) could clearly be distinguished from other peptides to which the responses were lower (Figure 4A). Virtually all of the 19 PGIA mice developed Abs against the first region of B'-domain as represented by cyclic peptide B'1, ^{254}CRRLGARLATTGQLYLAWQGGMDMC$_{278}$, which largely comprises linear peptides ^{263}TTGQLYLAWQGGMD$_{276}$ and ^{269}LAWQGGMDMCSAGWLA$_{284}$. Other apparent immunodominant epitopes were represented by linear peptide ^{178}HQCDAGWLADQTVRY$_{192}$ located in the B-domain, and linear peptide ^{316}TGYPDPSSRYDAICYT$_{331}$ located in the B'-domain. Cyclic epitope B2 (^{180}CDAGWLADQTVRYPIHTPREGC$_{201}$) and B'2 (^{278}CSAGWLADRSVRYPISKARPNC$_{301}$)

also scored relatively high in some PGIA mice. The immunodominant epitopes in the ELISA roughly correspond to immunodominant peaks in the B-cell epitope mapping experiment (Figure 1), although not all hits from the mapping experiment could be confirmed by ELISA. Compared to PGIA mice (Figure 4A), Abs to other, and the same, B cell epitopes were detected in the G1 domain in the sera from GIA mice (Figure 4B). Peptides to which Abs bound after induction of GIA included linear peptide ^{25}GSSLTIPCYFID$_{36}$, which had only an intermediate O.D. after PGIA induction. Mice that possessed Abs against epitope ^{25}GSSLTIPCYFID$_{36}$ also had Abs to the corresponding discontinuous peptide A; ^{24}LGSSLTIPCYFIDP$_{37}$-GG-^{104}LRSNDSGIYRCEV$_{116}$ (Figure 4B). Moreover, linear epitope ^{81}YQDKVSLPNYPA$_{92}$ gave a very high O.D. unseen with any of the other peptides either in PGIA or in GIA. Linear epitope ^{178}HQCDAGWLADQTVRY$_{192}$ and cyclic epitopes B2 and B'2 scored relatively high in GIA as well (Figure 4B), in a comparable manner as in PGIA (Figure 4A).

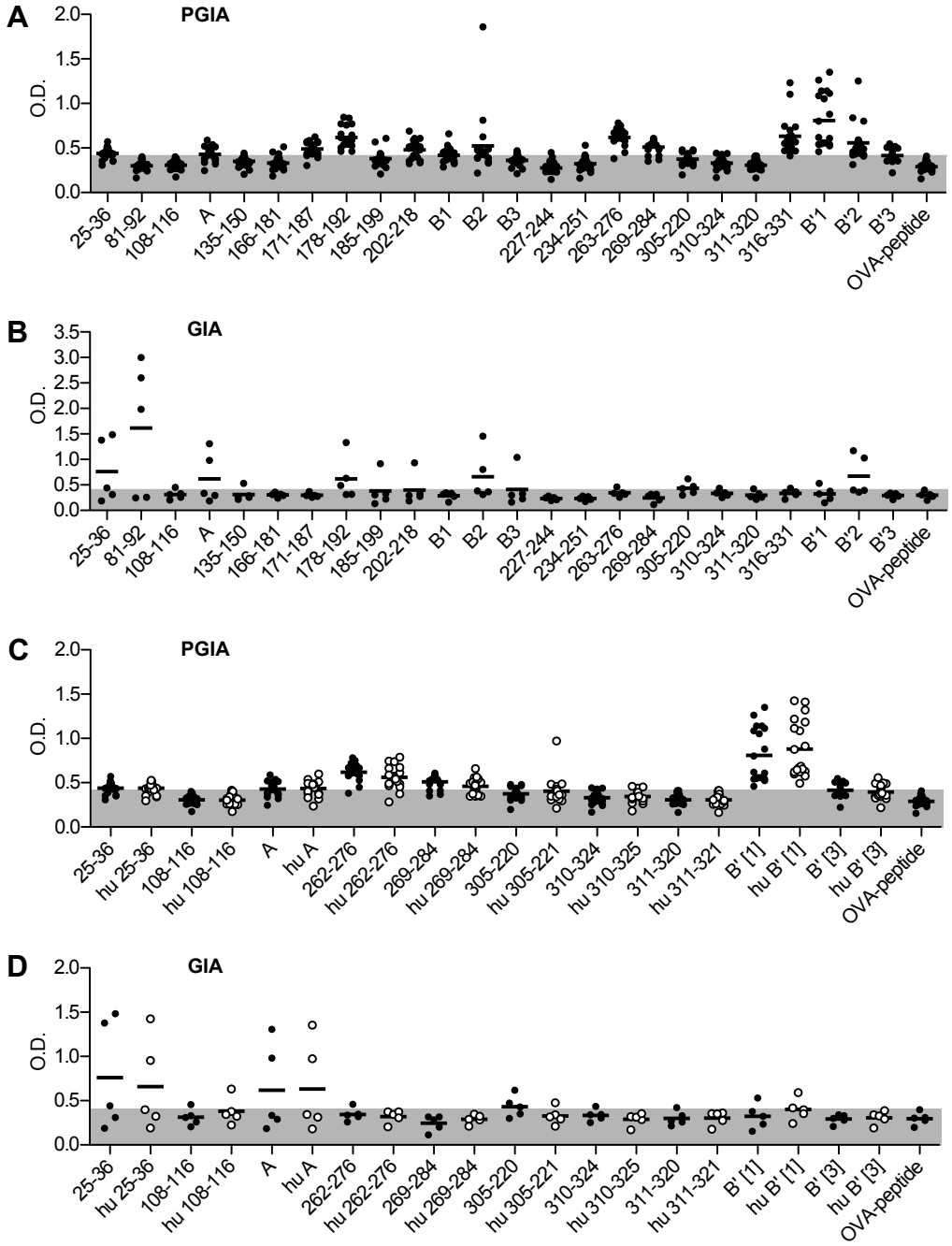
Side-by-side analysis of the binding of Abs to the human sequence analogs of the mouse-derived G1 peptide-epitopes, suggested that the Abs directed against mouse-derived sequences cross-react with human sequences in mice with either PGIA (Figure 4C) or GIA (Figure 4D). Comparison of epitope-recognition in single mice reveal that each individual mice developed Abs against a seemingly random combination of immunodominant epitopes, i.e., not all arthritic mice developed Abs against all peptides, and individual mice that showed relative high responses against one immunodominant peptide did not necessarily develop high antibody responses against other peptides (Figure 5).

Autoantibody isotypes

Abs directed against cyclic epitope ^{254}CRRLGARLATTGQLYLAWQGGMDMC$_{278}$ (B'1; relative high in PGIA and low in GIA) and linear epitopes ^{81}YQDKVSLPNYPA$_{92}$ (high in GIA; virtually absent in PGIA) and ^{178}HQCDAGWLADQTVRY$_{192}$ (moderate in both PGIA and GIA) were further characterized (Figure 6). Analysis of the epitope-specific autoAbs in the sera of arthritic PGIA mice shows that these immunoglobulins were mostly of the IgM isotype. Relative high IgM responses against specific epitopes were accompanied by very low (near detection limit) IgG responses (Figure 6A). Antibody isotype analysis of sera from arthritic GIA animals revealed that the humoral immune response directed against linear epitope ^{81}YQDKVSLPNYPA$_{92}$ consisted of switched IgG immunoglobulins in the majority of mice (Figure 6B). The Abs directed against other epitopes with intermediate responses in arthritic GIA animals (e.g. to linear epitope ^{178}HQCDAGWLADQTVRY$_{192}$) showed an isotype-pattern with, in contrast, dominantly IgM Abs similar to the Abs found in arthritic PGIA animals, although switched IgG immunoglobulins were occasionally found as well (Figure 6C).

Figure 4: Binding of immunoglobulins in sera of arthritic animals with synthesized G1 derived peptide epitopes.

(A and B) Binding of immunoglobulins of mice with PGIA (A) or GIA (B) to mouse G1-derived peptide epitopes (presented in Table 1) was determined by ELISA. (C and D) Side-by-side comparison of binding of immunoglobulins to mouse-specific peptide sequences (black circles) and their human homologous counterparts (white circles). OVA-peptide (irrelevant peptide) was included as a negative control and the gray area indicates the cut-off O.D.



level that is based upon the highest binding of immunoglobulins to OVA-peptide detected. Sera from naive control mice were included as negative controls, and no binding above background levels was detected to any of the peptides (Additional File 3). Data are representative of at least two independent ELISA measurements. The shown dataset includes 19 diseased PGIA mice (A and C) from earlier experiments and 5 GIA mice (B and D).

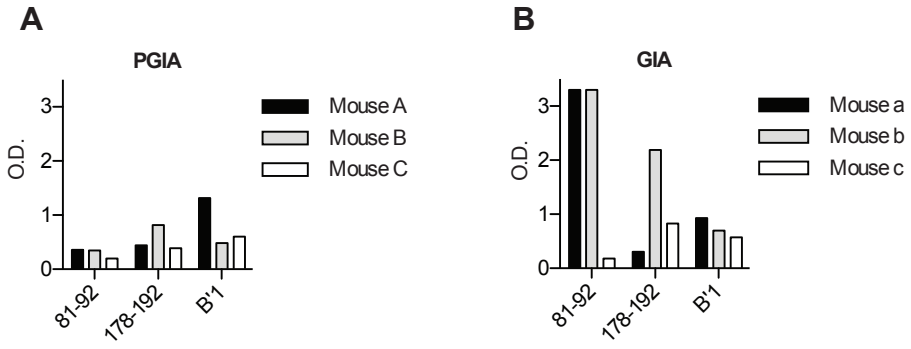


Figure 5: Differences in epitope-recognition between individual mice.

Three representative dominant epitopes (indicated on the x-axis) were selected to compare the antibody responses of three individual mice with PGIA (A) or GIA (B). Please note the heterogeneity in the epitope selection: mice with a relative high response against one specific epitope did not necessarily developed high responses against other epitopes.

RA-patients possess Abs against G1 derived epitopes

To investigate if RA patients possess Abs against G1 derived peptides in a similar manner as arthritic mice, we examined sera and synovial fluid by ELISA. Comparison of sera from RA-patients with sera from healthy controls revealed that the G1 peptide-specific Ab-levels in RA-patients only incidentally differed from those found in healthy controls (Additional File 5). Analysis of synovial fluids on the other hand, showed that a substantial number of RA patients (8 of 10) possess Abs against G1 derived peptides locally in the synovial fluid (Figure 7A), whereas these Abs were mostly absent from synovial fluid from patients diagnosed with osteoarthritis (Figure 7B).

Discussion

In this study we aimed to elucidate and compare the immunodominant B cell epitopes on the protein-backbone of the G1 domain of the self-Ag mouse PG after induction of PGIA and GIA. In addition, we tested the sera and synovial fluids of patients with RA or osteoarthritis for the presence of Abs against a selection of epitopes of the G1 domain of the self-Ag human PG. In summary, four B cell-specific immunodominant regions were identified in mice (schematically indicated in Figure 2); Abs to some of them were also detected in synovial fluids or RA patients:

I. The area directly around cysteine-residues 32 and 114 in the A loop (represented by linear epitope ^{25}GSSLTIPCYFID$_{36}$ and discontinuous epitope A).
II. Linear epitope ^{81}YQDKVSLPNYPA$_{92}$, partly overlapping with immunodominant T cell epitope ^{70}ATEGRVRVNSAYQDK$_{84}$ in human G1 domain.
III. Part of the B loop (represented by cyclic peptides B1 and B2, and linear epitopes that include epitope ^{178}HQCDAGWLADQTVRY$_{192}$).
IV. Part of the B' loop that is represented by cyclic peptide B'1 (and to a lesser degree B'2) and linear peptide ^{263}TTGQLYLAWQGGMD$_{276}$ (and ^{269}LAWQGGMDMCSAGWLA$_{284}$). This latter

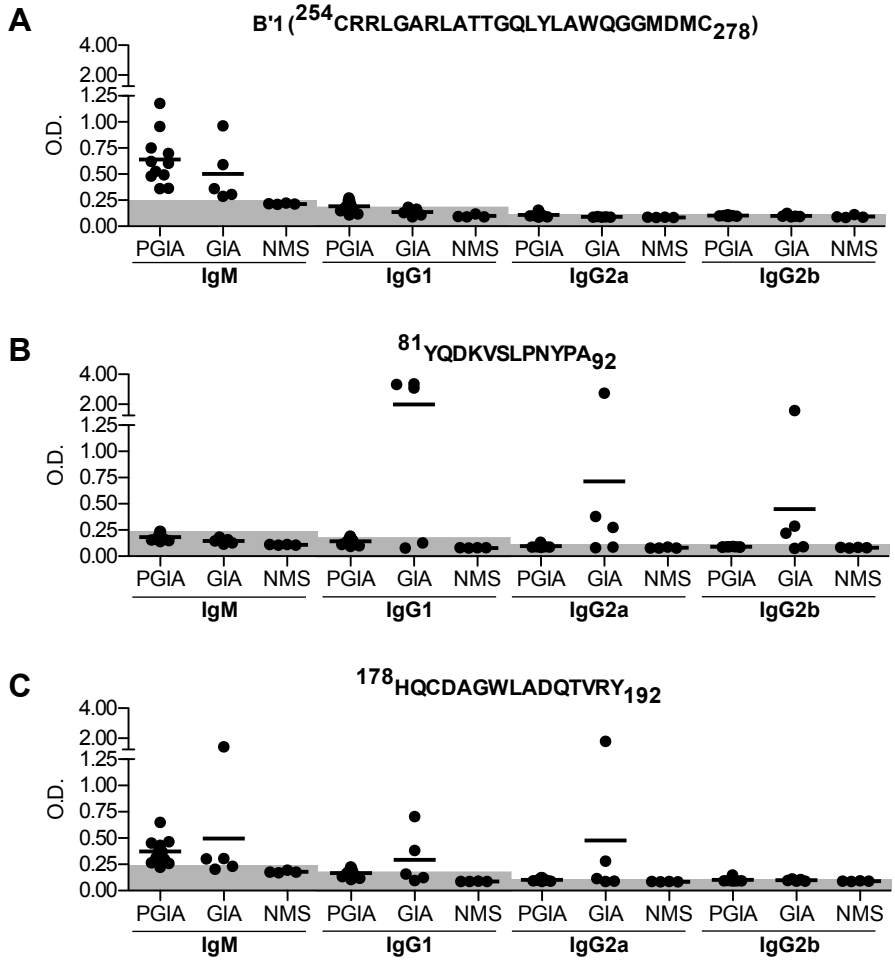


Figure 6: Immunoglobulin isotypes of Abs against G1 derived-epitopes after induction of PGIA and GIA. The presence of immunoglobulins isotypes specific for three selected epitopes in the sera of arthritic PGIA and GIA mice were analyzed and compared. Sera from naive control mice were included as negative control sera. OVA-peptide (irrelevant peptide) was included as a negative control peptide and the gray area indicates the cut-off O.D. level that is based upon the highest binding of immunoglobulins to OVA-peptide detected (Additional File 4).

epitope notably has a high sequence analogy with linear epitope ^{178}HQCDAGWLADQTVRY$_{192}$ from the previous immunodominant (CSAGWLA$_{284}$ vs CDAGWLADQTVRY$_{192}$) area, which indicates that cross-reactive Abs might be responsible for the binding of these two regions. In a 3D molecular modelling of the G1 domain (Figure 8), we found that all amino acids of the above identified immunodominant B cell regions/epitopes are represented as surface structures, and in contrast to other areas, all available for Ab-binding. Discontinuous peptide A is especially interesting, because both its units are on the surface (on both sides of the 3D structure), and not covered or hidden with either the interaction between the A loops of PG

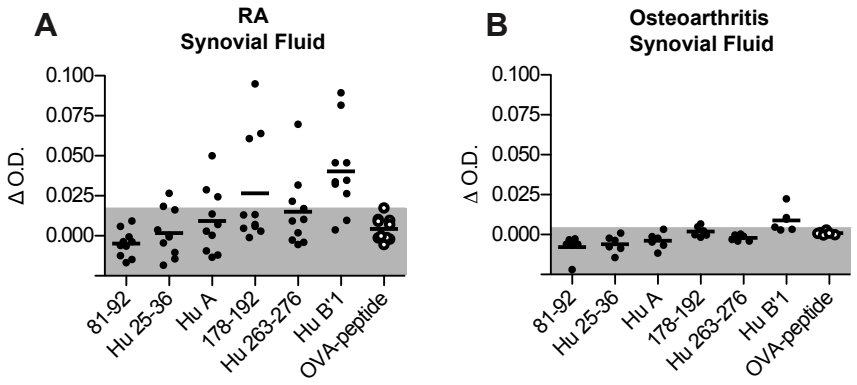


Figure 7: Abs in the synovial fluid from RA patients bind to G1 derived peptide epitopes.

Synovial fluid (n=9) from patients diagnosed with RA (A) or synovial fluid (n=6) from osteoarthritis patients (B) was analyzed by ELISA for Abs binding to six human G1 derived peptide epitopes. OVA-peptide (irrelevant peptide) was included as a negative control peptide and the gray area indicates the cut-off O.D. level that is based upon the highest binding of immunoglobulins to OVA-peptide detected. The binding of Abs is expressed as the O.D. after subtraction of the background levels (wells coated with avidin only). The background levels ($O.D._{avidin\ only}$) ranged between (A) 0.088 - 0.132 (Mean ± S.D.: 0.113 ± 0.014) and (B) 0.086 - 0.128 (Mean ± S.D.: 0.099 ± 0.016).

and link protein [41,42], nor the region of the hyaluronan-binding B loop [41]. Nevertheless, a high O.D. was only detected against the $^{25}GSSLTIPCYFID_{36}$ unit. Comparison of sera from arthritic animals indicated that diseased PGIA mice relatively often form Abs against the B and B' loops, whereas the A loop seemed to be immunodominant after induction of GIA. Immunoglobulins directed against the A loop in GIA were furthermore of the switched IgG isotypes, while Abs against the B loop in PGIA consisted mainly of the IgM isotype. A study by Dayer et al. [31] has shown before that both IgG and IgM directed against the protein-moiety of the G1-domain can deplete cartilage from the knees *in vivo,* but only the IgM monoclonal Ab (with unknown exact specificity) caused mononuclear cell infiltration and hyperplasia. The IgM Abs detected in PGIA thus may possess pathological roles and/or be involved in the development of experimental arthritis.

In another study, performed by Zhang et al. [43], arthritis was induced via hyperimmunization with bovine G1, and the B cell epitopes were subsequently mapped on the immunizing Ag itself. There is a significant amount of sequence analogy between the bovine, human and mouse G1 domain [44], and Zhang's B cell epitope mapping study showed that bovine G1's immunodominant areas are similar or resemble the ones on the self-Ag mouse G1 used in our study. Zhang et al. [43] also showed that Abs bound to mainly three areas when intact G1 was used for immunization, and to multiple other sides when G1 was depleted of its keratan sulfate side chains. Intact G1 induced Abs against (a) a peptide that consisted of the first 25 residues of mature G1 (partly overlapping with the area between position 17 and 25 that scores high in our epitope mapping but not with epitope $^{25}GSSLTIPCYFID_{36}$), (b) a 27 amino acid long peptide that overlapped with 9 (out of 12) residues of epitope $^{81}YQDKVSLPNYPA_{92}$

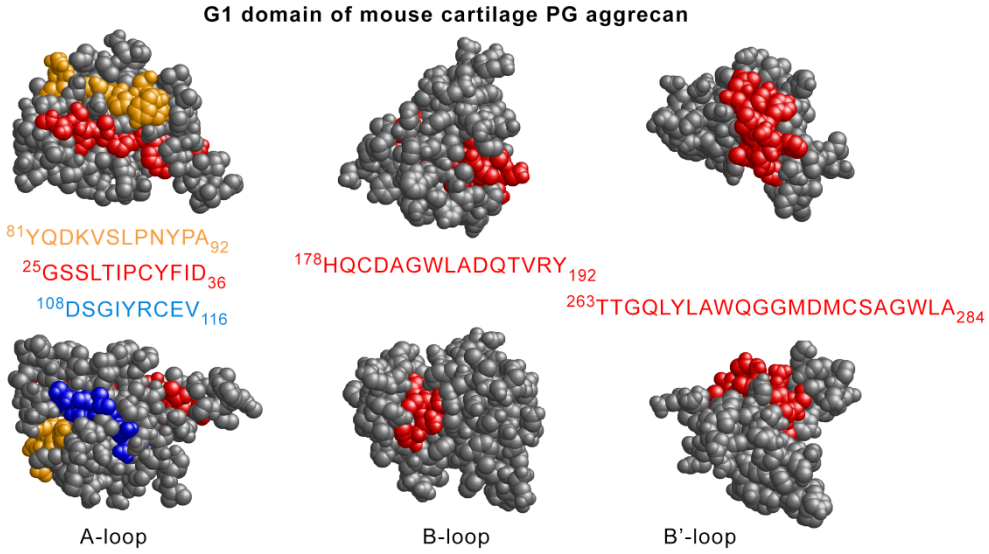


Figure 8: Molecular modeling of the mouse G1 domain shows that the antibody-binding areas are represented as surface structures

The immunodominant B cell epitopes were color-labeled in a 3D molecular model. The 3D model was rotated to a position where all amino acids were visible in either the upper and/or the lower 2D print. The three domains of the original model are shown separately in order to show the optimal angle in which all amino acids of the peptides can be seen. In loop A, the two arms of the discontinuous peptide are labeled in red and blue.

(immunodominant area II defined above) and (c) a 31 amino acid peptide from the B' loop that overlaps largely with immunodominant area IV. G1 devoid of keratan sulfate induced Abs against multiple other epitopes, the most prominent (highest O.D.) being a peptide from the B loop that corresponding with immunodominant area III.

Shi et al. [45], immunized mice with sf9-insect cell line-expressed human G1 domain and mapped the B cell epitopes on the same Ag template. This study found two immunodominant areas: First, a set of peptides covering the entire B loop scored relatively high, but a 24 amino acid long peptide that included the sequence of epitope ^{178}HQCDAGWLADQTVRY$_{192}$ in our study (immunodominant area III) clearly stood out. The second immunodominant area contained two peptides from the B' loop that corresponded with our immunodominant area labeled IV. Overall, the above listed studies indicate that the immunodominant B cell epitope areas in bovine, human and mouse G1 are quite similar. The notion that immunodominant areas can be protected by keratan sulfate side chains [43,46], which is an age-related posttranslational modification of the G1 domain [29,46,47] may further explain differences between the earlier studies and the differences seen between PGIA and GIA in the current study.

Humoral autoimmunity to citrullinated proteins in RA has gained increasing attention in recent years [48,49]. The G1 domain of human PG aggrecan contains 22 arginine residues (20 in the mouse G1 domain), of which 10 (both in the human and mouse G1 domain) are

on the surface of the 3D structure, thus all are potential sites of citrullination by peptidyl arginine deiminase-4 [50,51]. Recently, we found that PG aggrecan is citrullinated, especially in aging articular cartilage, and the citrullination sites were identified in the G1 domain of human PG aggrecan ([52] and manuscript in preparation). Future studies should point out whether citrullinated versions of the arginine-containing B cell epitopes in the G1 domain are present and are targeted in either (P)GIA or RA.

Our finding that the synovial fluid of RA patients contains autoAbs that bind G1 derived peptide-epitopes, suggests that Abs against PG's G1 domain play a role in RA as well. We found these autoAbs locally in the synovial fluid and clear differences between the presences of Abs were only incidentally detected when sera from RA patients were compared to those of healthy controls. This finding corresponds with other studies [11,46], in which autoAbs against PG core of the G1 domain were detected only in the synovial fluids but not in the sera of the same patients. These autoAbs are most likely produced by B cells in the inflamed rheumatoid joints.

Conclusions

Both human proteoglycan- and G1 domain-immunized mice develop arthritis and possess autoAbs to several B cell epitopes on the G1 domain of mouse PG. The most prominent B cell epitopes are contained with four immunodominant regions. Patients with RA were shown to possess autoAbs against corresponding regions in the human G1 domain in the inflammation site (the synovial fluid). This further validates the PGIA and GIA arthritis models and triggers the question whether the G1 specific Abs play an important role in the pathogenesis of PGIA/GIA, and possibly in human RA as well. Future studies are needed to elucidate the exact roles of these autoAbs and the B cells that produce them, and to investigate if they are possible targets for new diagnostic or therapeutic strategies in RA.

Contribution statement

BM: designed and performed research, analyzed and interpreted data (ELISA), discussion of results, drafted and revised the manuscript. RSB: designed and performed research, analyzed data and interpreted (epitope mapping), discussion of results, drafted and revised the manuscript. FB, RvdZ, WHW: conceived the project, designed research, discussion of results, and participated in writing and critically revising the manuscript. IdB, ISL,JS and AV: performed research (IdB: PGIA and GIA; ISL, JS: ELISA, AV: Figure 8) and participated in writing and critically revising the manuscript. FPJGL, JAGvR: undertook recruitment of patients and collection of patient samples and participated in writing and critically revising the manuscript. TTG, WvE: participated in critically revising the manuscript. All authors read, revised and approved the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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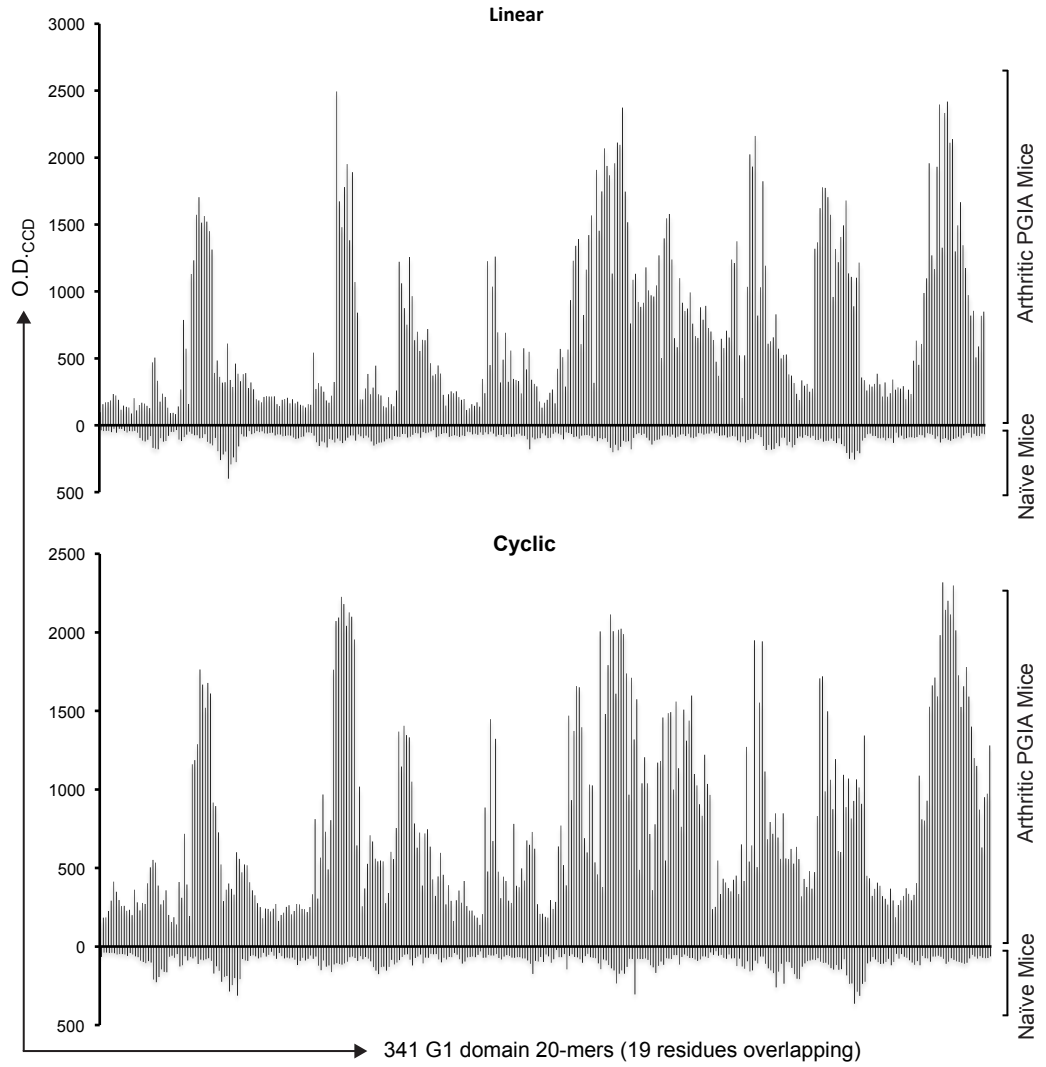
Additional File 1



Additional File 1:

Antibody binding to both linear (upper lane) and cyclic peptides (lower lane) derived from mouse G1 domain was determined. The binding of Abs with the 20-mer linear-fragments was highly similar to the binding to the corresponding 20-mer cyclic peptides.

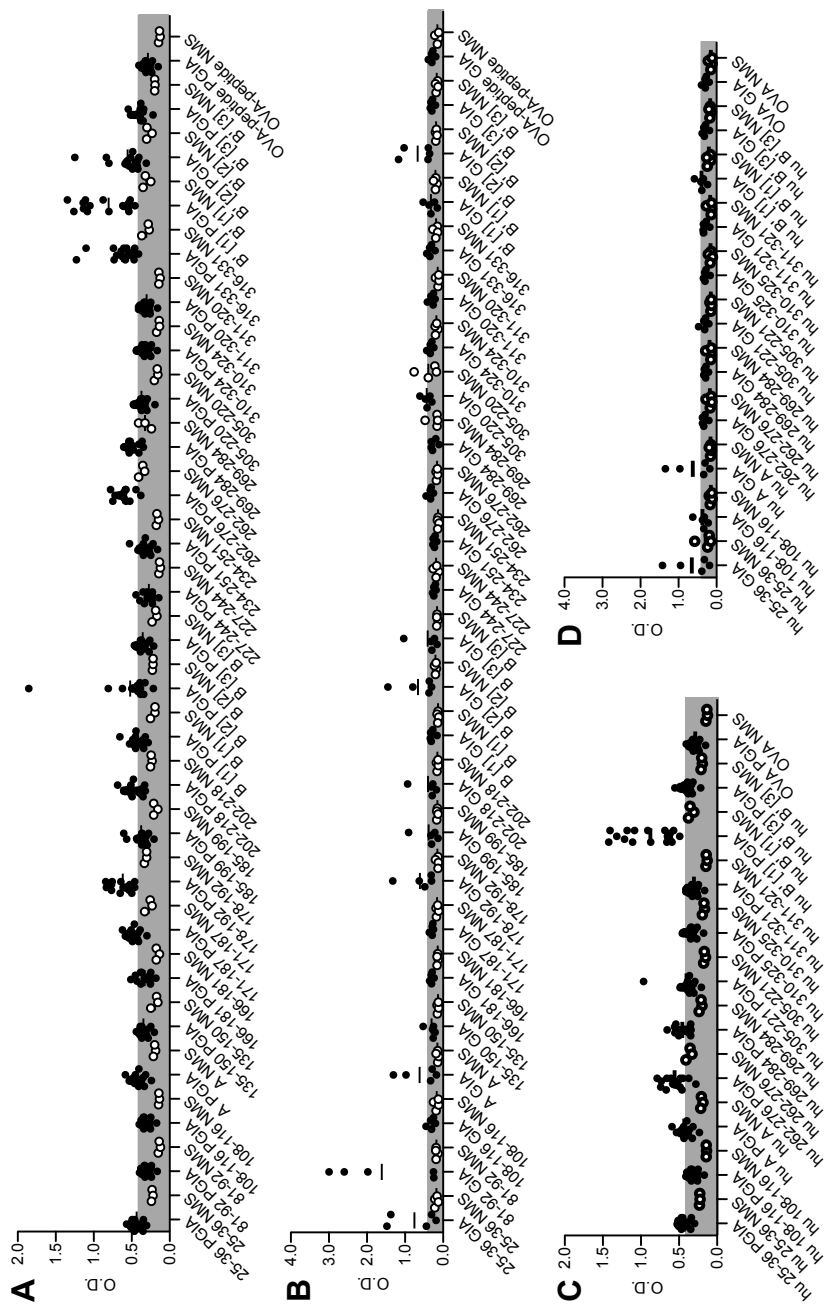
Additional File 2

Sequence of 20-mer:	O.D.	
^{16}QPSPLKVLLGSSLTIPCYFI$_{35}$	159	
^{17}PSPLKVLL**GSSLTIPCYFID**$_{36}$	1130	
^{18}SPLKVLL**GSSLTIPCYFID**P$_{37}$	1232	
^{19}PLKVLL**GSSLTIPCYFID**PM$_{38}$	1573	
^{20}LKVLL**GSSLTIPCYFID**PMH$_{39}$	1704	Derived epitope:
^{21}KVLL**GSSLTIPCYFID**PMHP$_{40}$	1514	$_{25}$GSSLTIPCYFID$_{36}$
^{22}VLL**GSSLTIPCYFID**PMHPV$_{41}$	1563	
^{23}LL**GSSLTIPCYFID**PMHPVT$_{42}$	1521	
^{24}L**GSSLTIPCYFID**PMHPVTT$_{43}$	1450	
^{25}**GSSLTIPCYFID**PMHPVTTA$_{44}$	1313	
^{26}SSLTIPCYFIDPMHPVTTAP$_{45}$	391	

Additional File 2:

A representative sample panel is shown which illustrates how a peptide-epitope was identified from the mapping data. The bold-printed amino acids are included in the epitope that was derived from this data since they represent the minimally required core sequence present in a set of overlapping peptides that were still recognized by Abs.

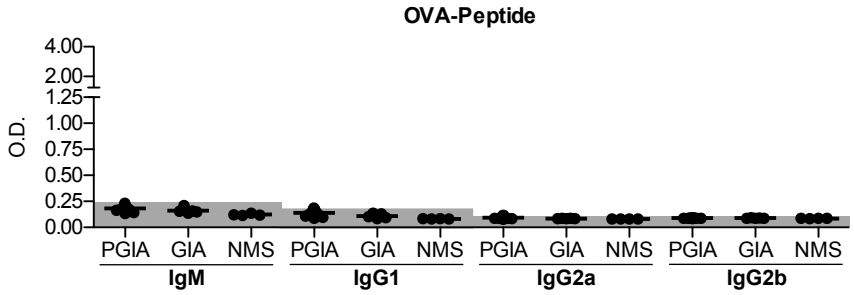
Additional File 3



Additional File 3:

Sera from naive control mice were included as negative controls in Figure 4 in the mail document, and no binding above background levels was detected to any of the mouse (A-B) or human (C-D) peptides. The shown dataset includes 19 diseased PGIA mice and 3 naïve control mice (A and C), 5 diseased GIA mice and 4 naïve control mice (B and D). OVA-peptide (irrelevant peptide) was included as a negative control and the gray area indicates the cut-off O.D. level that is based upon the highest binding of immunoglobulins to OVA-peptide detected.

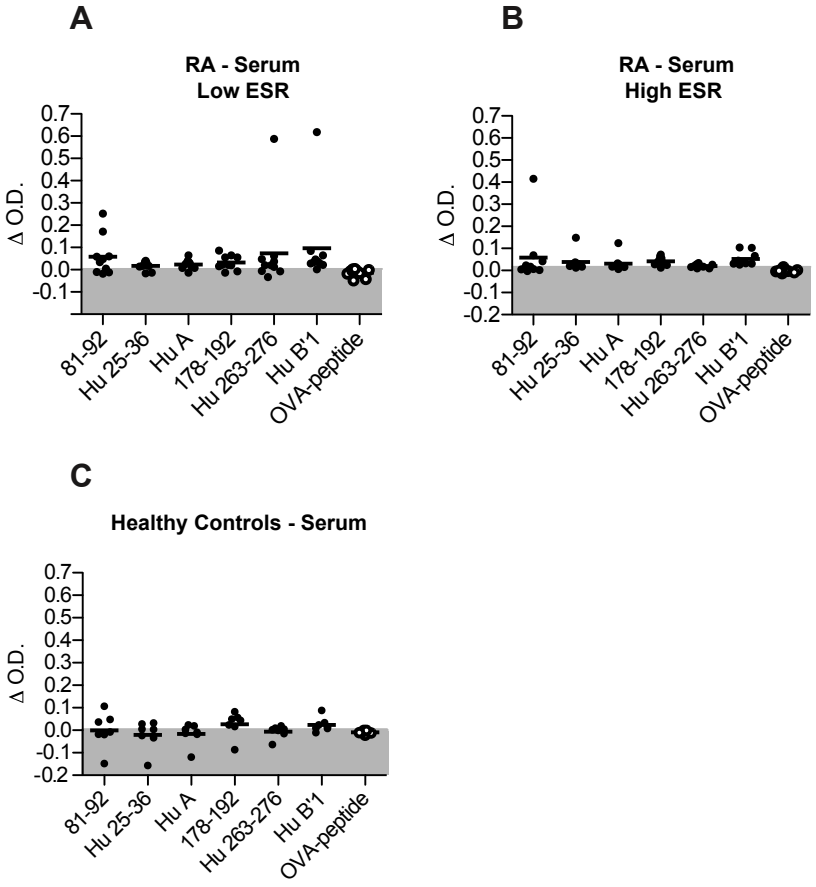
Additional File 4



Additional File 4:

OVA-peptide was included as negative controls/irrelevant peptide in Figure 6 in the mail document, and no binding above background levels was detected. The gray area indicates the cut-off O.D. level that is based upon the highest binding of immunoglobulins to OVA-peptide detected.

Additional File 5



Additional File 5:

Clear difference between the presence of Abs binding to six human G1 derived peptide epitopes were only incidentally detected when the sera from RA patients with a (A) low (n=10; left panel) or (B) high (n=10; right panel) ESR were compared to that of (C) healthy controls (n=7).

OVA-peptide (irrelevant peptide) was included as a negative control peptide and the gray area indicates the cut-off O.D. level that is based upon the highest binding of immunoglobulins to OVA-peptide detected. The binding of Abs is expressed as the O.D. after subtraction of the background levels (wells coated with avidin only). These background levels ranged between (A) 0.100-0.464 (Mean ± S.D.: 0.168±0.118), (B) 0.104-0.368 (Mean ± S.D.: 0.165±0.085) and (C) 0.129-0.417 (Mean ± S.D.: 0.258±0.107).

6

Summarizing Discussion

B cells were often thought of as simple precursors of end-stage effector cells that are merely in charge of antibody production. Research in the last decades has shown that B cells have important other roles as well, including their involvement in the regulation and functioning of T cell-mediated (autoimmune) diseases and host-protective immune responses. This thesis aimed to get a better understanding of the variety of roles B cell subsets play in immune regulation, and how the reciprocal interplay between B- and T cells shapes the outcome of an immune response. Knowledge on how specific B cell subsets influence other players of the immune network provides valuable information that can be used to develop and improve therapies aimed to restore the balance in dysregulated immune systems. In the following paragraphs, the data and results presented in this thesis will be summarized and put into perspective.

Compared to splenic B cells, peritoneal cavity B- and B-1a cells differentially activate T helper cells

Both **chapter 3** and **chapter 4** present research aimed at the elucidation of the potential role peritoneal cavity (PerC) B and B-1a cells play in the regulation of $CD4^+$ T helper (T_H) cells. **Chapter 3** focuses on the regulatory potential of B-1a cells functioning as antigen-presenting cells (APCs) when they present chicken ovalbumin (OVA)-derived peptide to OVA-specific T_H cells. OVA-specific T_H cells that are activated by PerC B-1a cells showed an enhanced potential to produce the cytokines interferon (IFN)-γ, interleukin (IL)-4 and IL-10, compared to the same T_H cells activated by splenic B cells. Next to this, activation of these antigen (Ag)-specific T_H cells by B-1a cells caused a cytokine environment that, compared to splenic B cells, contained increased levels of IL-10 and IL-6. These data show that PerC B-1a cells differentially regulate T_H cells compared to splenic B cells. It is hard to predict what effect the T_H cells that are activated by PerC B-1a cells would have on, e.g., a dysregulated immune system such as present in patients or mice suffering from autoimmune arthritis. A surplus of IFN-γ secreting T_H cells would likely aggravate experimental arthritis due to the T_H1-skewed nature of this autoimmune condition [1,2]. The increased levels of IL-6 that were found when B-1a cells activated T_H cells, also indicate that the communication between these cells could result in the aggravation of arthritis, since IL-6 is considered to be a pro-inflammatory cytokine in autoimmune conditions [3]. IL-4- and IL-10-secreting T_H cells, on the other hand, are likely to aid in the amelioration of such conditions. IL-4-secreting cells are characteristic players of a T_H2-skewed immune response and can therefore aid in the restoration of the T_H1/T_H2 unbalance present in experimental arthritis [1,2,4], as shown before by others [4,5]. IL-10-secretion is considered as a hallmark of classical immune regulatory cells that play important roles in the dampening of overactive immune systems [6]. In addition, IL-10-secreting T cells were previously described to help in the amelioration of experimental autoimmune arthritis [7]. The fact that PerC B-1a cell-activated T_H cells contained increased percentages of IL-10-producing cells, but decreased percentages of $FoxP3^+$ regulatory T cells ($T_{REG}s$), is not contradictory since Type 1 regulatory T cells (T_R1) are known to secrete IL-10 whereas they do no express the transcription factor FoxP3 [6]. At least some of the immunoregulatory effects that PerC B cells exerted on T_H cells were shown to be dependent upon the cytokine secreted by these B cells: data in chapter 3 shows that PerC B cells that activated T_H cells in

the presence of a neutralizing anti-IL-10 monoclonal antibody partially lost their potential to produce IL-10.

Activated peritoneal cavity B cells possess B_{REG} functions and regulate cytokine-production in T helper cells

The observation that PerC B cells induced IL-10-secretion by T_H cells (chapter 3), together with other observations presented in Appendix 1 of chapter 4, suggest that PerC B- and B-1a cells potentially possess functions that were previously only appointed to specific 'regulatory B cell' (B_{REG}) subsets, such as the transitional type 2-marginal zone precursor B-cell subset (T2-MZP) [8,9] and the B10 subset [10,11] described by others. **Chapter 4** provides more insight into the immunoregulatory capacity of B-1a cells functioning as B_{REG}s.
The data described in this chapter shows that the immunoregulatory potential of PerC B-1a cells crucially depends upon the activation status of the cell. Activation via CD40 triggering and short-term Toll-like receptor (TLR) 4 activation is an often-used activation-method for described functional B_{REG}s [12-14]. Activation of PerC B(-1a) cells via these stimuli results in prolonged IL-6 and IL-10 production. When these activated PerC B cells were co-cultured with activated proliferating T_H cells, the T_H cells showed a decrease in their ability to produce the pro-inflammatory cytokines IFN-γ and tumor necrosis factor (TNF)-α, when compared to co-cultures with activated splenic B cells. This *in vitro* immunoregulatory function of PerC B cells was so far only described for B cell subsets with well-defined B_{REG}s functions [15,16]. Non-activated (freshly isolated) PerC B cells did not display this immunoregulatory effect. Non-activated PerC B cells augmented the ability of $CD4^+$ T_H cells to produce TNF-α whereas the capacity to produce IFN-γ did not change compared to cocultures with splenic B cells. The production of IL-10 by CD40 and TLR-activated PerC B cells correlated with the above-described immunoregulatory B_{REG} functions of PerC B cells. This suggests that the immunoregulatory function is IL-10-mediated. In line with this suggestion, the co-cultures with T_H cells and non-activated PerC B cells were characterized with lower concentrations of IL-10 and higher concentrations of IL-2 and IL-6. It remains formally a possibility that activated PerC B cells induced T_R1-like cell properties in the T_H cell population, but preliminary data suggested that T_H cells produced little or no IL-10 under the conditions investigated.
These findings support a model in which activated PerC B cells co-cultured with T_H cells produce augmented amounts of IL-10 and subsequently favor the induction of a microenvironment that results in lower concentrations of pro-inflammatory IL-6 and a decreased amount of TNF-α and IFN-γ competent cells. Non-activated PerC B cells, on the other hand, produce an environment that is characterized by increased concentrations of IL-6 and increased amounts of TNF-α competent cells T_H cells.
Chapter 4 reports for the first time that CD40/TLR-activated PerC B cells possess regulatory B cell properties in a similar way as T2-MZP [15] and B10 B_{REG}s [16]. This finding raises an important question: is the B-1a cell subset in fact a regulatory B cell subset or can all types of B cell subsets perform regulatory B cell roles when activated in a specific way? Previous studies have suggested that the regulatory properties of B cells depend upon the activation-status of these cells, and less on the intrinsic properties of the B cell subset itself.

Lampropoulou et al. noticed that all B cell subsets (follicular [FO], transitional, marginal zone [MZ], B-1, PerC B cells and lymph node B cells) produce IL-10 when activated via TLR4 or TLR9 [17]. The study by Lampropoulou et al. suggested that the TLR-signal was responsible for the incitement of the immunoregulatory action whereas a signal delivered via CD40 was needed to amplify the initial population of IL-10-producing B cells [17,18]. Even though all B cells subsets are capable of IL-10 production after TLR triggering, other authors have noticed clear differences between the subsets. For example, immature and innate-like B cell subsets (that include B-1 cells) exhibit robust responses to TLR stimulation whereas the responses of other B cell subsets, such as the follicular B cells, are much more constrained [19]. Barr et al. furthermore described that, upon TLR4 and TLR9-signaling, FO B cells produce IFN-γ and IL-6, whereas the innate-like MZ and B-1 subset seem to be predisposed to produce IL-10 and IL-6 [20]. However, TLR-signaling in B cell subsets is not only involved in the initiation of B_{REG} functions. TLR-signals can also induce B cells to secrete pro-inflammatory cytokines, including IFN-γ, IL-6 and IL-12 [20-22], pathological autoantibodies [23,24] and it can result in the up-regulation of co-stimulatory molecules that aid in the activation of (self-reactive) T_H cells [chapter 3 and [20,25,26]]. The exact conditions required for the programming of B cell subsets into functional B_{REG}s are still largely unknown and are likely to include a variety of variables that are known to influence IL-10-production and TLR4-function such as, the availability of B cell activation factor (BAFF) [27,28], other pro-inflammatory cytokines such as IL-12 [29] and phosphatidylinositide 3-kinases [30,31].
In conclusion, TLR-activation of B cells under the right circumstances results in the secretion of anti-inflammatory IL-10. Multiple B cell subsets, among PerC B(-1a) cells possess B_{REG} functions, and evidence points towards the direction that a given B cell subset is not always *per se* intrinsically either pro- or anti-inflammatory. Our findings described in **chapter 4** are in line with this hypothesis.

Localization of peritoneal cavity B- and B-1a cells

B-1a cells are found in the spleen [32,33], in minimal amounts in the lymph nodes [33], but in relative large numbers in the body cavities (i.e., the pleural- and peritoneal cavity) [32,33]. In chapter 3 and chapter 4, the effect of PerC-derived B(-1a) cells on $CD4^+$ T_H cells was determined *in vitro*. We have isolated B-1a cells from the PerC since this is the ideal experimental source for relative large amounts of well-identifiable [32] B-1a cells. This approach raises the question if the investigated communication between B-1a and T cells is of physiological relevance.
Chapter 3 describes experiments that were performed to determine how PerC B(-1a) cells could come into contact with peripheral $CD4^+$ T_H cells *in vivo*. Experiments show that PerC B-1a cells can leave the PerC and subsequently end up in peripheral organs. However, it shows that only small amounts of PerC B-1a cells actually do so. This finding is in agreement with other studies that showed that PerC B-1a cells are continuously circulating in the bloodstream [34], but that there is minimal migration of PerC B-1a towards other organs, except for the pleural cavity [35]. B-1a cell migration patterns are different under inflammatory conditions, such as after i.v. or i.p. administration of LPS; [36-38] or during influenza virus infection [39]. Under these circumstances, PerC-derived B-1a cells have been shown to migrate towards the

spleen [36-38] or lungs and local lymph nodes [39] respectively. Potential migration of B-1a from the PerC during systemic autoimmune diseases has not been investigated so far, but it has been suggested that PerC B cells are activated locally in the PerC after administration of distant local inflammatory mediators [40], likely via cytokines and/or chemokines that end up in the circulation. Upregulation of splenic B_{REG}s in Experimental Autoimmune Encephalomyelitis (EAE) that express elevated levels of B-1 cell-associated marker CD11b [10] suggest that autoimmune conditions may cause B-1a cells to migrate from the PerC towards the spleen where they join or make part of the B10 B_{REG} population [10,11]. Other studies supporting the hypothesis that (PerC) B-1a cells are part of the B10 B_{REG} population are listed in supplementary data 1 of chapter 4.
Data in **Chapter 3** also shows that PerC B-1a cells do not necessarily need to leave the PerC in order to communicate with peripheral T_H cells. Instead, T_H cells can migrate into the PerC, engage with B cells at this location, and subsequently migrate towards other locations. T_H cells show one of the fastest migration rates [35] amongst *in situ* labeled PerC lymphocytes. PerC T cells are highly enriched in T cells with an Ag-experienced/memory phenotype [41,42], including T cells that were primed in other locations [43], but not with $FoxP3^+$ regulatory T cells [42]. Future research should indicate what the exact role is of the communication between B-1a cells and peripheral Ag-experienced/memory T_H cells that takes place locally in the PerC.
In summary, data from **chapter 3** shows that PerC B(-1a) cells may communicate with peripheral T cells in at least two different ways: (i) PerC B cells can migrate towards the periphery, and (ii) T_H cells can instead migrate towards the PerC where B-1a are located. Our data shows that PerC B-1a are likely to function as APCs and present Ag to T_H cells that enter the PerC, and one of the above-presented two mechanisms may facilitate cytokine-mediated immune-regulation of T_H cells by PerC B cells as well.

Innate-like peritoneal cavity B-1a cells may act as important immune-regulators of T-cell driven immune responses *in vivo*

The above-summarized data from chapters 3 and 4 suggests that PerC B-1a cells may be more powerful immune-regulators as previously thought. Compared to splenic B cells, PerC B(-1a) cells differentially activate T_H cells when serving as APC (**chapter 3**) and regulate the capacity of T_H cells to express cytokines (**chapter 4**). The latter findings are in line with a recent other report that describes the regulation of IFN-γ-producing T_H cells in experimental colitis by IL-10-producing PerC B cells [44]. PerC B(1-a) cells can either exert these immunoregulatory effect directly from the PerC (chapter 3) or after migrating out of the PerC (chapter 3). The latter has been described to take place under inflammatory conditions [36-39] and may take place during autoimmune conditions as well.
The innate-like broadly reactive BCR of B-1 cells [32,33], furthermore suggests that a relatively low percentage of B-1 cells has the potential to capture relative large amounts of Ag at early stages of an immune-response when the number of Ag-specific (follicular) B cells is still low [26,45,46]. Compared to conventional B cells, innate-like B cells furthermore possess elevated levels of the co-stimulatory molecules CD80/86 [chapter 4 and [47,48]], which are needed to

effectively present Ag to T_H cells at early time points of an immune-response [49]. Both these properties are reflected in the powerful capacities of B-1 cells to serve as APCs compared to other non-innate B cell subsets [46,47]. Despite the fact that relatively few B-1a cells are present in most organs, the APC-mediated immunoregulatory role of B-1a cells may thus be of considerable importance during early time-points that are decisive for the shaping and subsequent later development of the immune response [50].
B-1a cells are likely to play (APC- and cytokine-mediated) immunoregulatory roles in the maintenance of immune homeostasis as well; possibly in the presentation of gut-derived Ags to peripheral gut-Ag specific memory T_H cells as suggested in chapter 3, or via microflora-induced IL-10-mediated regulatory B cell roles [44,51]. Interestingly, a recent article has shown that B-1 cells presenting Ag to T_H cells *in vivo* caused rapid proliferation at first, but this process was followed by the deletion and/or induction of anergy [47] in a similar matter as when Ag is presented by steady-state tolerogenic dendritic cells as discussed in chapter 2. Other reports describe that B-1a cells play a role in the tolerogenic clearance of apoptotic cells expressing phosphorylcholine-related Ags that are recognized by natural antibodies (NAbs) and the B-1 cells producing them [52,53]. Apoptotic cells also promote both IL-10-producing [54] and NAbs-producing [54] functional B_{REG}s [54,55] that include B-1 cells [54, 55] via a mechanism that involves the stimulation of TLR9 [54,56]. This is in line with our finding in chapter 4 that TLR-activated PerC B cells possess regulatory B cell functions. On another note, it has been described that microflora-driven B-1-derived natural IgM has immunoregulatory roles in experimental colitis [57].
At present, it is hard to consolidate the above-presented observations of PerC B(-1a) cells and summarize their overall role in the murine immune system. Transgenic mouse models that lack or overexpress B-1 cells seem perfect tools to investigate the *in vivo* relevance of B-1a cells during autoimmune conditions but these models are often 'non-specific'. Mice that lack, e.g., functional Bruton tyrosine kinase (Btk) show defects in the development of B-1 cells, but this defect affects many other inflammatory events *in vivo* as well [58].

Successful B-T cell collaboration is required for the development of experimental arthritis

Proteoglycan-induced arthritis (PGIA) is a T-cell-dependent and antibody/B-cell-mediated autoimmune disease [59-62]. Similarly as in humans [3,21], B cell depletion therapy in mice is effective in reducing arthritis symptoms [68]. In this model, B-cells play a double role, since they are required as APCs for the induction of severe RA [61,63-65] and for the production of pathological autoantibodies [59,61,65-67]. On the one hand, B cells serving as APCs cause the promotion of autoreactive pathological proteoglycan (PG)-specific T_H cells that produce IFN-γ and IL-17 [61,63-65]. On the other hand, PG-specific T cells are required for the promotion of autoreactive B cells [59-62]. In this matter, PG-specific T cells serve as follicular helper T cells (T_{FH}) that are crucial in the development of PG-specific autoreactive B cells [65,69,70] and T_H1-skewed IFN-γ-producing T_H cells that aid in the formation of autoantibodies as well [71]. The interactions between T- and B cells thus cause a positive feedback loop that results in the development of severe (experimental) arthritis.

In **chapter 2** we investigated if the *in vivo* targeting of tolerogenic dendritic cells (DC) with the PG-derived arthritogenic T-cell epitope PG_{70-84} prior to the induction of PGIA resulted in alterations in the PG-specific T cell population that subsequently prevented the development of PGIA. The DCs were targeted in their steady (tolerogenic) state using an anti-DEC205 fusion antibody (αDEC205-PG) [72,73]. This tolerogenic vaccination strategy was successfully applied before to prevent the onset of EAE [74-76] and type 1 diabetes (T1D) [72,77]. This tolerogenic vaccination approach has been reported to induce both recessive tolerance (e.g., the deletion [78,79] and the induction of anergy [74,75] in Ag-specific T-cells) and dominant tolerance mechanisms. The latter involves the induction of regulatory T cells (T_{REG}s) that express FoxP3 as reported for pancreatic β-cell-specific T-cells [80], and non-self Ags [81-84]). $FoxP3^-$ T_{REG}s may be induced as well [75].
We studied the effects of tolerogenic DEC205-mediated vaccination on the pathogenic B- and T cells responses induced by immunization with PG in adjuvant. These adaptive immune responses precede the development of experimental arthritis's clinical symptoms. The data presented in chapter 2 shows that prophylactic administration of αDEC205-PG affects both the B- and T cell responses, as shown by lower percentages of B cells participating in the germinal center reaction (GC) and the reduced numbers of PG-specific T- and GC-supporting follicular helper (T_{FH}) cells that are present after treatment. The effect of tolerogenic DEC205-mediated vaccination on GC-development was not studied before. The detected impingements on the GC response and the subsequent detection of lower severity of experimental arthritis, fits well with the above introduced model in which B cells play an important double role in PGIA [61,63-65]. Lower amounts of B cells participating in GCs would namely result in both (i) lower amounts of Ag-specific B cells that can serve as APC for the promotion of pathogenic T_H cell subsets and (ii) lower amounts of pathogenic PG-specific autoantibodies. The latter has been shown in chapter 2. Both defects are likely to result in the reduced severity of arthritis. The findings described in chapter 2 are summarized in Figure 1.

Dominant and recessive tolerance mechanisms can prevent the development of autoantibodies

The exact mechanism of DEC205-mediated vaccination modulation of GC-formation could not be determined incontestably. Data from chapter 2 and other studies [82,85,86] very strongly suggest that anti-DEC205 fusion antibodies target steady-state (splenic $CD8^+$) $DEC205^+$ DCs, and not B cells directly. This indicates that T_H cells are likely affected before B cells are. The observation that alterations in the PG-specific T_H cell population are evident at time-points before GCs are formed (as shown in chapter 2) supports this notion.
It is less evident if the subsequent alterations in B cell development are the effect of dominant (i.e., actively suppressing T_{REG}s) or recessive (i.e., lower availability of arthritogenic T cells) tolerance mechanisms. Chapter 2 shows that the applied tolerogenic vaccination results in both increased percentages of PG-specific $FoxP3^+$ T_{REG}s and lower amounts of arthritogenic $FoxP3^-$ T_H ('T effector') cells. The relative increase in PG-specific $FoxP3^+$ T_{REG}s detected was likely the result of preferential deletion and/or reduced proliferative expansion of PG-specific arthritogenic $FoxP3^-$ T_H cells that resulted in a relative increase of $FoxP3^+$ T_{REG}s in

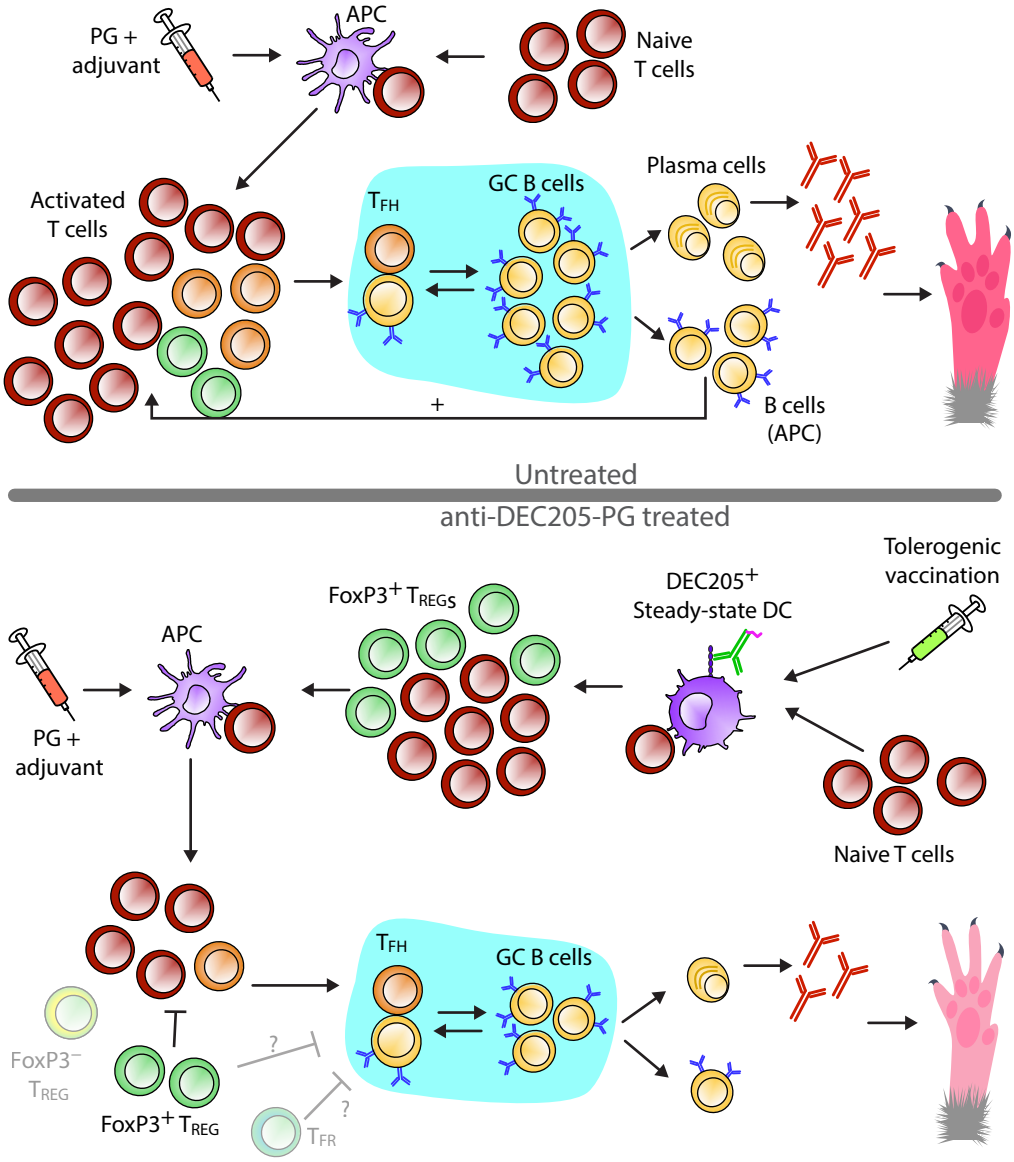


Figure 1: Schematic representation of the working mechanism of DEC205-mediated vaccination protecting against the development of severe PGIA.

Upper lane: Untreated mice are immunized with PG in adjuvant. This results in the activation of various T_H cells subsets, including T_{FH} cells. T_{FH} cells collaborate with GC B cells resulting in both autoantibody producing plasma cells and PG-specific B cells that may serve as APC for T_H cells. Severe PGIA is consequently observed. Lower lane: Prophylactic treatment with anti-DEC205-PG results in the activation of naive T_H cells by steady-state DCs. Subsequent proliferation of T_H cells results in a T cell population with (temporary) increased percentages of FoxP3$^+$ T_{REG}s. Activation of this T cell population by immunization with PG in adjuvant subsequently results

the residual PG-specific T cell population compared to the control treated groups. However, proliferative expansion of preformed Foxp3$^+$ T_{REG}-cells or *de novo* generation of T_{REG}-cells was not investigated and could not be formally excluded.
T_{REG}s involved in the suppression of autoantibody generation and/or GCs may be either conventional FoxP3$^+$ T_{REG}s [87-89], possibly FoxP3$^-$ T_{REG}s such as Type 1 regulatory T cells (T_R1) [90,91] and/or follicular regulatory T cells (T_{FR}) [92-94]. The T_{FR} subset expresses CXCR5, PD-1 and FoxP3, is thought to originate from thymic-derived natural T_{REG}s [92,94], and is specifically involved in the regulation of GCs [92-94]. The exact regulatory-mechanism is still a matter of debate [95]. In our studies, we were unable to precisely characterize and enumerate the PG-specific T_{FR} cell population that may play a role in the suppression of DEC205-mediated tolerogenic vaccination since only small amounts of PG-specific CD4$^+$ CD25$^+$ FoxP3$^+$ CXCR5$^+$ PD-1$^+$ cells were left after administration of anti-DEC205-PG due to the fact that most of the PG-specific T cells were deleted/anergized as mentioned above.
Importantly, our data strongly suggests that the decreased development of GCs in anti-DEC205-PG treated mice was mainly the result of recessive tolerance mechanisms. The reduced availability of PG-specific T_H cells in αDEC205-PG-treated animals correlated with smaller numbers of PG-specific T_{FH} cells and smaller GCs in a system where overall very low levels of PG-specific FoxP3$^+$ T_{REG}-cells were present. On the other hand, the higher T_{REG} : arthritogenic T_H ratio in anti-DEC205-PG treated mice indicates the possibility that the leftover arthritogenic T_H present were more hampered by T_{REG}s compared to T_H cells in control treated animals. The schematic representation of the above-discussed mechanism is summarized in Figure 1.

The G1 domain from proteoglycan aggrecan: an arthritogenic (self) antigen with B- and T cell epitopes

The above presented notion that a reduction in arthritogenic PG-specific T_H and T_{FH} cells results in decreased GC development raises the question how the reduction in the amount of T cells specific for a single T cell epitope can be responsible for the observed severe alterations in B cell and arthritis development. The $PG_{70\text{-}84}$ T cell epitope of which we made use in **chapter 2** is the strongest T cell epitope out of the three immunodominant T cell epitopes described in the G1 domain of PG [96-98]. Deletion of this epitope via molecular manipulation significantly diminished the subsequent immune response compared with the response seen with the native Ag [96]. Human PG (used for arthritis induction) is highly homologous to murine self-PG, and PGIA is based upon the putative cross-reaction between the B- and T cell reacting with both Ags [99-101]. The homology between both Ags is likely to limit the number of high affinity epitopes present in human PG.

in the induction of anergy and/or deletion of T_H cells, a smaller T_H cell population, lower amounts of T_{FH} cells, increased percentages of T_{REG}s and possibly increased amounts of T_{FR} cell and/or FoxP3$^-$ T_{REG}s as well. Decreased amounts of GC B cells, lower autoantibody titers and lower arthritis scores are consequently observed. All drawn lymphocytes represent PG-specific cells. Leukocyte drawings were adapted with permission from Ineke den Braber.

Immunization with synthetic peptides representing T-cell epitopes (single peptide or pooled peptides) has never induced arthritis [102], but a relatively short PG-derived fragment (G1 domain, ~340 amino acids of the PG core protein) did [102]. This suggests that indispensable arthritogenic B cell epitopes are additionally present in the T-cell epitope-containing G1 domain. The exact B cell epitopes are unknown. In **chapter 5**, we aimed to elucidate the B cell epitopes on the protein-backbone of the self-Ag G1 domain of mouse proteoglycan after immunization with human proteoglycan. The data show that both human proteoglycan- and G1 domain-immunized arthritic mice have autoantibodies to several B cell epitopes of mouse PG that are predominantly contained within four immunodominant regions. Comparison between the autoantibody response in PGIA and G1-induced arthritis (GIA) indicated that the characterized autoantibodies directed against the protein-backbone of G1 are mainly of the IgM isotype in PGIA and that some of these autoantibodies in GIA consist of switched isotypes as well. A previous study [103] has indicated that anti-G1 autoantibodies (with unknown exact specificity) of both the IgM and the IgG isotypes could deplete cartilage from the knees *in vivo* whereas only the IgM monoclonal antibody caused mononuclear cell infiltration and hyperplasia [103]. Although not investigated, the four immunodominant regions to which the detected autoantibodies are directed are likely also targets of Ag-specific B cells that can efficiently phagocytose PG and G1 via their B cell receptors for subsequent Ag-presentation to PG-specific T_H cells, including T_{FH} cells [45].

The fact that the study by Dayer et al. suggested that our detected autoantibodies have pathological roles in RA, made us investigate these antibodies in patients with rheumatoid arthritis (RA) as well. Data in chapter 5 show that the synovial fluid of RA patients contains autoantibodies that bind the human sequence analogs of the discovered murine PG G1 domain B cell epitopes. The autoantibodies binding these epitopes were only clearly detectable locally in the synovial fluid. This is in correspondence with other studies that investigated autoantibodies directed against the G1 domain [104,105]. B cells present in the inflamed arthritic joints are likely the producers of these antibodies, and the local presence of these autoantibodies suggests that they may play a role in the pathogenesis of human rheumatoid arthritis. Future studies are needed to elucidate the exact roles of these autoantibodies and to investigate if they are possible targets for new diagnostic or therapeutic strategies in rheumatoid arthritis in a similar manner as antibodies directed to, e.g., citrullinated- and carbamylated Ags [106].

Concluding remarks

In conclusion, the research described in **chapter 3** and **chapter 4** of this thesis indicates that PerC-derived B- and B-1a cells, differentially regulate the capacity of T_H cells to secrete cytokines via two different mechanisms: (i) PerC B-1a cells can serve as APC and differentially activate T_H cells, and (ii) activated PerC B cells can perform B_{REG}-functions. PerC B(-1a) cells can communicate with peripheral T_H cells after (i) migrating from the PerC to, e.g., the spleen or (ii) when peripheral T_H cells enter the PerC. These findings indicate that PerC B(-1a) cells can be powerful immune-regulatory cells that potentially play important roles in the prevention and regulation of autoimmune diseases.

Chapter 2 shows that alteration in the PG-specific T_H cell population can severely impair

the GC reaction responsible for the development of autoreactive PG-specific B cells. Evidence indicates that the decreased availability in arthritogenic PG-specific T_{FH} cells is most likely responsible for the subsequent lowered production of PG-specific autoantibodies. The amount of PG-specific B cells that can serve as arthritogenic APCs, which are needed for the full activation of pathogenic T_H cells and the development of severe arthritis, is presumably diminished as well. **Chapter 5** demonstrates that the B cell epitopes to which PG G1 domain specific autoantibodies are directed are mainly contained within four immunodominant regions. Both arthritis mice and patients with rheumatoid arthritis have antibodies against these B cell epitopes, suggesting that they may play a role in the pathogenesis of these autoimmune diseases.

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List of Abbreviations
Nederlandse samenvatting
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List of Abbreviations

3D	Three-dimensional
Ab(s)	Antibody(/-ies)
ABTS	2,2'-azino-di-3-ethylbenzthiazoline sulfonate
Ag(s)	Antigen(s)
APC(s)	Antigen-presenting cell(s)
ASCs	Antibody-secreting cells
autoAbs	Autoantibodies
BCL6	B cell lymphoma 6
BCR	B cell receptor
Blimp1	B-lymphocyte-induced maturation protein 1
B_{REG}	Regulatory B cell
CCD	Charge-coupled device
CCR	C-C chemokine receptor
CD	Cluster of Differentiation
CD40L	CD40 Ligand
CpGs	oligodeoxynucleotides with unmethylated deoxycytidine-deoxyguanosine dinucleotides
CSR	Class switch recombination
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DC(s)	Dendritic cell(s)
DDA	Dimethyldioctadecylammonium bromide;
DZ	Dark Zone
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EF	Extra Follicular
ELISA	Enzyme-linked immuno sorbent assays
ESR	Erythrocyte sedimentation rate
Fc	Fragment crystallizable
FDC(s)	Follicular dendritic cell(s)
FO	Follicular
FoxP3	Forkhead box P3
GCs	Germinal Center(s)
GIA	G1-induced arthritis
hi	high
HRP	Horseradish peroxidase
i.p.	intraperitoneal(ly)
i.v.	intravenous(ly)
ICOS	Inducible T cell costimulator
ICOSL	Inducible T cell costimulatory ligand
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
LN(s)	Lymph node(s)
lo	low
LZ	Light Zone
MBC(s)	Memory B cell(s)
MHC-II	Major histocompatibility complex class II molecules
MZ	Marginal Zone
NAbs	Natural Antibodies
O.D.	Optical density
PAMPs	Pathogen-associated molecular patterns
PB(s)	Plasmablast(s)
PBS	Phosphate-buffered saline
PC(s)	Plasma cell(s)
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand 1
PerC	Peritoneal cavity
PG	Proteoglycan
PGIA	Proteoglycan-induced arthritis
pMHC-II	Peptide-major histocompatibility complex class II molecules
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
rhG1	recombinant human G1
S.D.	Standard deviation
SHM	Somatic hypermutation
SPF	Specific Pathogen Free
T2-MZP	Transitional type 2-marginal zone precursor B cell subset
TCR	T cell receptor
T_{FH}	Follicular helper T cell
T_{FR}	Follicular regulatory T cell
T_H	T helper
T_H1	Type 1 T helper cell
T_H17	Type 17 T helper cell
T_H2	Type 2 T helper cell
TLR(s)	Toll-like receptor(s)
TNF	Tumor Necrosis Factor
T_R1	Type 1 regulatory T cell
WT	Wild Type

Nederlandse Samenvatting

Het immuunsysteem en de rol van B-cellen en T-cellen

Het immuunsysteem heeft een aantal belangrijke functies: het beschermt tegen indringende pathogenen (zoals bacteriën en virussen), het vernietigt foutief geproduceerde lichaamseigen cellen (bijvoorbeeld kankercellen) en het ruimt afvalstoffen op. Het immuunsysteem bestaat onder andere uit verschillende soorten witte bloedcellen, die als een netwerk met elkaar samenwerken.
T-helpercellen (afgekort T_H-cellen; ook wel $CD4^+$ T-cellen genoemd) zijn witte bloedcellen die worden gezien als voornamelijk immuunsysteem-regulerende cellen. De voornaamste taak van T_H-cellen is om er voor te zorgen dat andere witte bloedcellen goed worden geactiveerd en daardoor hun specifieke taak naar behoren kunnen uitvoeren.
Andere soorten witte bloedcellen, zoals macrofagen en dendritische cellen (DCs) kunnen pathogenen fagocyteren (opnemen), lyseren (verteren) en vervolgens T_H-cellen activeren. Dit laatste gebeurt wanneer een klein stukje eiwit (oftewel een peptide) afkomstig van een pathogeen wordt gepresenteerd en wordt herkend door een 'T_H-cel receptor' op een T_H-cel. Een receptor is een speciaal soort eiwit op een cel dat een signaal kan opvangen om een cel vervolgens actief te laten worden. Een stof die een immuunreactie oproept (zoals een peptide of een groter eiwit) wordt een antigeen genoemd. Elke T-cel heeft zijn eigen antigeen-specifieke T-cel receptor (TCR). Fagocyterende cellen die antigenen presenteren aan T_H-cellen worden antigeen-presenterende cellen (APCs) genoemd. APCs kunnen alleen effectief peptiden presenteren indien zij zijn geactiveerd. Een APC wordt bijvoorbeeld geactiveerd wanneer receptoren op het celoppervlak van de APC een chemische structuur van een bacterie herkennen. Een receptor genaamd Toll-Like Receptor 4 (TLR4) herkent bijvoorbeeld lipopolysaccharide (LPS) dat aanwezig is op het celmembraan van bepaalde bacteriën.
B-cellen zijn witte bloedcellen die zich kunnen ontwikkelen tot antilichaam-producerende cellen welke plasmacellen worden genoemd. Antilichamen (ook wel antistoffen genaamd) zijn eiwitten die onder andere bescherming bieden door pathogenen onschadelijk te maken. Elke B-cel heeft een eigen B-cel receptor (BCR) op zijn celoppervlak. BCRs herkennen, net als TCRs, slechts een beperkte hoeveelheid antigenen. BCRs hebben echter geen APC nodig om geactiveerd te worden: een B-cel kan direct een eiwit (of een ander molecuul) herkennen. Omdat B-cellen een belangrijke taak vervullen bij de productie van antilichamen worden ze voornamelijk gezien als taak-uitvoerende cellen en in mindere mate als immuunsysteem-regulerende cellen.

T_H-cellen reguleren B-cellen en de antilichaamproductie

De meeste B-cellen hebben signalen nodig van T_H-cellen voordat zij zich kunnen ontwikkelen tot een plasmacel. In een typische situatie wordt bijvoorbeeld een bacterie eerst herkent door een APC zoals een DC. De DC fagocyteert en lyseert de bacterie en knipt daarbij de eiwitten van de bacterie in kleine peptiden. Daarmee maakt deze cel de individuele bacterie onschadelijk. Vervolgens presenteert de DC een peptide afkomstig van deze bacterie aan een T_H-cel. Om dit te kunnen doen, verpakt de DC het van bacterie-afkomstige peptide in een speciaal eiwit. Dit eiwit wordt het major histocompatibility complex (MHC) genoemd.

De combinatie van het MHC met daarin het peptide kan vervolgens worden herkend door de TCR van een T_H-cel. Indien dat gebeurt wordt de T_H-cel geactiveerd, vermenigvuldigt deze zich en veelal specialiseert de T_H-cel zich gelijktijdig om een speciaal type immuunreactie te helpen ondersteunen. Een T_H-cel kan zich bijvoorbeeld specialiseren in het ondersteunen van B-cellen bij de antilichaamproductie. T_H-cellen die dat doen worden 'folliculaire helper T-cellen' (T_{FH} cellen) genoemd.
Tegelijkertijd worden ook de B-cellen geactiveerd. Een B-cel herkent een bacterie met zijn BCR. Na herkenning fagocyteert en lyseert de B-cel de bacterie. De B-cel fungeert vervolgens ook als APC. Wanneer de TCR op een T_{FH}-cel een peptide herkent dat een B-cel presenteert in zijn MHC krijgt de B-cel extra signalen van de T_{FH}-cel (co-stimulatoire signalen genoemd). Een co-stimulatoir signaal wordt bijvoorbeeld verkregen wanneer de receptor genaamd CD40 (op het celoppervlak van de B-cel) een interactie aangaat met het molecuul CD40L, dat aanwezig is op het celoppervlak van de T-cel. Deze signalen zijn vereist voor de verdere ontwikkeling van de B-cel tot een plasmacel.
De antilichamen, welke uiteindelijk worden geproduceerd door de volledig ontwikkelde plasmacel, herkennen hetzelfde eiwit dat de BCR van de B-cel herkende die zich tot plasmacel heeft weten te ontwikkelen.

Immuunregulatie door T-cellen en auto-immuniteit

Een correct functionerend immuunsysteem beschermt zonder schade aan het lichaam zelf aan te richten. B- en T-cellen ondergaan een ontwikkelingsproces dat er in principe voor zorgt dat de BCRs en TCRs alleen kunnen reageren met lichaamsvreemde eiwitten en peptiden.
Indien B- en/of T-cellen onverhoopt toch reageren op lichaamseigen eiwitten (met een 'zelfantigeen'), kunnen speciale regulatoire T_H-cellen (T_{REG}-cellen) er voor zorgen dat deze immuunresponse wordt beperkt ("downregulatie van de immuunresponse"). T_{REG}-cellen kunnen dit onder anderen doen door de signaalstof (cytokinen genaamd) interleukine 10 (IL-10) uit te scheiden. Veel witte bloedcellen hebben IL-10-receptoren die de aanwezigheid van IL-10 herkennen. De herkenning van IL-10 heeft veelal een downregulerende werking op deze cellen.
In een immuunsysteem wat uit balans is, wordt een foutieve immuunreactie tegen een zelfantigeen soms niet downgereguleerd. Er kan dan een auto-immuunziekte ontstaan. Het immuunsysteem valt dan als het ware het eigen lichaam aan. Dit is bijvoorbeeld het geval bij patiënten met multiple sclerosis (MS) of reumatische artritis (RA). In RA reageren de BCRs en de TCRs van de B-cellen en T-cellen met de lichaamseigen eiwitten in de gewrichten. De B-cellen ontwikkelen zich tot antilichaamproducerende cellen en de binding van antilichamen aan de eiwitten in de gewrichten van RA patiënten spelen een belangrijke rol in de pathogenese van deze auto-immuunziekte.

Verschillende soorten B-cellen

In muizen komen, net als bij mensen, verschillende soorten B-cellen voor. B-cellen die zijn gespecialiseerd om zich te kunnen ontwikkelen tot plasmacel worden in de muis 'B-2 cellen' genoemd. B-2 cellen bevinden zich voornamelijk in de milt en in de lymfeklieren. In de muis bestaan er daarnaast ook nog marginale zone B-cellen en B-1 cellen. Deze twee soorten B-cellen

zijn gespecialiseerd in andere functies. Zij kunnen bijvoorbeeld effectief fungeren als APC en staan bekend om het feit dat ze grote hoeveelheden cytokinen kunnen produceren, inclusief het immuunsysteem-downregulerende IL-10. Marginale zone B-cellen bevinden zich in een speciale locatie van de milt en B-1 cellen bevinden zich in groten getale in de buikholte van de muis. In de buikholte zijn relatief weinig B-2 cellen te vinden.

B-cellen reguleren T_H-cellen

Ondanks dat B-cellen niet bekend staan om hun immuunsysteem-regulerende functies, heeft onderzoek in de laatste decennia laten zien dat B-cellen wel degelijk de functies van T_H-cellen beïnvloeden. Samengevat kunnen B-cellen T_H-cellen beïnvloeden via twee processen: (i) B-cellen kunnen fungeren als APC en de T_H-cel daarmee op een speciale manier activeren/laten specialiseren en (ii) B-cellen kunnen cytokinen afscheiden en daarmee de werking van andere witte bloedcellen reguleren. Recent ontdekte IL-10-producerende B-cellen met immuunsysteem-downregulerende effecten worden regulatoire B-cellen (B_{REG}-cellen) genoemd. B_{REG}-cellen beïnvloeden vaak de functies van T_H-cellen. In bepaalde situaties spelen B_{REG}-cellen vergelijkbare rollen als T_{REG}-cellen. De immuunsysteem-downregulerende werking van deze cellen zijn van belang in het voorkomen en/of het verminderen van auto-immuunziekten.

Dit proefschrift

Het doel van het onderzoek beschreven in dit proefschrift is om (i) de immuunsysteem-regulerende rollen van de verschillende soorten B-cellen en (ii) de bidirectionele immuunregulatie tussen B- en T-cellen, welke bepalend is voor het verloop van een immuunreactie, beter te beschrijven. Kennis van deze aspecten is van belang voor het ontwikkelen en verbeteren van therapieën gericht op het herstellen van de balans in een ontregeld immuunsysteem.
Om het beschreven onderzoek goed te kunnen begrijpen is het belangrijk te realiseren dat een correct functionerende immuunreactie het lichaam tegen pathogenen beschermt. Immuunreacties gericht tegen zelfantigenen kunnen echter schadelijk zijn omdat ze auto-immuunziekten veroorzaken. Auto-immuunziekten vinden vaak plaats in de context van een overgeactiveerd immuunsysteem. Downregulatie van het immuunsysteem kan auto-immuunziekten dus voorkomen en/of verminderen.

Hoofdstuk 2 beschrijft hoe een tolerogeen vaccin er voor kan zorgen dat muizen geen RA meer kunnen ontwikkelen. Het toegepaste tolerogene vaccin zorgt er voor dat een peptide afkomstig van proteoglycaan (een eiwit in de gewrichten) door onvolledig geactiveerde DCs aan T_H-cellen wordt gepresenteerd. Onvolledig geactiveerde DCs blijken ook de T_H-cellen met een TCR specifiek voor proteoglycaan-peptide onvolledig te activeren. Ook ons '*in vivo*' onderzoek (d.w.z. uitgevoerd in levend materiaal) laat zien dat deze T_H-cellen niet worden geactiveerd zoals dat gebeurt in ongevaccineerde muizen die wel RA ontwikkelen. Tevens ontstaan er verhoogde hoeveelheden T_{REG}-cellen met een TCR specifiek voor proteoglycaan-peptide. Het onderzoek laat zien dat B-cellen zich vervolgens niet meer kunnen ontwikkelen tot antilichaam-producerende plasmacellen, dat de muizen minder antilichamen aanmaken die met proteoglycaan reageren en dat de dieren behandeld met het tolerogene vaccin vrijwel

geen RA meer ontwikkelen. Antigeen-presentatie door niet geactiveerde DCs als gevolg van een tolerogene vaccinatie is dus voordelig in deze context omdat het de pathogenese van experimentele auto-immune RA remt.
In de toekomt kunnen tolerogene vaccins er wellicht voor zorgen dat mensen geen auto-immuunziekten meer ontwikkelen. Voordat het zo ver is moet er echter veel meer onderzoek worden gedaan.

Hoofdstuk 3 beschrijft onderzoek naar de verschillen in T_H-cellen die zijn geactiveerd door B-1 cellen uit de buikholte en B-2 cellen uit de milt. In dit onderzoek treden de B-cellen op als APC. Het onderzoek laat zien dat de T_H-cellen die '*in vitro*' (d.w.z. in het laboratorium; in een kweekschaaltje) door B-1 cellen uit de buikholte zijn geactiveerd meer van de cytokinen interferon-γ (IFN-γ), IL-4 en IL-10 produceren in vergelijking met de T_H-cellen welke zijn geactiveerd door B-2 cellen uit de milt. *In vivo* onderzoek liet zien dat T_H-cellen in het lichaam van de muis kunnen migreren naar de buikholte waar zij vervolgens worden geactiveerd door B-1 cellen. Dit geeft aan dat ons *in vitro* onderzoek wellicht een situatie beschrijft die ook in het lichaam plaats kan vinden.

Hoofdstuk 4 laat zien dat geactiveerde B-cellen uit de buikholte (voornamelijk B-1 cellen) kunnen functioneren als Breg-cel. B-cellen uit de milt (voornamelijk B-2 cellen) doen dit niet. B_{REG}-cellen downreguleren het immuunsysteem. Dit kan voordelig zijn in de context van een auto-immuunziekte, omdat het immuunsysteem bij patiënten met een auto-immuunziekte als het ware overgeactiveerd is.
In dit onderzoek zijn de beide B-cel soorten *in vitro* geactiveerd met een artificieel signaal via de co-stimulatoire receptor CD40 en met het bacteriële LPS. We hebben ontdekt dat de geactiveerde buikholte B-cellen veel immuunsysteem-downregulerend IL-10 produceren, terwijl milt B-cellen dat niet doen.
Wanneer geactiveerde buikholte B-cellen *in vitro* samen worden gekweekt met geactiveerde T_H-cellen, onderdrukken deze B-cellen vervolgens T_H-cellen in de productie van twee immuunsysteem-stimulerende cytokinen: tumornecrosefactor-α (TNF-α) en IFN-γ. Niet geactiveerde buikholte B-cellen en milt B-cellen bezitten deze voordelige immuunsysteem-onderdrukkende eigenschappen niet. Niet geactiveerde buikholte B-cellen bleken zelfs in staat te zijn de productie van het immuunsysteem-stimulerende cytokine TNF-α door T_H-cellen te verhogen.
Bij patiënten met auto-immuunziekten worden soms met behulp van een moderne therapie (Rituximab) alle B-cellen verwijderd. Dit onderzoek geeft aan dat deze therapie in de toekomst misschien nog beter zou kunnen werken indien bepaalde soorten geactiveerde B-cellen niet zouden worden verwijderd.

Hoofdstuk 5 beschrijft een onderzoek naar de antilichamen die aanwezig zijn in muizen en patiënten met RA. Antilichamen gericht tegen de verschillende soorten eiwitten in de gewrichten spelen een belangrijke rol bij de pathogenese van RA. Antilichamen tegen het eiwit proteoglycaan waren echter nog relatief onbekend. In hoofdstuk 5 hebben we onderzocht waar de antilichamen gericht tegen het eiwit proteoglycaan precies aan binden. Een stukje eiwit waaraan een antilichaam bindt, wordt een epitoop genoemd. Na opheldering van de

epitopen, hebben we bovendien ontdekt dat antilichamen gericht tegen deze epitopen zowel aanwezig zijn in muizen waarin RA is geïnduceerd als in het gewrichtsvocht van patiënten met RA. Kennis van deze antilichamen zou in de toekomst mogelijk gebruikt kunnen worden voor diagnostische doeleinden, bijvoorbeeld voor het karakteriseren van de verschillende soorten RA.

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Curriculum vitae

Bram Margry werd geboren op 30 januari 1981 te Boxtel. In 1998 behaalde hij zijn havodiploma aan de Bracbant havo in Boxtel en begon hij met de opleiding Chemie aan het Hoger Laboratoriumonderwijs van de Fontys Hogescholen in Eindhoven. Hij liep stages in de onderzoeksgroep van Prof. dr. Michael Carpenter in het toenmalige Institute for Materials aan de University at Albany - State University of New York en in het Philips Natuurkundig Laboratorium te Eindhoven. In 2002 behaalde hij zijn bachelordiploma en begon hij aan het masterprogramma Drug Innovation van de Universiteit Utrecht. Als onderdeel van zijn studies verrichte hij onderzoek naar de potentiële immunomodulatoire eigenschappen van diverse plantenextracten. Dit onderzoek vond plaats in het departement biogene medicinale chemie onder leiding van dr. Kees Beukelman. Daarnaast liep hij stage aan de faculteit Chemie van de Universidad Nacional te Heredia (Costa Rica) en verrichte hij een literatuuronderzoek naar biopiraterij. In 2004 behaalde hij zijn masterdiploma, en daarna verhuisde hij naar Costa Rica waar hij werkzaam werd als docent Scheikunde op de Country Day School te Escazú. In 2008 verhuisde hij naar Taiwan waar hij zijn loopbaan als docent voortzette op de Pacific American School te Hsinchu. In 2009 startte hij als assistent in opleiding (aio) op de divisie Immunologie van het departement Infectieziekten en Immunologie aan de Universiteit Utrecht. Het onderzoek dat hier plaatsvond, onder begeleiding van Dr. Femke Broere en Prof. dr. Willem van Eden, staat beschreven in dit proefschrift.