Role of Progranulin in Humoral Immunity

Linda Cassis

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DIRECTOR

Prof. Andrea Cerutti

iCREA

Fundación IMIM

Mount Sinai School of Medicine

DEPARTMENT OF EXPERIMENTAL AND HEALTH SCIENCES

Universitat Pompeu Fabra Barcelona

To my family

Acknowledgments

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Thesis abstract

Human spleen is continually exposed to blood-borne antigens derived from autologous apoptotic cells and commensal bacteria. This chronic stimulation of the marginal zone (MZ) results in the generation of a steady-state antibody response that occurs under non-inflammatory conditions. Immunoregulatory signals, still poorly understood, are required to avoid continuous inflammation.

Our group identified a population of splenic neutrophils called B cell-helper neutrophils (N_{BH} cells) that contribute to the induction of steady-state antibody responses in the MZ[1]. N_{BH} cells express B cell-activating and immunoregulatory factors, including progranulin (PGRN).

PGRN is an anti-inflammatory protein highly expressed at sites constantly exposed to antigens. It was shown to regulate several processes, including embryogenesis, neuronal survival, and wound repair. However, the role of PGRN in the immune response is still largely unknown. Here we show that PGRN actively participates in the pre-immune and post-immune responses against splenic microbial antigens, regulating the frequency and/or function of innate and adaptive immune cells such as neutrophils, dendritic cells, T and B cells. These findings suggest that PGRN functions as an endogenous adjuvant that may facilitate the development of novel strategies for modulating protective immune responses against invading pathogens.

Resumen de la tesis

El bazo humano está continuamente expuesto a antígenos provenientes de la sangre derivados de células apoptóticas autólogas y bacterias comensales. Esta estimulación crónica de la zona marginal (ZM) resulta en la generación de una respuesta de anticuerpos que se produce de forma fisiológica bajo condiciones no inflamatorias. Para evitar la inflamación continua, se requieren señales inmunorreguladoras, todavía poco conocidas.

Nuestro grupo identificó una población de neutrófilos esplénicos llamada neutrófilos ayudantes de células B (células N_{BH})[1] que contribuyen a la inducción de anticuerpos en la ZM en condiciones fisiológicas. Las células N_{BH} expresan factores activadores de las células B y factores inmunorreguladores, incluyendo progranulina (PGRN).

PGRN es una proteína antiinflamatoria altamente expresada en lugares constantemente expuestos a antígenos. Regula varios procesos, incluyendo la embriogénesis, la supervivencia neuronal, y la reparación de heridas. Sin embargo, el papel de PGRN en la respuesta inmune sigue siendo en gran medida desconocido. En este estudio demostramos que PGRN participa activamente en las respuestas pre- y post-inmunes contra antígenos microbianos en el bazo, regulando la frecuencia y / o la función de células inmunitarias innatas y adaptativas como neutrófilos, células dendríticas, células T y B. Estos hallazgos sugieren que PGRN actúa como un adyuvante endógeno que puede

facilitar el desarrollo de nuevas estrategias para modular la respuesta inmunitaria protectora contra patógenos invasores.

Preface

More than 2.5 million neonates and infants die every year from blood-borne infections by encapsulated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae, Neisseria meningitidis*, *Escherichia coli* and *Staphylococcus aureus*. These bacteria express on their surface capsular polysaccharides (CPSs), repetitive carbohydrate units that are poor activators of conventional follicular B cells[2]. This B cell subset generates long-lasting antibodies with high affinity for antigen through a T cell-dependent (TD) mechanism requiring the formation of a germinal center (GC).

By contrast, CPSs predominantly activate marginal zone (MZ) B cells that are strategically positioned at the interface between the splenic immune system and the circulation. Compared to follicular B cell that need 5-7 days upon infection to generate high affinity antibodies, MZ B cell provide quick protection by producing in 1-3 day period short-lived antibodies with lower affinity for the antigen, in a T cell-independent (TI) manner[3,4].

For this reason, vaccines containing multiple unconjugated (or native) CPSs generate protective antibodies in adults, but not in children, who have a functional immaturity of the splenic MZ, as well as in asplenic or splenectomized patients[5].

By contrast, vaccination with protein-conjugated CPSs induces the generation of protective antibodies through a TD

pathway that could circumvent these limitations. However, it is too expensive for broad use in developing countries and elicits limited protection in T cell-deficient individuals. In addition, conjugated vaccines contain CPS from only three serotypes of encapsulated bacteria, whereas unconjugated vaccines contain CPS from up to 23 serotypes and thus provide broader protection.

For this reason the development of novel, more effective and less expensive vaccine strategies is urgently required, especially for children, asplenic and splenectomized patients or individuals with an impaired function of MZ B cells, due to common variable immunodeficiency (CVID), HIV infection and malaria, among other pathological conditions. The aim of this work was to determine a role for PGRN in the immune responses that take place in human spleen, both in homeostasis and in response to TD and TI antigens. The observation that PGRN was capable of enhancing antibody responses against TI antigens such as lipopolysaccharides (LPS) and phosphorylcholine (PC) and in response to immunization with TD antigens strongly suggest that PGRN may function as an adjuvant able to increase vaccine effectiveness in unresponsive patients.

On the other hand, several studies support the contribution of PGRN to the development of autoimmune diseases, such as systemic lupus erythematosus (SLE), systemic vasculitides, rheumatoid arthritis. In some patients suffering

from these conditions, neutralizing antibodies against PGRN have been detected, suggesting that PGRN could constitute a regulatory factors preventing the onset of the disease[6].

The involvement of dendritic cells (DCs) in autoimmunity is complex, but some evidence sustains that changes in DC functionality might induce inflammation and/or autoimmune manifestations, by activating self-reactive T cells or because of an inappropriate regulatory T (Treg) cell induction[7].

So far, no data are available about a PGRN-mediated negative regulation of DC function in autoimmunity. However, the findings that PGRN can reduce the secretion of pro-inflammatory cytokines and increase the production of IL-10 in DCs could represent a new mechanism adopted by the immune system to avoid autoimmune disorders caused by DC hyperactivation.

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INTRODUCTION

1. THE IMMUNE SYSTEM

1.1. Innate and adaptive immune responses

Pathogens usually enter a foreign body through the skin or mucosal surfaces[8]. When invasion occurs, proper cells of the immune system known as leukocytes (commonly known as white blood cells) sense their presence and thereafter remove them by mounting protective responses characterized by a progressively increasing specificity[9]. The cells, the mechanisms and the molecules that control the early and the late reactions to invading microorganisms belong to the **innate** and **adaptive immune system**, respectively. Leukocytes are broadly comprised of granulocytes (also known as polymorphonuclear leukocytes), monocytes, macrophages, dendritic cells (DCs), mast cells and lymphocytes. Granulocytes include neutrophils, eosinophils and basophils, whereas natural killer (NK) cells, invariant NKT (iNKT) cells, T cells and B cells belong to lymphocytes.

While granulocytes, monocytes, macrophages, DCs, mast cells and NK cells play essential roles in the innate immune system, T cells and B cells functionally participate in the adaptive immune system. Specific subsets of T and B cells, including iNKT cells, $\gamma\delta$ T cells, peritoneal B-1 cells and splenic marginal zone (MZ) B cells occupy an intermediate position[10]. Granulocytes, monocytes, macrophages and DCs cooperate with soluble components of the innate immune system such as complement proteins to mount nonspecific protective responses that occur very rapidly but do not confer long-lasting protection[11]. These responses promote the

initial containment of invading microbes by inducing an inflammatory reaction that triggers the recruitment of granulocytes and monocytes from the circulation to the site of infection[12].

Inflammation is further amplified by cytokines and chemokines secreted at a later stage by lymphocytes that cooperate with the innate immune system to mount specific protective responses conferring long-lasting protection. Specifically, antigen-activated $CD4^+$ T cells differentiate into multiple antigen-specific T helper (Th) effector cell subsets that enhance the recruitment and activation of granulocytes and macrophages, collaborate with B cells to induce antibody production, and enhance the antimicrobial activity of epithelial cells[13]. In contrast, antigen activated $CD8^+$ T cells develop into cytotoxic T lymphocytes (CTLs) that specifically kill infected cells to prevent the spreading of invading microbes such as viruses throughout the body[14]. Finally, antigen-activated B cells differentiate into plasma cells (PCs) that secrete antigen-specific antibody molecules belonging to the IgM, IgG, IgA or IgE class, each of them associated with specific effector functions[15].

1.2. Pattern recognition receptors

Innate cells promote immune responses by recognizing and responding to microbes through nonspecific germline encoded pattern recognition receptors (PRRs). PRRs have a broad reactivity for a large number of bacterial and viral molecules known as pathogen associated molecular patterns (PAMPs) or microbe-associated molecular patters, which include cell wall components such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic

acids, lipoproteins and β-glucan, but also viral signatures like single-strand RNA (ssRNA), double-strand RNA (dsRNA) and non-methylated deoxyribocytidinephosphateguanosine (CpG) DNA[16]. PRRs can be found in different compartments: on the cell surface and in intracellular organelles (transmembrane PRRs), in the cytoplasm (cytoplasmic PRRs), or released in the extracellular environment (soluble PRRs) (**Fig. 1**).

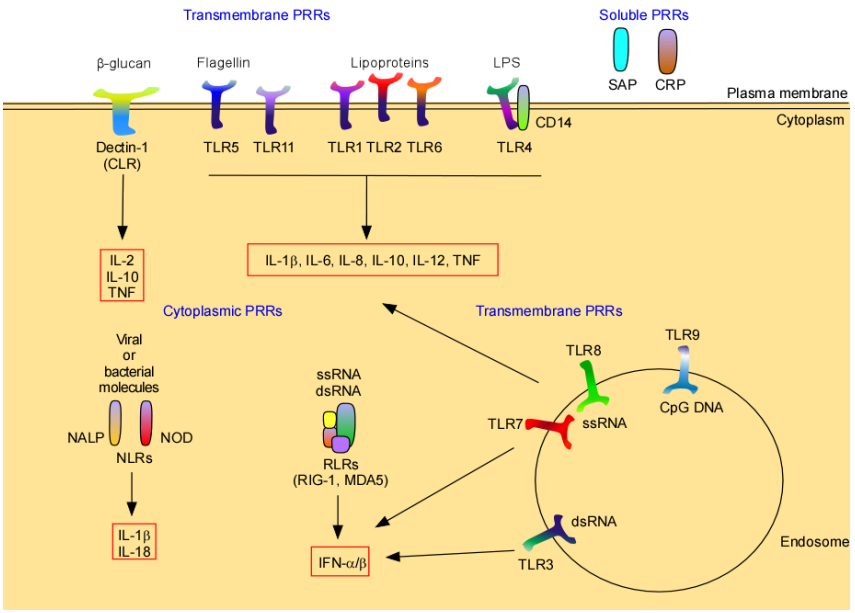

Figure 1. Mechanisms underlying microbial sensing. The immune response begins after the recognition of microbial intruders by PRRs belonging to the innate immune system. Transmembrane PRRs include Toll-like receptors (TLRs, from 1 to 11) and C-type lectin receptors (CLRs), including Dectin-1 and many other molecules. TLRs induce inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, IL-12 and TNF as well as anti-inflammatory cytokines such as IL-10. Moreover, TLR3, TLR7, TLR8 and TLR9 elicit the production of antiviral cytokines, including IFN-α and IFN-β (IFN-α/β). CLRs such as Dectin-1 induce the secretion of IL-2, IL-10 and TNF after recognizing β-glucan associated with fungi. Soluble PRRs include serum amyloid P (SAP) and C-reactive

protein (CRP) and cytoplasmic PRRs include NOD-like receptors (NLRs) such as NLR3 (or NALP) and NOD (nucleotide oligomerization domain) proteins, which induce the production of inflammatory cytokines such as IL-1β and IL-18 after recognizing various viral and bacterial molecules, including CpG DNA and muramyl dipeptide. Furthermore, cytoplasmic PRRs include retinoic-acid-inducible gene 1-like receptors (RLRs) such as RIG-1 and MDA5, which trigger the production of antiviral IFN-α/β after sensing viral ssRNA and dsRNA, respectively. *Figure from Knowles' Neoplastic Hematopathology, 2012.*

Transmembrane PRRs include Toll-like Receptors (TLRs) and C-type Lectin Receptors (CLRs).

-TLRs are type I transmembrane proteins composed by an extracellular portion containing leucine-rich repeats (LRRs) and a cytoplasmic domain with a conserved region called Toll/IL-1 receptor (TIR) domain[17,18].

Ten different TLRs have been identified in human (TLR1 to TLR10) and twelve in mouse (TLR1 to TLR9**,** TLR11**,** TLR12 and TLR13). They are located on the plasma membrane with the exception of TLR3, TLR7 and TLR9 that are expressed in the endosomal compartment[19]. TLR2 is essential for the recognition of a variety of PAMPs including bacterial lipoproteins, peptidoglycan and lipoteichoic acids from Gram-positive bacteria. TLR3 binds to virus-derived double-stranded RNA. TLR4 is mainly activated by LPS. TLR5 detects bacterial flagellin and TLR9 is involved in the response to unmethylated CpG DNA. TLR7 and TLR8 recognize single-stranded RNA[20] and small synthetic antiviral molecules[21]. TLRs are also able to heterodimerize with one another and their specificity is increased by various adaptor and accessory molecules. For example, MD-2 and CD14 can form a complex with TLR4 in response to LPS[22].

After recognizing specific cognate ligands, TLRs recruit TIR domain containing adaptor proteins such as MyD88 and TRIF to initiate downstream signaling events that trigger activation of various transcription factors, including NF-κB[23,24,25]. This pathway induces the generation and secretion of inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-12, tumor necrosis factor (TNF)), antiviral cytokines such as interferon (IFN)-α, chemokines, and antimicrobial peptides. In general, TLR signalling initiates an inflammatory response that causes recruitment of neutrophils, activation of macrophages, and induction of IFN-stimulated genes that help the killing of intracellular pathogens, including viruses. In addition, TLR signalling promotes the maturation of DCs, which in turn initiate the adaptive immune response by presenting processed antigens to T cells and unprocessed antigens to B cells[26].

-CLRs comprise a large family of receptors that bind to carbohydrates in a calcium-dependent manner and are involved in fungal recognition. The lectin activity of these receptors is mediated by conserved carbohydrate-recognition domains (CRDs). This family includes, among others, DEC205, macrophage mannose receptor (MMR), Dectin-1, Dectin-2 and DC-SIGN[27].

Cytoplasmic PRRs include nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic-acid-inducible gene 1-like receptors (RLRs).

-NLRs constitute a major class of intracellular PPRs. Twenty-two NLRs have been identified in humans. NLRs sense infection and stress by recognizing various bacterial, viral and danger molecules (including non-methylated CpG DNA, dsRNA and uric acid). Their engagement can induce an inflammatory response, autophagy or

cell death[28]. Several chronic inflammatory or autoimmune disorders are associated to genetic mutations in NLRs[29,30].

-RLRs constitute a family of cytoplasmic RNA helicases including RIG-1 and MDA5 that are critical for host antiviral responses, since they recognize ssRNA and dsRNA, respectively, leading to production of IFN-α and IFN-γ in infected cells[31,32].

Soluble PRRs are a heterogeneous group of molecular families, which represent functional ancestors of antibodies**.** They include collectins, ficolins, and pentraxins. They play a key role as effectors and modulators of innate responses in mice and humans[33,34].

2. HUMORAL RESPONSES IN THE SPLEEN

Development and activation of immune cells take place in primary and secondary lymphoid organs, respectively. The **primary lymphoid organs** in adults are comprised of the bone marrow and the thymus. Their principal function is the generation of mature B and T lymphocytes, respectively. The **secondary lymphoid (or peripheral) organs** include the lymph nodes, the spleen and the mucosal-associated lymphoid tissues (MALTs) such as tonsils and Peyer´s Patches. The main function of these organs is the recognition of the antigens and the initiation of the immune response[35].

In this thesis we will focus on immune responses occurring in secondary lymphoid organs, and in particular on spleen that represents the most important organ for antibacterial and antifungal immune reactivity.

2.1. Structure and function of the spleen

The spleen is an organ positioned in the abdomen and connected to the stomach. It is organized in two regions called the **white pulp** and the **red pulp**, which are separated by an interface called the **marginal zone (MZ)**. In human, the uniqueness of this organ is due to its structure and to its localization in the circulatory system, since part of the bloodstreams ends in the **perifollicular area**, the zone that separates the MZ from the red pulp[36] (**Fig. 2**).

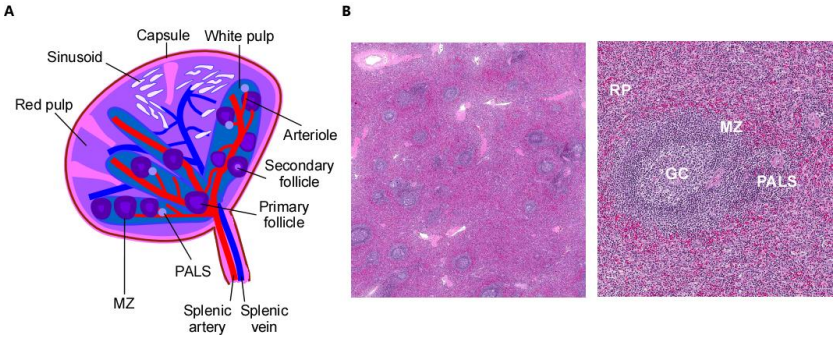

Figure 2. Structure of the spleen. **A**: Diagram of the spleen. The splenic artery enters the organ and branches into arterioles that terminate in the red pulp. The white pulp includes lymphoid structures called periarteriolar lymphoid sheath (PALS), which are composed of T cells organized around a central arteriole. The white pulp also includes primary follicles with no germinal center and secondary follicles with germinal center. Each follicle is surrounded by a large MZ that is in direct contact with the open circulation of the red pulp. Sinusoidal vessels drain blood from the red pulp into the venous system. **B**: Light micrographs of spleen sections stained with hematoxylin and eosin. The red pulp (RP) is filled with erythrocytes from circulating blood, whereas the white pulp contains numerous lymphoid follicles surrounded by a clear MZ area and with or without germinal center (GC). The MZ contains constitutively activated B cells that are larger than the small naïve B cells lodged in the follicular mantle. Therefore the MZ stains paler than the follicular mantle. Original magnification, x2 (left image) and x20 (right image). *Figure from Knowles' Neoplastic Hematopathology, 2012.*

The white pulp corresponds to the lymphoid compartment, which consists principally of two separated regions: the **T-cell zone** and the **B-cell zone**. The T-cell zone (also named the periarteriolar lymphoid sheath, PALS) is the region where T cells reside and can interact with interdigitating DCs and transiting B cells to develop adaptive immune responses. The B-cell zone is organized in follicles surrounded by a large MZ that undergo antigen-specific B-cell clonal expansion and immunoglobulin isotype switching upon

interaction with follicular DCs, DCs and Th cells. Specific chemokines, such as chemokine (C-C motif) ligand 19 (CCL19) and chemokine (C-X-C motif) 13 (CXCL13) are responsible for the correct organization and maintenance of the white pulp by attracting to their specific domains T and B cells, respectively.

In general, the white pulp is involved in the generation of antibody secreting cells (ASCs) that protect the body against blood-borne bacterial, viral, and fungal infections. The red pulp serves mostly to filter blood and recycle iron from aging red blood cells, but it is also a site where plasmablast (PBs) and PCs lodge, after they differentiate in the follicles of the white pulp. This extrafollicular antibody production leads to a rapid entry of antibodies in the bloodstream[36].

2.2. Splenic cell subsets

Leukocytes in the spleen include various subsets of T and B cells, DCs, macrophages, neutrophils and innate lymphoid cells (ILCs) that exert discrete functions. Here I will provide a brief description of each subset, while B cells and DCs will be discussed in more detail in part 2.3 and part 3, respectively.

T cells

Most studies about T cells are performed using mouse models or by the analysis of the peripheral blood compartment and very few information is available about their distribution and function in tissues, due to the scarce availability of such samples.

Circulating T cells frequently gain access to secondary lymphoid organs in search for their cognate antigens. Trafficking and positioning of lymphocytes is guided by stromal cell networks, integrin and chemokines such as CCL19 and CCL21 that bind CCR7 on T cells[37]. Both naïve and memory T cells remain in the secondary lymphoid organs for approximately 6-18h while scanning for antigen before returning to the circulation via the lymphatic vessels. Once primed by the antigen, the majority of activated T cells die within few hours, while a subset of T cells develop into long-lived memory T cells that persist in the tissue. A recent analysis of several human lymphoid tissues revealed that memory $CD4^+$ T cells represent the main population in donors aged 30 or older as they accumulate through life[38]. In this regard, one critical limitation of mouse models is that they cannot completely reflect the effect of exposure to multiple pathogens for decades.

According to their phenotype and the molecules produced, T cells can be subdivided in several effector subsets, including the following ones:

- T helper type 1 (Th1) cells are involved in the defense against intracellular viral and bacterial pathogens. They secrete inflammatory cytokines such as IFN–γ, IL-2, IL-10, and TNF and

depend on the expression of the transcription factor T-bet. The differentiation and expansion of Th1 cells are driven by cytokines including IL-12 and IFN-γ[39]. It has been largely demonstrated that exaggerated Th1 responses can be associated with rheumatoid arthritis, multiple sclerosis, and other autoimmune diseases[40].

- T helper type 2 (Th2) cells protect against large extracellular pathogens and are responsible for allergic responses, promoting the activation of eosinophil and mast cells. They mostly produce IL-4, IL-5, IL-9, IL-13, and IL-17E/IL-25. GATA-3 is the master transcriptional regulator of Th2 cells[41].

- Follicular helper T (Tfh) cells are highly involved in the generation of antibody-mediated immune responses, since they trigger the formation and maintenance of germinal centers (GCs) through the expression of CD40 ligand (CD40L) and the secretion of IL-21 and IL-4. They can be identified by the presence on the surface of CX-chemokine receptor type 5 (CXCR5) along with ICOS and/or PD-1[42].

- Regulatory T (Treg) cells comprise 5-10% of total $CD4^+$ cells. These cells are responsible for maintaining immune homeostasis by controlling the activation of Th cells and antigen presenting cells (APCs). Foxp3 expression is the most commonly used marker for Treg cells in mice and humans. Treg cells mediate their suppressive function through a variety of different mechanisms. They inhibit T cell responses through the secretion of IL-10 and TGF-β. They can also modulate DC function by binding CD80 and CD86 molecules via surface CTLA-4[43]. Moreover, $Foxp3^+$ Treg cells have been found in T-B area borders and within GCs of

human lymphoid tissues where they can directly suppress B cell antibody responses[44,45].

B cells

The spleen contains distinct B cell lineages, including *follicular* and *MZ* B cells. Follicular B cells recirculate and participate mainly in T cell-dependent (TD) immune responses[46]. MZ B cells reside between the MZ and red pulp, capture antigens carried in the blood via complement receptors, and promote principally T cell-independent (TI) responses, but in certain conditions they are also able to induce TD immune reactions[47]. In human, another abundant population of B cells has been identified, the *memory B cell* subset.

Macrophages

In mice, splenic macrophages can be classified in *red pulp macrophages* and within the reticular framework of the marginal zone, in *MZ macrophages* (MZMs) and *metallophilic macrophages* (MMMs). *Red pulp macrophages* cells are specialized to phagocytose aging erithrocytes and to regulate iron recycling and release. By contrast, ER-TR9^{+} MZMs and MOMA-1^{+} MMMs, which respectively populate the outer and inner MZ in mice, are involved in the elimination of certain types of blood-borne bacteria and viruses, thanks to the involvement of specific pathogen receptors[48]. For instance, the C-type lectin SIGN-R1, present on MZMs, mediates the recognition of pneumococcal saccharides and is essential for *S. pneumoniae* clearance[49]. Similarly, CD169, present on MMMs, has been implicated in the binding of meningococci[50].

The MZM and MMM subsets in mice are not present in humans. However, macrophages expressing CD68 (lysosome/macrosialin) and CD169 have been identified in the perifollicular area of human spleen[51,52] although their function remains to be established.

Dendritic Cells

Mouse DCs are composed by at least two classical subsets: $CD8\alpha^{+}CD11b^{-}$ cells, present in T-cell zones and responsible for the uptake of dying cells and cross-presentation of antigens to $CD8^{+}$ T cells, and $CD8\alpha^{-}CD11b^{+}$ cells, preferentially found in the red pulp and MZ, expressing major histocompatibility complex class II glycoprotein (MHCII)-peptide complexes and presenting antigens to $CD4^{+}$ T cells[53].

In humans, at least four populations of myeloid DCs and one population of plasmacytoid DCs (pDCs) have been found, but how they regulate immune responses is still under investigation[54].

Neutrophils

Neutrophils belong to the family of innate immune cells, since they are the first cells that migrate from the bone marrow to the site of infection or inflammation to eliminate microbes and necrotic cells. In inflamed tissues, they phagocyte and kill the bacteria principally through the production of reactive oxygen species (ROS) and the formation of neutrophil extracellular traps (NETs) containing extracellular DNA, enzymes and antimicrobial peptides.

Over the last 10 years, it has become evident that neutrophil have a lifespan longer than originally thought and that their function is not exclusively associated to the innate phase of the immune response, but can also influence adaptive immunity[55]. Neutrophils initiate and amplify innate and adaptive immune responses by establishing bidirectional interactions with monocytes, macrophages, DCs, NK cells, and T cells through contact-dependent and contact-independent mechanisms[56]. Neutrophil-derived myeloperoxidase (MPO) inhibits activation and migration of DCs to the lymph nodes, thereby reducing inflammatory T-cell responses[57]. On the other hand, monocyte-derived DCs maturation is induced, at least in vitro, by human neutrophils through contact-dependent interactions involving CD18 and CEACAM1. Those mature DC are able to induce T-cell proliferation and polarization towards a Th1 cell phenotype [58,59].

Neutrophils and T cells can reciprocally attract to the site of inflammation, by releasing different chemoattractant factors, including CXCL9 or CXCL8, respectively[60]. In addition, activated human $CD4^+$ and $CD8^+$ T cells have been shown to upregulate CD11b, CD64, and CD62L on neutrophils and delay neutrophil apoptosis, mainly via the secretion of IFN-γ and granulocyte–macrophage colony-stimulating factor (GM-CSF)[61]. Moreover, human and mouse neutrophils were shown to cross-present exogenous antigens *in vitro*, and injection of mice with antigen-pulsed neutrophils promoted the differentiation of naive $CD8^+$ T cells into cytotoxic T cells[62].

Mouse and human neutrophils exhibit many features that are typically reserved for DCs, including expression of DC markers (HLA-DR, CD1c, and CD11c), IL-12 cytokine production upon stimulation with TLR agonists, and APC function to present various forms of antigens to naive T cells[63].

Finally, our group identified a population of neutrophils called B cell-helper neutrophils (N_{BH} cells) that populate the perifollicular zone of the spleen after postnatal mucosal colonization by microbes, in the absence of inflammation. N_{BH} cells form NET-like structures that allow them to interact with MZ B cells, inducing immunoglobulin class switching recombination (CSR), somatic hypermutation (SHM) and antibody production[1,64]. Accordingly, neutropenic patients present fewer and hypomutated MZ B cells. N_{BH} cells have a distinct gene expression profile and phenotype, and express immunoregulatory factors, including B cell-activating factors like B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL, **Fig. 3**)[1].

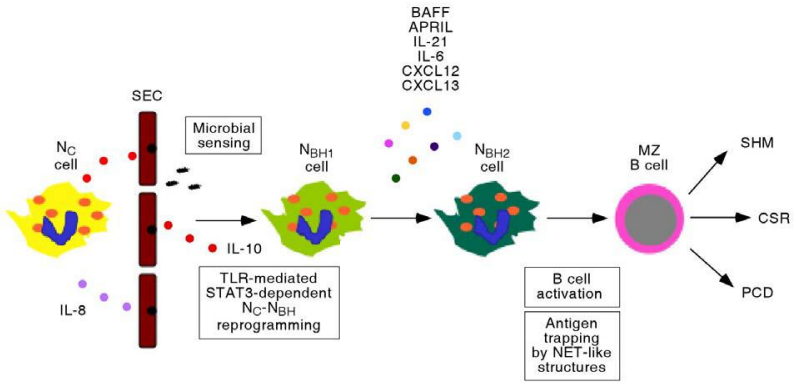

Figure 3. Proposed model for N_{BH} cell reprogramming and function. N_C cells home to the spleen under homeostatic conditions in response to microbial products undergoing splenic filtration after systemic

translocation from mucosal surfaces. In the presence of TLR signals, sinusoidal endothelial cells (SECs) release chemokines such as IL-8 to induce recruitment of N_C cells as well as STAT3-inducing cytokines such as IL-10 to induce reprogramming of NC cells into N_{BH} cells. These latter include N_{BH1} and N_{BH2} subsets with distinct phenotype, gene expression profile and B-helper activity. N_{BH} cells trigger SHM, CSR, plasma cell differentiation (PCD), including IgM, IgG and IgA production, by activating MZ B cells through a mechanism involving BAFF, APRIL and IL-21 release as well as formation of antigen-trapping NET-like structures. Production of B cell-attracting chemokines such as CXCL12 and CXCL13 as well as PCD-inducing cytokines such as IL-6 may also play a role. Interaction of N_{BH} cells with MZ B cells would facilitate the formation of a circulating pre-immune repertoire of antimicrobial immunoglobulins to TI antigens. *Figure from Puga et al.*[1]

Innate lymphoid cells

Innate lymphoid cells (ILCs) are a new family of developmentally related cells that are involved in innate immunity, tissue development and remodelling. It includes, among others, NK cells that mediate early immune responses against viruses and cancer cells, and lymphoid tissue-inducer (LTi) cells, essential for the formation of lymph nodes during embryogenesis[65].

Our group recently described a subset of ILCs in the MZ and perifollicular area of the spleen that regulate B cell-helper neutrophil homeostasis and activate them via GM-CSF. In addition, splenic ILCs enhance antibody production in MZ B cells in response to TI antigens, through BAFF, APRIL, CD40L and Notch ligand Delta-like 1 (DLL1). This study indicates that splenic ILCs

are able to orchestrate innate-like antibody production at the interface between the immune system and the circulatory system[64,66].

2.3. B cell responses

Each of the above mentioned population participates in the B cell-mediated immune responses that take place in the spleen. Such responses can be divided into innate-like responses and adaptive responses. In general, the white pulp is involved in the initiation of adaptive immune responses, while the marginal zone participates to both processes, by interacting with the key effectors of innate and adaptive immunity.

Antibody responses can be broadly separate in TD and TI[67]. The former require the interaction between T and B cells and the formation of a GC to initiate antibody production. TD responses are induced by soluble proteins or peptides associated with MHC molecules. By contrast, TI responses do not require T cells and can be promoted by two different classes of antigens. TI-1 antigens induce immune responses both in adults and neonates and include LPS, part of Gram-negative bacteria cell wall. By contrast TI-2 antigens normally induce antibody responses in neonates only after 24 months and are bacterial polysaccharides from encapsulated bacteria such as *S. pneumonia*, *N. meningitidis*, and *H. influenza*. However, several antigens such as proteins on pathogen surfaces are considered both TI and TD[67] (**Fig. 4**).

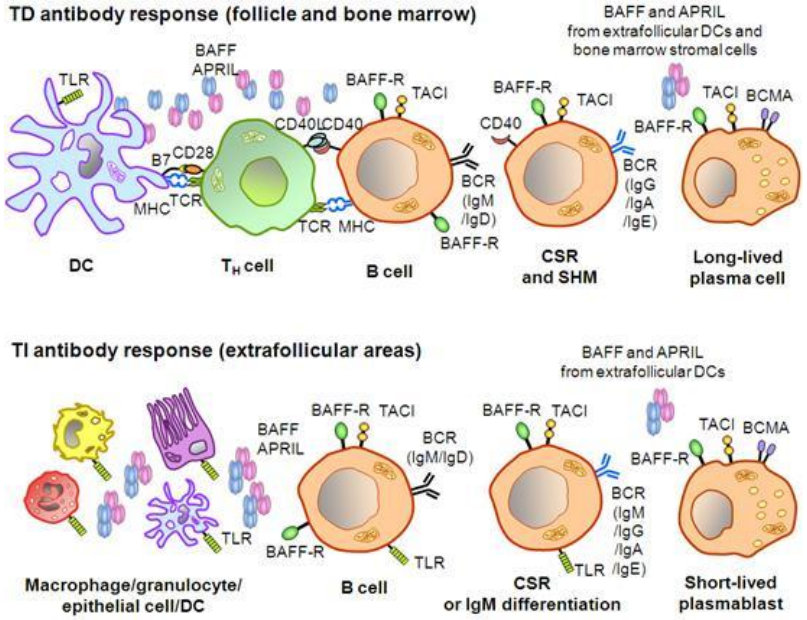

Figure 4. T cell-dependent and –independent antibody responses. TD antibody responses involve the ligation of the B cell receptor (BCR) and the engagement of CD40 on follicular or bone marrow B cells by CD40L expressed on $CD4^+$ Tfh cells that have been activated by antigen presentation on MHC-II molecules from those B cells. CD40L-CD40 interaction activates CSR and SHM, which eventually leads to immunoglobulin class switching from IgM to IgG, IgA or IgE and the differentiation of these B cells into PCs. This process mostly takes place in the GCs of secondary lymphoid organs, such as lymph nodes, spleen and Peyer's patches. BAFF and APRIL secreted by DCs can further augment TD antibody responses. TI antibody responses involve the activation of B cells by BCR ligands as well as BAFF and APRIL secreted by multiple immune cell types, such as DCs, monocytes, macrophages, epithelial cells (ECs) and granulocytes. This process mostly takes place in extrafollicular areas such as those in the splenic MZ and intestinal lamina propia. BAFF and APRIL induce CSR of these B cells by binding to their receptor TACI, which induces signals that activate NF-κB signaling and AID function. The B cells can undergo CSR from IgM to IgG, IgA and IgE and differentiate into PCs. BAFF secreted by the various cell types can promote the survival of these PCs by binding to the BAFF receptor (BAFF-R) expressed on these cells. *Figure from Chen K.*

Before describing splenic B cell-mediated immune responses, a brief overview of antibody structure and function will be provided.

2.3.1. Antibody structure and function

Antibodies are heterotetrameric structures composed of two identical heavy (IgH) and light (IgL) chains held together by disulfide bonds. Each IgH chain contains a variable (V) region and a constant (C) region, the latter determining the isotype (or class) of the antibody and thus its specific effector function[68]. Briefly, mammals produce five classes of immunoglobulins (Igs): IgM, IgD, IgG, IgE, and IgA, encoded by the μ, δ, γ, ε, and α constant regions, respectively[69] (**Fig. 5**).

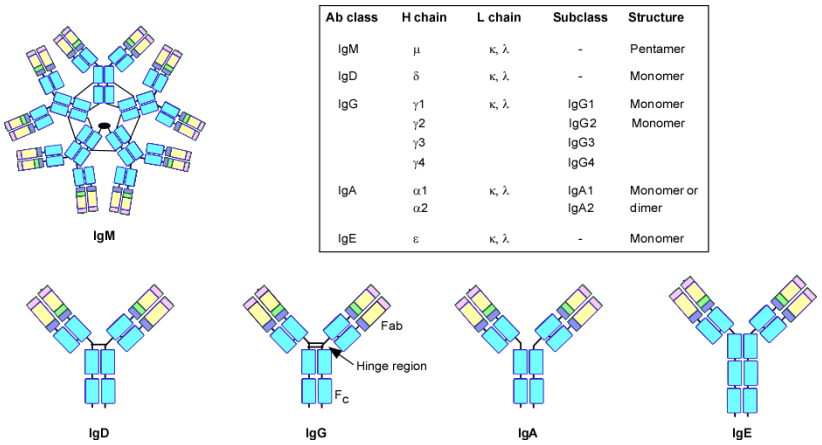

Figure 5. Structure of antibodies. Antibodies (or immunoglobulins) are heterotetrameric molecules composed of two identical IgH chains (blue) and two identical IgL chains (yellow) that are held together by disulphide bonds (black lines). Each IgH chain contains a V region encoded by a V_HDJ_H exon and a C region encoded by Cμ, Cδ, Cγ, Cα or Cε exons, which determine the IgM, IgD, IgG, IgA and IgE isotype (or class) of an

antibody. Cγ and Cα exons can be further divided into Cγ1, Cγ2, Cγ3, Cγ4, Cα1 and Cα2 exons, which determine the IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2 subclass of an antibody. Similar to IgH chains, IgL chain contain a V region encoded by a V_LJ_L exon and a C region encoded by Cκ or Cλ exons. The hinge region connects the Fab fragment, which is the antigen-binding portion of the antibody, with the Fc fragment, which mediates the effector functions of the antibody. While IgD, IgG and IgE antibodies are exclusively produced as monomeric molecules, IgM and IgA antibodies are produced as either monomeric or oligomeric molecules, including IgM pentamers and IgA dimers. Transmembrane IgM and IgA are always monomeric, whereas secreted IgM and IgA can be either monomeric (circulating IgA) or oligomeric (circulating IgM, mucosal IgM and mucosal IgA). *Figure from Knowles' Neoplastic Hematopathology, 2012.*

IgM is expressed on the plasma membrane of the B lymphocytes as a monomer and serve as surface B cell receptor (BCR)[70]. Serum IgM represents approximately 10% of total serum immunoglobulins, can form pentamers in mammals and predominates in primary immune responses to most antigens[71]. It accounts for the majority of natural antibodies present in our circulation, and is characterized by a low affinity but a high avidity for the antigen. It is the most efficient complement fixing immunoglobulin but it is also involved in neutralization and opsonisation[72].

IgD can be expressed as a monomer in the cell membrane, in the cytoplasm or can be secreted[73]. Transmembrane IgD is expressed by mature naive B cells prior to antigenic stimulation and CSR, by MZ B cells and by IgD producing B cells in the upper aerodigestive MALTs and peripheral blood of healthy individuals. In addition to blood, soluble IgD is also present in human nasal, lacrimal, salivary, mammary, bronchial, pancreatic, and cerebrospinal fluids and in the amniotic fluid of pregnant women. Amounts of IgD are

also present in intestinal mucosal secretions. Secreted IgD can bind to many pathogenic microorganisms and their products, such as *rubella virus, measles virus* or *diphtheria toxin*, strongly supporting the notion that IgD has protective functions against these pathogens perhaps by contributing to immune exclusion or neutralization, but not complement activation[74]. In this regard, circulating IgD has been showed to interact with basophils through a calcium-fluxing receptor that induces antimicrobial, opsonizing, inflammatory and immunostimulating factors[75].

IgG is expressed by memory B cells as a surface BCR and released by class-switched PCs as part of the secondary immune response to an antigen. It constitutes approximately 75% of total immunoglobulins in human serum. It is composed of four subclasses: IgG1, IgG2, IgG3, and IgG4[76].
Of note, IgG is the only class able to cross the placenta in humans, being largely responsible for protection of the newborn during the first months of life[77]. All IgG subclasses can easily perfuse tissues to perform neutralization, opsonization and complement fixation[78].

IgA can be expressed as a surface BCR but also secreted by some memory B cells. In serum, it exists in both monomeric and dimeric forms, representing approximately 15% of the total serum Igs[79]. Circulating IgA is mostly composed of IgA1, while mucosal IgA encompasses both IgA1 and IgA2 subclasses. Secretory IgA is a dimer abundant in mucosal secretions that is involved in defense mechanism against some local infections[80]. The principal function of secretory IgA is to neutralize pathogens and toxins without causing inflammation due to its inability to fix and activate complement cascade[81,82].

IgE is found on the membrane of some memory B cells, but also in serum and mucosal secretions, although in much smaller quantities than other immunoglobulins. It participates both in primarily defence against parasitic invasion and in allergic reactions. Elevated production of IgE is present in subjects with the atopic conditions, asthma, allergic rhinitis, and atopic dermatitis. IgE has a high affinity for FceRI expressed by mast cells, eosinophils and basophils. Binding of the antigen to FceRI-IgE complex induces the release of proinflammatory and anti-microbial molecules such as histamine and proteases[83].

2.3.2. Marginal zone and marginal zone B cells

MZ was originally identified in 1901 as a distinguishable collection of cells surrounding splenic follicles but the name ´marginal zone´ was attributed to the structure that separates the white pulp from the red pulp by MacNeal only in 1929[84].

The anatomy of mouse and human spleens is mostly similar, even though the human MZ is more structured, with well-defined outer and inner layers surrounded by a large perifollicular zone. Also the splenic microvasculature shows remarkable differences in these two species. In mice, where the MZ resides in the outer white pulp of the spleen between the marginal sinus and the red pulp, antigens reach the MZ passing through the marginal sinus. By contrast, human spleen lacks a marginal sinus and antigen-carrying blood directly drains into capillaries of the red pulp and perifollicular zone[36].

The fact that MZ is not fully functional in the first years after birth explains why children less than 2–3 years of age are more susceptible to infections by encapsulated bacteria and present defects in long-lasting protection towards the polysaccharide pneumococcal vaccine[85,86]. Similarly, inefficient antibody response to invasive pneumococcal disease in aged individuals is associated with altered functions of the MZ[87]. Finally, splenic dysfunction or splenectomy increase the risk of infection with *Streptococcus pneumonia*, *Heamophilus influenza*, *Neisseria meningitis* and other encapsulated bacteria, normally attributed to a defect in circulating MZ B cells and a lack of IgM and IgG antibody responses against these pathogens[88].

Different cell types are strategically located in the MZ to quickly respond to blood-borne pathogens (bacteria and viruses), and in particular to TI antigens. Mouse MZMs express the C-type lectin SIGNR1 that efficiently binds polysaccharide antigens and several viruses[89], and the type I scavenger receptor MARCO that can recognize many pathogens, including *Escherichia Coli* and *Staphylococcus Aureus*. Both molecules are proposed to activate MZ B cells[90]. MMMs express SIGLEC1, which can bind sialic-acid residues at the cell surface of pathogens[91]. They are the main producers of IFN-α and IFN-β after viral challenge, whereas the key function of MZMs is to interact with antigens and present them to MZ B cells[92].

Along with DCs, macrophages and neutrophils, MZ B cells have been described as one of the major population of innate-like lymphocytes that cross over the conventional boundaries between

the innate and adaptive immune systems. They bridge the temporal gap required for the production of high-affinity antibodies by follicular B cells. Whereas in mice MZ B cells are confined to the MZ of the spleen, in human they recirculate freely and can be found also in the inner wall of the subcapsular sinus of lymph nodes, in the epithelium of tonsillar crypts and in the subepithelial area of MALTs, including the subepithelial dome of intestinal Peyer's patches[4,93,94].

Mouse and human MZ B cells can be distinguished from $IgM^{hi}CD21^{hi}IgD^{hi}CD23^{hi}$ follicular naïve B cells as being $IgM^{hi}CD21^{hi}IgD^{lo}$ and $CD23^{neg}$. In humans, MZ B cells are $CD27^{+}$ memory B cells harboring a prediversified immunoglobulin repertoire[95]. Many MZ B cells express high levels of TLRs and polyreactive BCRs that bind to multiple microbial molecular patterns and allow providing efficient immune surveillance to the circulatory system. After interacting with antigens exposed on macrophages, DCs or neutrophils, and in the presence of co-stimulatory signals from other cells, MZ B cells rapidly differentiate into PBs that produce large amounts of IgM^{1} (**Fig. 6**).

Mouse blood $CD11c^{lo}$ DCs have been shown to play a necessary and sufficient role in the initiation of a TI immune response against blood-borne *S. pneumoniae*, bringing intact bacteria to the spleen, providing critical survival signals to MZ B cells, and promoting their differentiation into IgM-secreting PBs[96]. MZ are also able to undergo SHM and CSR and to produce IgG and some IgA in response to pathogens and commensal antigens[1,64].

Although MZ B cells are typically responsible to mount effective responses to TI antigens, the expression of high levels of MHC class II and co-stimulatory molecules render these cells able to present antigens to Th cells and promote extrafollicular antibody production[97]. Song el al. showed for the first time that entry of MZ B cells to the white pulp through MZ can initiate adaptive responses mediated by GC reactions, through activation of T cells[98].

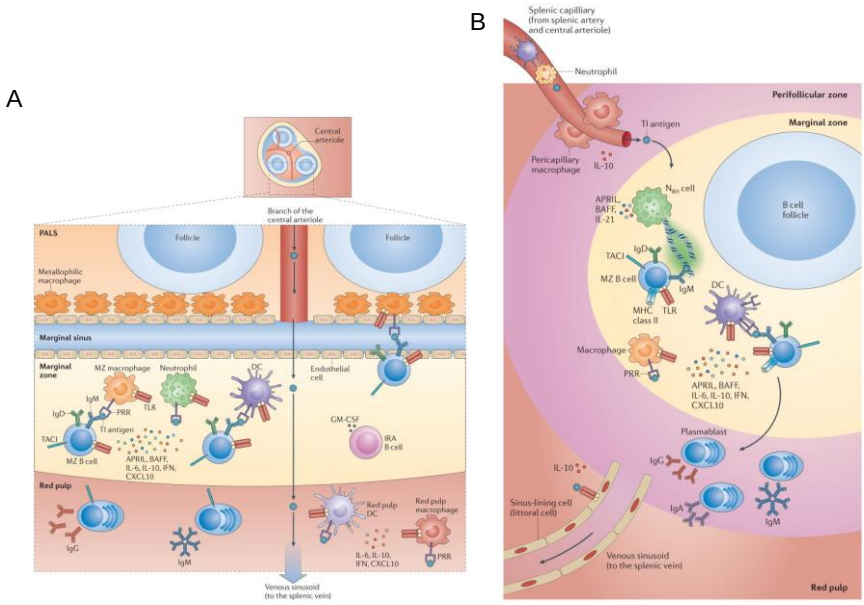

Figure 6. (A) T cell-independent responses by mouse MZ B cells TI antigens are captured by metallophilic macrophages and MZ macrophages after entering the splenic MZ via the marginal sinus. Alternatively, TI antigens are captured by DCs and neutrophils in the circulation. Innate response activator (IRA) B cells may enhance the survival and activation of these antigen-capturing cells by releasing GM-CSF. Antigen-sampling cells stimulate MZ B cells via the BCR, the TLRs and TACI. TACI delivers signals that induce CSR and antibody production after ligating BAFF and APRIL, which are released by antigen-capturing cells in response to microbial TLR ligands. Antigen-capturing cells, including red pulp DCs and macrophages, also secrete IL-6, IL-10, type I IFN and CXCL10, which cooperate with BAFF and APRIL to promote the

differentiation and survival of plasmablasts secreting IgM or class-switched IgG. Arrows indicate the path followed by antigens through the spleen. (**B) T cell-independent responses by human MZ B cells.** TI antigens are thought to enter the MZ through the perifollicular zone. Once in the MZ, they may be captured by NET-like structures emanating from N_{BH} cells Antigen capture may also involve reticular cells, macrophages, sinus-lining cells and DCs. In addition to making TI antigens available to the BCR and TLRs on MZ B cells, antigen-capturing cells release BAFF and APRIL, which engage TACI on MZ B cells. N_{BH} cells also release IL-21, thereby inducing CSR, SHM and antibody production in MZ B cells. The generation of PBs secreting IgM or class-switched IgG and IgA involves the production of IL-6, IL-10, IL-21 and CXCL10 by antigen-capturing cells. Arrows indicate the putative path followed by antigens through the spleen. *Figure from Cerutti et al.* [4]

2.3.3. Germinal center

B cell follicles can be classified as quiescent **primary follicles**, composed of largely naïve B cells migrating in search of their cognate antigen, and activated **secondary follicles**, which contain a **GC** full of B cell blast undergoing events associated with antibody affinity maturation[99,100] (**Fig. 7**).

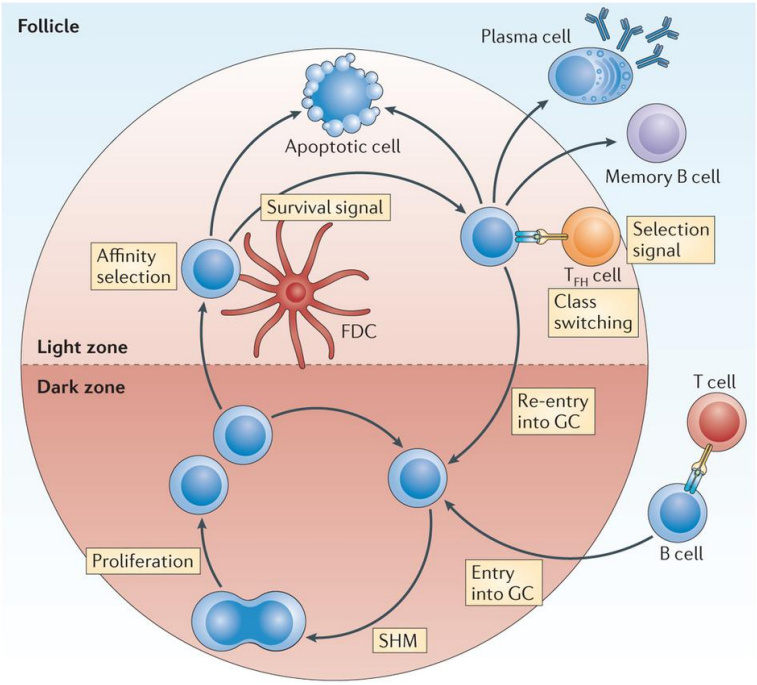

Figure 7. The germinal center reaction. Naïve B cells capture native antigen from subcapsular sinus macrophages and paracortical DCs through the BCR (both IgM and IgD molecules) and subsequently establish a cognate interaction with Tfh cells located at the boundary between the follicle and the extrafollicular area. After activation by Tfh cells via CD40L and cytokines such as IL-21, B cells enter either an extrafollicular pathway to become short-lived IgM-secreting PBs or a follicular pathway to become GC centroblasts. In the dark zone of the GC, centroblasts undergo extensive proliferation, express AID and induce SHM and CSR from IgM to IgG, IgA or IgE. After exiting the cell cycle, centroblasts differentiate into centrocytes that interact with FDCs located in the light zone of the GC. FDCs expose immune complexes containing native antigen to the BCR and centrocytes with low-affinity for antigen die by apoptosis, whereas centrocytes with high affinity for antigen differentiate to long-lived memory B cells or PCs expressing high-affinity and class-switched antibodies. Memory B cells recirculate, whereas PCs migrate to the bone marrow. *Figure from Heesters et al.*[101]

Small soluble antigens gain access to B cells after entering the follicle through a specialized transport system known as follicular

conduit network[102]. In the B cell zone, follicular DCs (FDCs) first present antigen to naïve B cells that after interacting with the antigen, migrate to the T-B border, the boundary of the follicle with the T-cell zone[103].

Similarly, initial activation of antigen-specific T cells is thought to occur in the T cell-zone through encounters with IL-12 producing DCs. Like activated B cells, at least some activated T cells, named early Tfh cells, increase the expression of the chemokine receptor CXCR5 and migrate to the B cell follicle in response to the chemokine CXCL13, a CXCR5 ligand produced by FDCs[104]. After the encounter with antigen presenting DCs, Tfh-committed T cells enhance also their expression of B cell lymphoma 6 (BCL-6), the master regulator of both Tfh cell and GC B cell programme.

In the T-B zone, B cells then present antigens in the form of antigenic peptides to antigen-specific Tfh cells, which, in turn, deliver survival signals to the cognate B cells. At this stage, B cells begin to divide. Some B blasts migrate to extrafollicular sites and differentiate into low-affinity IgM-secreting PBs[3]. Other B blasts move to the center of the follicle to form a GC[105,106]. Upon upregulation of CXCR5 on their surface, also activated Tfh move to the center of the follicle and become GC Tfh cells. GC Tfh cells sustain the proliferation, differentiation, diversification and selection of centroblasts and centrocytes, by expressing high levels of CD40L and IL-21[107].

Centroblasts undergo clonal expansion in the dark zone of the GC, thereby pushing naive IgM^+IgD^+ B cells to a peripheral area of the follicle called *mantle zone*[108]. They also undergo SHM and

CSR, two Ig-diversifying processes that are highly dependent on the enzyme activation-induced cytidine deaminase (AID)[109]. Then, centroblasts exit cell cycle and migrate to the FDC-rich zone called the *light zone* as small and non dividing **centrocytes** that can be distinguished phenotypically on the basis of high CD83 and CD86 surface expression with low CXCR4 expression[110]. Centrocytes take up antigen presented by FDCs, from which they also appear to receive survival signals. After binding antigen, centrocytes establish a cognate interaction with GC Tfh cells[111,112]. Centrocytes that receive T-cell-derived survival signals then proceed to become either long-lived PCs or memory B cells, which can protect the host against re-exposure or help clear persistent primary infections[113–115].

Centroblasts and centrocytes express IgM or IgG or IgA together with CD19, CD27, CD38 and CD10, and the GC-associated transcription factor Bcl-6[116,117]. Of note, centroblasts also express CD77, whereas centrocytes do not[116].

T-cell help is thus central to GC maturation and centrocyte selection in the light zone and therefore critical for the genesis of a high affinity and long-lasting antibody response, and for the formation of quality memory B cells. Indeed, centrocytes that express a low affinity BCR die by apoptosis and then are engulfed by resident phagocytes known as tangible body macrophages[108,118]. The high susceptibility of centroblasts and centrocytes to apoptosis is due to lack of the intracellular anti-apoptotic factor Bcl-2 and the expression of intracellular Bcl-2 family members with pro-apoptotic activity[119]. This feature allows their elimination in the absence of engagement of BCR by high-affinity antigens. Engagement of CD40 in GC B cells by CD40L on

Tfh cells up-regulate the expression of the death-inducing receptor Fas[120]. In addition, Fas ligand (FasL) expressed by Tfh cells further promote the elimination of low-affinity GC B cells[121,122]. Only GC B cells expressing a BCR with high affinity for antigen survive thanks to strong “rescue” signals generated by the BCR[123].

Interestingly, Schwickert et al. showed that GCs are dynamic and plastic microenvironment that can be reutilized during immune responses to unrelated antigens[124]. Several independent studies support a likely role of abnormal GC reactions in the pathogenesis of human autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis, and thus could lead to the identification and development of new therapeutic targets[118].

2.3.4. Immunoglobulin diversification

Mature B cells emerge from the bone marrow with fully competent IgM and IgD antibodies that serve as surface BCR molecules for antigen, generated through an antigen-independent process called V(D)J recombination that requires RAG proteins[125]. It is also in the bone marrow where tolerance checkpoints take place. Self-reactive clones are extinguished by apoptosis or receptor editing, whereas non-autoreactive cells are selected to further develop and mature at the periphery[126].

In secondary lymphoid tissues, BCR engagement by the antigen leads to an additional diversification of the immunoglobulin genes to optimize the response to a specific pathogen, through two antigen-dependent processes called SHM and CSR. These

processes occur only in B cells, and they are regulated by developmental and environmental stimuli. They are essential, and their impairment results in immunodeficiency. Although the molecular signatures are distinct, all these processes are initiated by the single B cell–specific factor AID, strongly expressed by centroblasts and centrocytes[109]. Both CSR and SHM are primarily detected in the GC, but studies showed that AID is also expressed in extrafollicular large activated B cells[127].

Class Switch Recombination

CSR irreversibly detaches an expressed heavy chain variable (VDJ) region from the constant (C) region of the IgM and joins it to a downstream C region of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgE, deleting the DNA in-between. Thus, CSR determines how the antigen will be removed by the immunogobulin without affecting the specificity of antigen-recognition[128].

Cytokines have been shown to direct the synthesis of specific constant heavy chain (CH) genes. Thus, while IL-4 and IL-13 preferentially induce IgG4 and IgE[129–131], TGF-β is mainly responsible for IgA1 and IgA2 synthesis[132].
In addition, CD30 engagement effectively downregulates CSR to all downstream isotypes by inhibiting targeted CD40-mediated DNA recombination to Cγ, Cα, and Cε in human $IgM^{+}IgD^{+}$ B Cells[133].

CSR must be tightly regulated because abnormal IgG and IgA production favours the onset of autoimmune disorders such as

SLE[134], while increased switching to IgE leads to atopic disorders such as asthma and atopic dermatitis[135].

Somatic Hypermutation

SHM is a programmed process of point mutation targeting the variable regions of immunoglobulin genes that allows the clonal selection of B cells that express immunoglobulin receptors with the highest affinity for foreign antigens exposed on FDCs. The C regions and the rest of the genome remain untouched. Together, SHM and clonal selection not only increase antibody affinity, but also provide a dynamic response to pathogens that are simultaneously undergoing continuous mutation and selection[136]. Of note, while mouse MZ B cells primarily express non-mutated immunoglobulin variable (IgV) genes, human MZ B cells undergo SHM at a very early developmental stage through a mechanism that does not require the presence of an anatomical MZ[137]. In addition, human MZ B cells accumulate more mutations through antigen-dependent and antigen-independent mechanisms in both children and adults. This mechanism guarantees the presence of a highly diversified repertoire of ready-to-use MZ B cells[95,4].

3. DENDRITIC CELLS

DCs are highly specialized accessory cells that were firstly described in the 1970s by Ralph Steinman and Zanvil Cohn, as a novel subset of adherent cells with an elongated and stellate morphology, characterized by the presence of branched neuron-like cytoplasmic projections (or dendrites). Their principal function is to transmit to lymphocytes all the information derived from pathogens or endogenous danger signals[138].

Despite strong evidence that DCs are functionally distinct from other leukocytes, there is no single cell-surface antigen able to identify this heterogeneous family of cells. The lack of common markers, together with their low frequency in the body (1-2% of the total leukocytes), have made difficult for several decades the identification of a DC precursor, as well as their purification and characterization[139]. DC subtypes differ in their morphology, origin, location, migration patterns, expression of PRRs, and functional features. All these parameters determine the type and quality of the immune response that has to cover the vast array of pathogens encountered by the immune system[140]. However, no clear consensus has yet been reached among immunologists for DC subset nomenclature[141].

3.1. Dendritic cell subsets

A first subset of DCs is observed only in response to inflammatory or microbial stimuli and differentiates from Ly6C^{hi} monocytes recruited to the site of inflammation[142]. They are referred to as

inflammatory DCs (infDCs). Most inf-DCs are $CD11c^{int}$ $CD11b^{+}$ HLA-DR^{+} $Ly6c^{+}$ but can also express CD206, GM-CSF receptor (GM-CSFR), Mac-3/CD107b, FceRI, and CD64. Several studies demonstrated that inf-DCs can present antigens to $CD4^{+}$ T cells but they can also cross-present exogenous antigens to $CD8^{+}$ T cells[143]. Inf-DCs have been described also in human to participate to several pathological conditions, such as atopic dermatitis, psoriasis, and rheumatoid arthritis. Despite a quite well characterized phenotype, there are still limited data on the functional properties of human inf-DCs[143]. A recent work showed that inf-DCs are the main inducers of Th17 cells in arthritic joints, and possibly in other inflammatory settings, through the secretion of IL-23[144].

Human **$CD14^{+}$ DCs** are found in tissues (such as liver, kidney, heart, connective tissues) and lymph nodes and are a subset of $CD11c^{+}$ myeloid cells originally described as 'interstitial DCs'. $CD14^{+}$ DCs were found to be unique in their ability to induce the generation of Tfh cells[145] and the differentiation of naive B cells into IgM-secreting PCs[146].

Two other major groups of DCs can be defined under steady state conditions according to their primary location and their migration pattern. DCs that reside in non-lymphoid tissues and migrate to the draining LNs via lymphatic vessels are referred to as **migratory DCs (mig-DCs).** By contrast, DCs that reside throughout their life cycle in lymphoid organs but may also be circulating at very low frequency in the blood are denoted as **lymphoid tissue-resident DCs (LT-DCs**). LT-DCs are found in lymphoid organs such as lymph nodes, spleen and thymus. This subset principally includes

two populations of cells, the ***plasmacytoid dendritic cells (pDCs)*** and the ***classical dendritic cells (cDCs)*.**
Mig-DCs will not be discussed in this thesis, while pDCs and cDCs will be described indvidually.

3.1.1. Plasmacytoid dendritic cells

pDCs develop in the bone marrow and accumulate principally in blood and lymphoid tissues, entering the lymph nodes from the blood[147] . They were firstly described in human as cells with a plasma cell-like morphology, specialized in rapid and massive secretion of IFN-α/β in response to virus and/or virus-derived nucleid acids[148,149].

The subsequent identification of the murine counterpart[150] led to a deeper knowledge of pDC development and function. They are rare cells (0.3-0.5% in lymphoid organs), characterized by low levels of CD11c, MHC class II and costimulatory molecules. They can be identified by the surface expression of B220/CD45RA, SiglecH and Bst2/PDCA-1. In addition, pDCs express TLR7 and TLR9 that allow them to respond promptly to viral infections, producing 1,000 fold more IFNα than any other cell[151]. Upon TLR engagement they can also secrete TNF and IL-12 and acquire the capacity to present antigens. Altogether, these features define a population of cells that function as a link between innate and adaptive immunity, by promoting the recruitment and/or activation of nearly all immune cell types, including NK cells and PCs[147].

In humans, pDCs typically lack myeloid antigens and are distinguished by the expression of CD123, CD303 and CD304. In steady state, due the low levels of costimulatory and MHC class II molecules and the reduced phagocytic activity, pDCs are poor stimulators of $CD4^+$ T cells, and seem to be involved in inducing T cell anergy and Treg cell formation[152]. Upon stimulation pDCs acquire APC capacity and can activate naïve $CD4^+$ T cells[153].

3.1.2. Classical dendritic cells

CDCs populate lymphoid (spleen, lymph nodes and bone marrow) and nonlymphoid tissues, such as connective tissue and intestine. A key feature of cDCs is their short half-life (approximately 3–5 days) and their continuous replacement from bone marrow precursors, a process dependent on the cytokine Flt3L[154]. cDCs have enhanced capacity to sense injuries, environment or cell-associated antigens and to present them to T lymphocytes. Although different subtypes exist, they share some important features.

Subtypes

Spleen and lymphoid organs contains two major subsets of cDCs: the $CD8^+$ $CD11b^-$ and the $CD8^-CD11b^+$ DCs. $CD8^+$ DCs represent 20-40% of spleen and lymph node DCs, express high levels of Flt3 and proliferate in response to Flt3L. $CD11b^+$ DCs lack CD8 expression and also proliferate to Flt3L, although to a lesser extent. The $CD8^+$ DCs in lymphoid organs mediate efficient cross-

presentation to cytotoxic T cells[155], whereas the $CD11b^+$ subset is preferentially involved in MHC class II- restricted antigen presentation to $CD4^+$ Th cells[156].

In the spleen, $CD8^+$ DCs are concentrated in the T-cell areas. In contrast to mig-DCs that arrive to the lymph nodes in a mature state, LT $CD8^+$ DCs are phenotypically immature in the steady state and mature upon stimulation with microbial products[155]. They closely relate to nonlymphoid tissue $CD103^+$ cDCs[157]. By contrast, $CD11b^+$ DCs reside in the MZ of most laboratory mice, but can migrate into the T-cell zones in response to microbial products. Among $CD11b^+$ DCs, two different populations can be identified, based on the expression levels of the endothelial cell-specific adhesion molecule (ESAM). $ESAM^{hi}$ DCs are also $CD4^+$, $CD11c^{high}$, $Flt3^{high}$ and are required for efficient T cell priming. By contrast, $ESAM^{lo}$ DCs are $CD4^{low}$, $CD11c^{low}$, and $Flt3^{low}$ and are phenotypically closer to, but distinct from, monocytes and macrophages[158].
Lymph nodes contain two extra DC subtypes that are not normally found in spleen, which probably enter through the lymphatic system. $CD4^-CD8^-CD11b^+$ DCs are present in all lymph nodes, express moderate levels of CD205, and are considered the mature form of tissue interstitial DCs. Finally, another DC subtype has been found only in skin-draining lymph nodes, expresses high levels of langerin, and is believed to be the mature form of Langerhans cells.

Human lymphoid tissues are poorly described, but principally contain pDCs and myeloid DCs, in steady state. $CD11c^+$ Myeloid DCs correspond to mouse ‘classical’ DCs and may be classified

into $CD1c^+$ (0.6% of PBMCs) and $CD141^+$ (<0.05% of PBMCs) DCs. Genome-wide expression analysis showed that human $CD141^+$ DCs are more related to mouse $CD8^+$ DCs, whereas human $CD1c^+$ are homologous to $CD11b^+$ DCs[159]. They both lack CD14 or CD16, markers that in human distinguish DCs from tissue resident $CD11c^+$ monocytes.

$CD1c^+$ DCs are the major population of human mDCs not only in lymphoid organs, but also in blood and peripheral tissues. They express CD11b, CD13, CD33, CD172 (SIRPα) and CD45RO[160]. They have been shown to readily stimulate naïve $CD4^+$ T cells and to secrete high amounts of IL-12 in response to TLR ligation[161].

$CD141^+$ DCs uniquely express the lectin Clec9A[162] and the chemokine XCR1. They produce high amount of IL-12 and IFN-α in response to TLR3 agonists[163] and cross-present antigens derived from dead or necrotic cells to $CD8^+$ T cells[164].

Antigen processing and presentation

CDCs can present endogenous and exogenous antigens associated to both MHC-I and MHC-II molecules. Self- or pathogen-derived antigens that are synthesized within the cell classically bind to MHC class I molecules and are presented to $CD8^+$ T cells, whereas exogenous antigens derived via endocytic uptake are loaded onto MHC class II molecules for presentation to $CD4^+$ T cells[165]. Some DCs are also specialized to process exogenous antigens into the MHC class I pathway, through a process called cross-presentation[166]. By contrast, autophagy defines another occurring process whereby intracellular antigens

are assembled on MHC class II molecules and presented to $CD4^+$ T cells[167].

Antigen processing and presentation are influenced by the maturation status of the cell. Typically, immature DCs can efficiently capture and present antigens, but do not stimulate T cells as mature DCs do. Immature DCs migrate to sites of microbial infection or tissue damage in response to a large spectrum of inflammatory chemokines produced by local cells, including CCL3, CCL5 and CCL20. At these sites, DC maturation occurs. CDCs recognize general molecular signatures associated with microbes through a broad array of germline-encoded PRRs, including TLRs and CLRs with endocytic function[168]. Microbial recognition not only enhances the internalization of intruding microbes via pinocytosis and endocytosis, but also triggers the release of immunostimulating and inflammatory cytokines (IL-6, IL-12, TNF) as well as antimicrobial factors (defensins, IFN-α/β) that limit the spread of the infection[169].

Migration

Mature DCs move via the afferent lymphatics from peripheral sites of infection or inflammation to the T cell areas of secondary lymphoid organ or mucosa-associated lymphoid follicles[170]. Peripheral cDCs migration via afferent lymphatics is CCR7 dependent and cDCs utilize CCL19 and CCL20 (at T cells do) to enter the lymph nodes[171].

Intratissue migration of LT-DCs, although seemingly also CCR7 dependent, remains less well understood. Recruitment of splenic

$CD11b^+$ cDCs to the bridging channel of the MZ is controlled by the chemotactic receptor EBI2[172].

3.1.3. Dendritic cells in human spleen

While dendritic cells in mouse spleen are perhaps the most extensively studied DC type, little has been published concerning the human equivalent, due to their rarity in blood and reduced accessibility of the tissue.

In human spleen, DCs represent 0.1-1.8% of total splenocytes. McIlroy and collegues analyzed DCs from different organ transplant donors and they found that most of DCs are $CD11c^+$ $HLA\text{-}DR^+$ and localize at the periphery of the white pulp, in the T-cell zone and in the B-cell zone[173]. The so-called "MZ DCs" at the edge of the white pulp might correspond to the $CD11b^+$ DCs in mouse, cells able to sample blood-borne antigens as they pass through the marginal zone. However, they do not share the same anatomic localization, since in human spleen they surround both the T-cell zone and the B-cell zone. The T-cell zone include some activated DCs and thus could represent the site where maturation occurs.

Steinman's group further characterized splenic DCs using DEC205 as a marker. CD205 is an endocytic receptor involved in the efficient presentation of antigens. They observed that $DEC205^+$ DCs are present in a border region between the red and white pulp, and are distinct from $CD169^+CD209^+CD206^+$ phagocytes[174].

Few years later, Mittag et al. analyzed the phenotype and function of human spleen DC subsets by comparing them with circulating DCs. Among the $CD11c^{+}HLA\text{-}DR^{+}$ DCs, they identified three different populations: $CD1b/c^{+}$, $CD141^{+}$ and $CD16^{+}$ $CD11c^{+}$ cDCs. They also found a subset of HLA-DR $CD11c^{-}$ $CD304^{+}$ pDCs. The proportion of $CD1b/c^{+}$, $CD141^{+}$ and $CD304^{+}$ is similar to that found in peripheral blood[54].

TLRs are differentially expressed by the four subsets, with TLR7 and TLR9 being expressed exclusively by pDC. By contrast, TLR4 is more expressed by $Cd1b/c^{+}$ and $CD16^{+}$ DCs, and TLR3 by $CD1b/c^{+}$ and $CD141^{+}$ DCs. Finally, they observed that both circulating and splenic cDCs secrete IL-12p70 and are able to cross-present exogenous antigens to $CD8^{+}$ T cells[54].

3.2. Dendritic cell-T cell crosstalk

Once in the secondary lymphoid organs, mature DCs undergo cognate interaction with rare antigen-specific T cells to induce their progression into cell-cycle, activation, and differentiation into distinct effector subsets that orchestrate both cellular and humoral immune responses[175]. T-cell activation and proliferation might lead to immunity or to tolerance. The typology of Th cell differentiation depends on several factors, such as the affinity between the TCR and the MCH molecules presenting the antigen, the CD28 ligation by the co-stimulatory molecules CD80 and CD86, the production of polarizing cytokines by local immune and stromal cells, and the expression of lineage-specific transcription factors by antigen-activated $CD4^{+}$ T cells (**Fig. 8**)[176].

Cognate DC-T cell interaction involves the “instruction” and the “priming” of naïve T cells by DC cytokines such as IL-6, IL-10, IL-12, IL-23, IFN-α and TGF–β[169]. Although the DC signals that govern T-cell polarization have not all been determined, the production of IL-12 is critical for the Th1 response and involves the separate control of the production of the p40 and p35 components. Indeed, Th1 response is severely impaired in subject carrying mutations in the genes encoding for IL-12p40 subunit or IL-12 receptor[177]. Human IL-12 also induces higher expression of IL-21, ICOS, CXCR5 and Bcl-6 on activated Tfh cells. Thus, subjects lacking the expression of functional IL-12 receptor β1 chain (IL-12Rβ1), show reduced frequency of Tfh cells and memory B cells, and also impaired GC formation in lymph nodes[178].

The ability of DCs to induce specific types of T cell responses reflects the type of maturation signals they receive at the time of antigen encounter. TLRs play a crucial role in determining the Th response[176]. Whereas TLR4 engagement by LPS strongly induce IL-12 secretion, TLR2 ligands like Pam3Cys facilitate Th2 cell polarisation, by inhibiting IL-12p70 production[179,180]. Moreover, Zymosan, a TLR2 ligand, induces DCs to secrete abundant IL-10 but little IL-6 and IL-12p70, thus promoting tolerogenic T cell responses[181].

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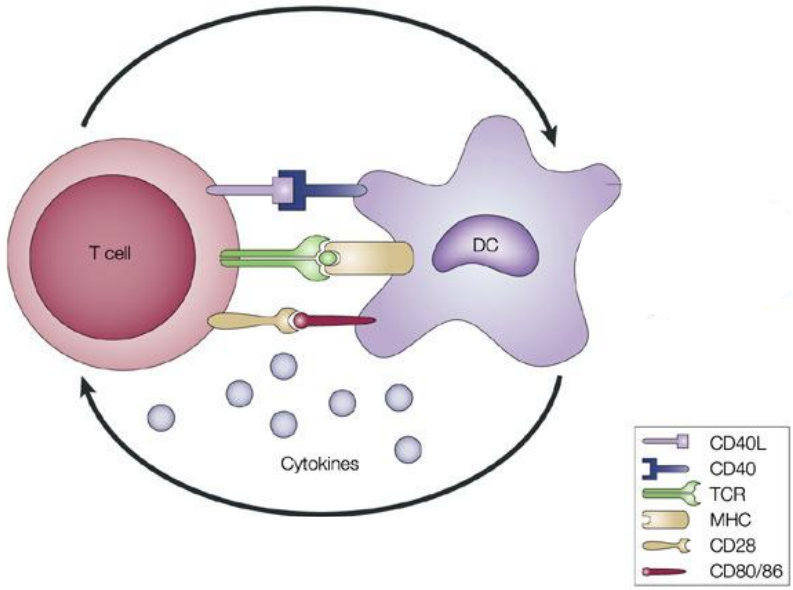

Figure 8. DCs serve as the bridge between innate and adaptive responses. The interaction between DCs and T cells is driven by the recognition of antigen-loaded MHC molecules on DCs and TCRs with high affinity for the complex on T cells. Costimulatory signals (that is, CD28 recognition of CD80/CD86) and the production of pro-inflammatory cytokines are required for the full activation of antigen-specific T cells. The expression of costimulatory molecules and cytokines by DCs are tightly regulated processed that occur after encounter with the antigen. *Figure modified from O'Hagan et al.*[182]

DCs also participate in the maintenance of peripheral tolerance by downregulating T cell responses against self-antigens[183].

Of note, different subsets of T cells have distinct requirements for costimulatory signals. Indeed, compared to naïve T cells, effector and memory T cells require less costimulatory signals to become activated. For this reason, effector and memory T cells can be activated not only by professional APCs such as activated DCs but also by less specialized APCs such as activated macrophages and B cells.

However, because of the plasticity in Th subsets[184] and the existence of several heterogeneous DC populations, many questions remain unanswered about the function of DCs in in driving the appropriate T cell response.

3.3. Dendritic cell-B cell crosstalk

Recent studies suggest that different myeloid DC subsets can regulate humoral immunity in humans, although the understanding of their role is still an underdeveloped research area[185]. In vitro generated DCs have been shown to influence B cell proliferation, isotype switching, and PC differentiation[186].

Studies with pDCs isolated from human blood and tonsils demonstrate that pDCs are directly involved in B cell responses. IFN-α and IL-6 secreted by pDCs upon stimulation with *Influenza virus* promote the differentiation of CD40-stimulated B cells into antibody-secreting PCs[187]. In addition, pDCs activated with TLR9 ligand are also capable of inducing TLR9-triggered naïve B cells to differentiate into IgM-producing PCs[188].

pDCs might also contribute to the humoral immunity through cross-talk with mDCs. IFN-α/β, as well as CD40 ligand, induce mDCs and monocytes to express BAFF and APRIL that, together with IL-10 or TGF-β, promote B cell survival, proliferation, and CSR[189]. Similarly, murine mDCs *in vivo* exposed to IFN-α support antibody responses through the differentiation of naïve $CD4^+$ T cells towards Tfh cells[190].

$CD14^+$ dermal DCs also indirectly promote CSR and antibody production in naïve B cells by inducing the differentiation of naïve $CD4^+$ T cells into Tfh cells[191]. Tfh cells secrete the cytokine IL-21 that drives the growth, differentiation, and isotype switching of B cells[192]. In mouse, IL-6 derived from DCs appears to be the major cytokine involved in the induction of IL-21 in $CD4^+$ T cells[193]. In contrast, in humans, it has been showed that DC-derived IL-12, traditionally viewed as a potent inducer of Type 1 response, promotes the development of IL-21-producing $CD4^+$ T cells[194]. Accordingly, the injection of IL-12 into tumor sites of head and neck of cancer patients resulted in the activation of B cells in the draining lymph nodes[195].

Another study showed that a population of $CD11c^{high}$ DCs that does not associate with T cells has a critical role in supporting PB differentiation in response to TI antigens[196]. On the same line, transfer to naive mice of immature bone marrow-derived DCs pulsed with *streptococcus pneumoniae in vitro*, elicites both TD and TI immunoglobulin reponses *in vivo*[197].

Finally, a population of blood-derived $CD11c^{lo}$ DC has been described to capture and transport antigens to the spleen and be responsible for initiating and supporting the TI immune response, through the release of BAFF and APRIL[96].

4- PROGRANULIN AND SECRETORY LEUKOCYTE PROTEASE INHIBITOR

4.1 Progranulin

Progranulin (PGRN), also known as granulin-epithelin precursor (GEP)[198], proepithelin (PEPI)[199], acrogranin[200], and PC cell-derived growth factor (PCDGF)[201] was identified from different sources by several independent laboratories.

PGRN has emerged as a pleiotrophic growth factor with widespread effects thought the body, participating in embryogenesis[202], wound repair[203], maintenance of neuronal survival[204] and cartilage development[205]. Due to its ubiquitous and multifaceted nature, PGRN was shown to be involved in many types of disease processes, like oncogenesis[206], autoimmune disorders[6,207–209], inflammation[210] and neurodegerative diseases[211]. It is abundantly expressed in epithelial cells, endothelial cells, neurons, chondrocytes and immune cells such as neutrophils, macrophages, and DCs.

PGRN is a 593-amino acid protein, typically secreted in a highly glycosylated 90 KDa form, composed by 7 ½ repeats of cystein-rich motifs[212]. It is cleaved in the intergranulin linkers into seven 6-KDa peptides called granulins (GRN: P,G,F,B,A,C,D,E). The cleavage is mediated by many proteinases, including matrix metalloproteinase (MMP) 9,12 and 14, proteinase 3 and elastase[203,213–215]. GRN peptides have a unique structurally defined motif of six disulfide bonds[216]. Both PGRN and GRN peptides

possess biological activity, and they often exert distinctive and contrasting functions.Thus, factors controlling the conversion of full-length PGRN, generally anti-inflammatory, into GRN peptides appear to be pivotal (**Fig. 9**).

Okura et al. showed that apolipoprotein (APO) A-1, a major component of high density lipoprotein (HDL), binds to PGRN and suppresses its conversion into proinflammatory GRNs. This mechanism could explain the reduction of serum HDL during the acute phase of coronary events[217]. Secretory leukocyte protease inhibitor (SLPI) is one of the smallest serine proteinase inhibitors in plasma. Zhu and colleagues identified interactions among elastase, SLPI and PGRN, showing that PGRN escapes from elastase-mediated digestion when bound to SLPI. SLPI-deficient mice show defects in wound healing, which can be overcome by exogenous PGRN. SLPI can protect PGRN by two distinct mechanisms: by binding the substrate and by binding the proteinase[203].

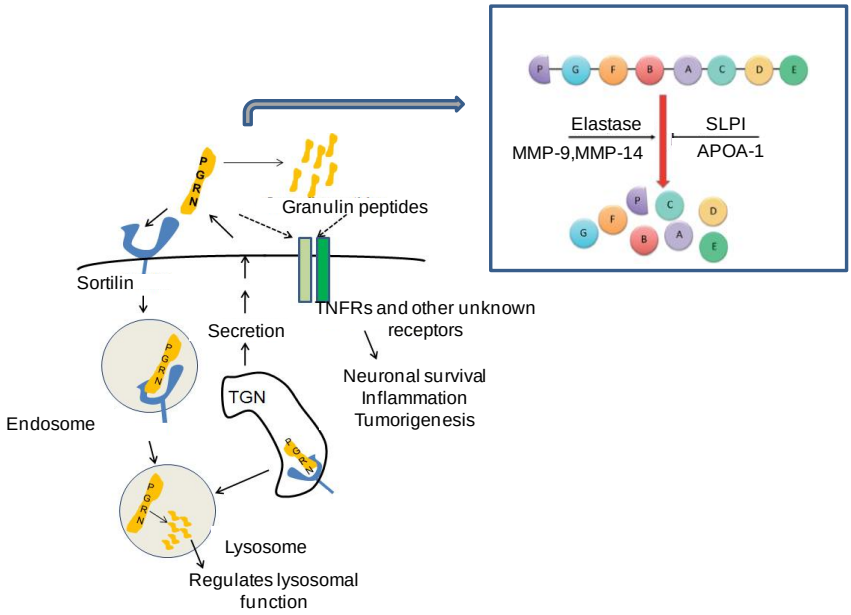

Figure 9. Progranulin and granulin peptides. Progranulin (PGRN) is sorted form trans-Golgi network (TGN) to secretory pathway or to lysosomes through its interaction with sortilin. PGRN functions both intracellularly in the lysosomes and extracellularly upon binding to tumor necrosis factor (TNF) receptors (TNFRs) and possibly to other still unknown receptors, to promote neuronal survival and regulate inflammation and tumorigenesis. After secretion, full-lenght PGRN can be proteolycally cleaved between the granulin (GRN) domains by serine proteases like elastase, metalloproteinase-9 (MMP-9) y metalloproteinases-14 (MMP-14). PGRN processing can be inhibited by secretory leukocyte protease inhibitor (SLPI) or apolipoprotein (APO) A-1. *Figure modified from Demorrow et al*.[218]

4.1.1. Progranulin in immune system

PGRN binds with several proteins at different levels, ranging from extracellular receptors to cytoplasmic and nuclear components, probably achieving different conformations by combining its GRN domains[219].

PGRN or its products GRNs seem to participate in antigen presentation, either binding to purified HLA-DR11α,β dimers[220], and to macrophage TLR9. In this latter study, the authors propose that progranulin serves to deliver CpG-ODN to TLR9 in endolysosomes, potentiating TLR-mediated immune responses. However, they do not distinguish between PGRN and GRN peptides[221,222].

PGRN and its processed fragments have opposing physiological effects on neutrophils and epithelial cells. GRN B can promote epithelial cytostasis and neutrophil recruitment by inducing the secretion of IL-8, while PGRN promotes epithelial proliferation and inhibits respiratory burst and proteinase release in human neutrophil activated with TNF. These results strongly contribute to the understanding of the early and late events in wound healing and repair[203]. One mechanism by which PGRN and GRNs promotes tissue repair is to increase cell proliferation and migration, signaling through PIK3 and MAPK pathways[223].

Cleavage of PGRN is also a mechanism by which proteinase 3 and neutrophil elastase enhance immuno complex-mediated neutrophil infiltration and activation, two events that occur in several human diseases, like autoimmune disorders, infections or hypersensitivity reactions[213].

The importance of PGRN in innate immunity was also confirmed in PGRN-deficient mice, since they respond to infection by *Listeria monocytogenes* with excessive and prolonged inflammation in spleen, liver and brain. In addition, bone marrow-derived macrophages isolated from these mice produce more pro-inflammatory mediators and less IL-10 in response to TLR ligands[224].

Despite the variety of roles and the mechanisms of action of PGRN, only recently its possible binding receptors have been identified. Interactions between PGRN expressed by microglias and the neuronal receptor sortilin have been reported to be important for internalization of full-length PGRN and for its neurotrophic activity[225]. In 2011 the TNF receptors (TNFRs) were isolated from a two-hybrid cDNA library searching for PGRN binding proteins. The authors reported that PGRN directly binds to TNFR1 and TNFR2 and acts as an antagonist of TNF. Collagen-induced arthritis, an autoimmune disease strongly mediated by TNFR signaling, is entirely reverted by the administration of recombinant PGRN[226]. In another study, they extended the previous finding by demonstrating that cysteine-rich domain 2 (CRD2) and CRD3 of TNFR are important for the interaction with PGRN.
On the same line, it has been proposed a therapeutic function for PGRN in the control of inflammatory skin diseases such as dermatitis that are strongly dependent on TNF[227].

However, other data reported no evidence for a direct physical or functional interaction between PGRN and TNFRs, nor an alteration of LPS-induced inflammatory gene expression or cytokine

secretion in bone marrow-derived macrophages from *Grn*$^{-/-}$ mice[228,229].

4.1.2. Progranulin as prognostic marker and therapeutic target

Frontotemporal Dementia (FTD) is one of the most common forms of dementia before 65 years of age. It is a clinical syndrome characterized by cerebral atrophy (usually most severe in the frontal lobes), neuron loss and microglia activation[230]. FTD patients experience abnormalities of personality, behavior and/or prominent language disorder, such as progressive non-fluent aphasia or semantic dementia. Almost 5-10% of patients suffering FTD present mutations in the gene encoding for PGRN. Genetic analysis of chromosome 17q21 led to identification of 70 pathogenic mutations that include frameshift, splice-site, nonsense, signal peptide and missense mutations. Most of these are predicted to result in premature termination codons, namely functional null alleles or in a non-functional or unstable protein[231]. In patients with null mutations, there is about 75% reduction of PGRN in plasma. In addition, it has been recently published that PGRN deficiency is also associated with an increased risk of late-onset Alzheimer's disease (AD) in mice[232]. While AD principally affects memory centers in the hippocampus and temporal cortex, FTD is associated with greater injury to cells in the frontal cortex, causing behavioural and personality changes. Strategies to elevate endogenous levels of PGRN in FTD patients as well as in AD patients could achieve therapeutic relevance in humans. However, due to the tumorigenic nature of PGRN, these strategies should

also be able to modulate its clearance in the event of adverse effects.

PGRN was also identified as a marker for chronic inflammation in type 2 diabetes and visceral obesity. In patients with visceral obesity, higher PGRN serum concentrations are associated to an increased macrophage infiltration into adipose tissue, suggesting that PGRN could act as a chemoattractant molecule that promotes the cross-talk between adipocytes and macrophages[233,234].

Finally, high levels of PGRN expression are also found in several human cancers including breast cancer, clear-cell renal carcinoma, invasive ovarian carcinoma, glioblastoma, adipocytic teratoma, and multiple myeloma[204].

4.1.3 Progranulin-sensitive signalling pathways

A few of the downstream pathways modulated by PGRN and its peptides have been described. PGRN can affect pathways that are related to cellular growth and maintenance. For instance, in breast cancer lines, PGRN promotes the expression of cyclin D1[235].

PGRN can also induce the phosphorylation of proteins, such as shc and p44/42 MAPKs, and modulate the phosphatidyl inositol-3 (PI3) cascade by promoting phosphorylation of phosphatidyl inositol-3 kinase, protein kinase B/AKT, and p70S6 kinase[198,235,236].

PGRN inhibits the atherosclerotic inflammatory process induced by LPS by activating the Akt/eNOS signaling pathway In vascular endothelial cells (HUVEC), suppressing their expression of VCAM-1, ICAM-1, MCP-1, and TNF and inhibiting monocyte migration via inhibition of the NF-κB pathway[237].

In bone marrow-derived macrophages, PGRN blocks TNF-induced phosphorilation of IKK and IκBα , NF-κB nuclear translocation and activation of NF-κB -dependent genes. In these settings, also p38, JNK and ERK1/2-members of the MAPK family are involved in TNF-mediated inflammation[226]. ERK1/2 and its target genes, including JunB transcription factor, are also required for PGRN-mediated chondrocyte differentiation, rendering PGRN a potential targets for treatment of cartilage disorders and arthritic conditions[238].

4.2. Secretory leukocyte protease inhibitor

SLPI is an 11.7-kDa cationic serine protease inhibitor purified from parotid secretions and sequenced in 1986[239]. It is composed of 2 highly homologous domains of 53 and 54 amino acids and contains 16 cysteine residues that assemble into eight disulfide bridges.

SLPI is constitutively expressed at many mucosal surfaces and in skin, as well as in blood, seminal plasma, cervical mucus, and bronchial secretions where it is conventionally thought to play an important role in the defence of epithelial surfaces against proteolytic damage. It is produced by a variety of cells including neutrophils, granulocytes, monocytes/macrophages and epithelial

cells[240]. Although the major physiologic substrate for SLPI is neutrophil elastase[241], it can also inhibit the proteases chymotrypsin, trypsin, and cathepsin G[203].

Elastase and cathepsin G are involved in extensive pathological tissue distruction following several disorders, including delayed and chronic wound healing. Mice null for the gene encoding SLPI showed increased inflammation and elastase activity after cutaneus injury, indicating that SLPI is a crucial molecule in the control of tissue destruction[242]. Recently, reduced levels of SLPI have been identified in myeloid cells and plasma from patients with severe congenital neutropenia (SCN) caused by autosomal-dependent mutations in neutrophil elastase. Furthermore, inhibition of SLPI in $CD34^+$ bone marrow hematopoietic progenitors from healthy donors significantly reduces myeloid differentiation, proliferation and survival. The authors proposed a reciprocal regulation among SLPI and elastase, suggesting SLPI as a new myelopoiesis regulatory factor[243].

SLPI is also an anti-retroviral, anti-bacterial, anti-fungal and anti-inflammatory protein. When delivered to animals in which bacterial products induce arthritic lesions, it profoundly inhibits proteolytic tissue destruction and joint inflammation[244]. SLPI was also shown to contribute to the anti-HIV-1 activity of human saliva in vitro by interfering with the entry of the virus in the cell[245], independently of its anti-protease activity. The anti-inflammatory nature of SLPI has been investigated in different settings. Samson et al. reported that in mouse, DCs in cervical lymph nodes express higher amounts of SLPI compared with DCs from peripheral lymph nodes. Bone marrow-derived DCs from *SLPI*$^{-/-}$ mice release more inflammatory

cytokines in response to LPS. Moreover, *SLPI*$^{-/-}$ mice fail to maintain tolerance to nasal OVA application in the presence of low doses LPS. All together these results indicate that the expression of SLPI in mucosa-draining lymph nodes might contribute to protecting the mucosal system to continuous microbial pressure[246].

Accordingly, B cells exposed to exogenous SLPI, including intraepithelial B cells, contain SLPI in both the cytoplasm and nucleus. In those B cells, SLPI inhibits CSR by interfering with the NF-κB activation and with the upregulation of AID induced by viral RNA, BAFF and IL-10. In this scenario, SLPI may function as a negative feedback protein restraining the intraepithelial production of potentially pathogenic IgG and IgA antibodies, critical for host defence at mucosal sites of entry[247].

Bone marrow-derived DCs increase SLPI mRNA in response to TLR ligands[248], and transfection of macrophages with SLPI suppresses LPS-induced activation of NF-κB and production of nitric oxide and TNF[249]. In monocytes, SLPI can bind NF-κB regions within IL-8 and TNF promoters, reducing p65 binding and cytokine production[250]. These data suggest that in myeloid cells, subjected to positive and negative regulation, SLPI might exert its effect by providing a negative signal.

Finally, increased expression of SLPI has been reported in a wide variety of human cancers, including breast, lung, ovarian and colorectal carcinomas, and glioblastoma[251] .

CHAPTER II

AIMS

AIMS

This project has been developed to investigate the mechanisms by which progranulin (PGRN) modulates innate and adaptive immune responses.

AIM 1: to determine the localization and sources of PGRN in human and mouse spleen.

AIM 2: to explore the influence of PGRN on immune cell activation and function, with a special focus on neutrophils and dendritic cells.

AIM 3: to dissect the involvement of PGRN in humoral responses, in homeostasis and in response to immunization with T cell-independent and T cell-dependent antigens.

CHAPTER III

MATERIALS AND METHODS

Human samples

Spleens from organ donors and individuals undergoing post-traumatic splenectomy without clinical signs of infection or inflammation and normal histology were used for functional assays and indirect fluorescent-antibody (IFA) staining. Cells were also obtained from peripheral blood of healthy donors (HD), patients with neutrophil disorders (ND) and patients with frontotemporal dementia (FTD) due to mutations in *GRN* gene. ND patients: age 23±10, neutropenia due to unknown mutation; FTD patients: age 64±9, null mutation in GRN gene (p.C366fsX1, n=4). Spleens and blood samples from patients were obtained from Hospital Clinic de Barcelona and blood samples from HD from Institut Hospital del Mar d'Investigacions Mèdiques (IMIM). Paraffin-embedded tissue sections used for immunohistochemical (IHC) staining from healthy subjects were obtained from local tissue repositories from Hospital del Mar and Hospital Sant Joan de Deu. The Ethical Committee for clinical investigation of IMIM and Hospital clinic de Barcelona approved the use of tissue and blood samples. Prior to collection, signed informed consent was obtained from the patient or his/her parent or guardians. All the blood and tissue samples were coded and relevant clinical information remained anonymous.

Animals

Spleen, bone marrow and peripheral blood were collected from healthy wild type or *Grn*$^{-/-}$ C57BL6/6 mice. All mice were housed in specific pathogen–free conditions. Male mice were used at 8–12 weeks of age. All tissue extractions from mice and rats were in

accordance with approved protocols from the Institutional Animal Care at PRBB.

Cell isolation

Human splenocytes were obtained by perfusion using phosphate buffer solution followed by separation using Histopaque-1077 gradient (ficoll technique, Sigma). PBMCs (peripheral blood mononuclear cells) were also obtained by ficoll technique.

After gradient separation, $CD15^+CD16^+$ N_C cells and N_{BH} cells were sorted from the pellet fraction of whole PBMCs or splenocytes by flow cytometry with BD FACSAria II (BD Biosciences, > 98% purity). Splenic $CD19^+$ B cells, $CD14^+$ macrophages, $CD3^+$ T cells, $lineage^-CD56^+$ NK cells and $lineage^-CD11c^+HLA\text{-}II^+$ splenic (spDCs) and circulating dendritic cells (cDCs) were sorted by flow cytometry.

$CD14^+$ monocytes were magnetically isolated (Miltenyi Biotec) from blood mononuclear cells. Briefly, PBMCs were resuspended in an appropriate amount (~$50x10^6$ cells/ 500 µL PBS + 2% FBS (fetal bovine serum). FcR blocking reagent (20 µL) were added to the cells during 15 minutes at 4°C. The PBMCs were then labeled with a biotinylated monoclonal antibody to human CD14 (Southern Biotechnology) for 10 min at 4°C. After washing with PBS, cells were incubated with anti-biotin MicroBeads (20 µL) for 15 min at 4°C. Cells were washed again, resuspended in MACS buffer (PBS 0,5% FBS 2mM EDTA) and applied onto the column. The column was washed for three times with 3mL of MACS buffer. Finally, the column was removed from the separator and the cells were collected by pipetting 5 mL of MACS buffer onto the column and

flushing out the magnetically labeled cells by firmly pushing the punger into the column.

Mouse splenocytes were obtained after mechanical dissociation and processing through a 70-µm cell strainer. Bone marrow cells were obtained from both femours and tibias, by flushing the contents with 2 ml of PBS using a 1-ml insulin syringe. Erythrocytes from spleen or bone marrow suspension were lysed with ammonium chloride–potassium phosphate buffer.

$Ly6G^{hi}CD11b^{hi}$ neutrophils and $CD11c^{high}$ dendritic cells were sorted by flow cytometry.

Viable human and mouse leukocytes were determined by trypan blue exclusion and counted using a Neubauer chamber.

Cultures and Reagents

Human $CD14^{+}$ monocytes were cultured in 6 wells plates ($3x10^6$ cells/3ml/well), in complete medium (RPMI/10% FBS, 5 mM Glutamine and 10 mM penicillin-streptomycin) during 5 days with 20 ng/ml GM-CSF and 40 ng/ml IL-4. At day 3 the cultures were supplemented with 500 µl complete medium with GM-CSF and IL-4. At day 5 cells differentiated to immature dendritic cells (iDCs) were washed and used for in vitro stimulation. Immature DCs were seeded in round bottom 96 well plates ($2x10^5$ DCs/ 200 µl complete medium), cultured alone or with 10 µM PGRN (R&D) and 1 µg/ml SLPI (R&D). After 2 hours, 10 ng/ml LPS was added for the following 5 hours or 24 hours (mature DCs). Supernatant was collected for ELISA assays, cells were washed and used for FACS analysis, qRT-PCR assay or co-culture experiments.

$CD45RA^+CD45RO^-$ naive or $CD45RA^-CD45RO^+$ memory $CD4^+$ T cells were purified from peripheral blood of HD (as described before), stained with 1μM Carboxyfluorescein succinimidyl ester (CFSE, CellTrace CFSE Cell Proliferation Kit, Invitrogen) and seeded (2 x 10^5/ well) in 96-well plates in the presence of mature DCs for 5 or 3 days (ratio 10:1), respectively. As positive control cell were cultured with anti-CD28 antibody (5 μg/ml) in wells pre-coated overnight with anti-CD3 antibody (1 μg/ml, data not shown). As negative control cells were cultured with complete medium alone (data not shown). At day 3 or day 5 supernatant was collected and the proliferative response was determined at day 5 (naive T cells) or day 3 (memory T cells) by FACS.

Flow cytometry

Freshly isolated cells were incubated with total human IgG (Fc blocking reagent Miltenyi Biotec) at 4 °C with various combinations of antibodies (**Tables 1, 2**). Cell survival was assessed by 4'-6-diamidine-2'-phenylindole (DAPI, Boehringer Mannheim) staining or with Annexin-V Apoptosis Detection Kit II (BD Pharmingen). To stain intracellular cytokines, mouse splenocytes were firstly incubated with labeled antibodies to specific surface molecules, permeabilized and fixed with Cytofix/Cytoperm kit (BD Pharmingen), and further incubated with labeled antibodies to specific intracellular antigens. All gates and quadrants were drawn to give ≤ 1% total positive cells in the sample stained with isotype control antibodies. Cells were acquired using a FACSCalibur or a FACS LSR II (BD Biosciences) and analyzed using the FlowJo 7.6.5 software (Tree Star).

Immunohistochemistry (IHC)

Formalin-fixed paraffin embedded tissue sections with a thickness of 3-4 μm were stained with the LSAB+ System-HRP (DAKO) to stain PGRN and SLPI. For PGRN, HRP signal was amplified with the TSA kit AF-488 (Molecular Probes), followed by incubation with anti-flurescein-HRP. Sections were counterstained with haematoxylin.

Fluorescence microscopy and confocal microscopy

OCT-frozen splenic tissues cells were fixed and washed as reported[1,64] and stained with various combinations of antibodies (**Tables 1, 2**). Biotin-conjugated antibodies were detected with streptavidin-conjugated Alexa Fluor 488, 546 or 647 (Life technologies). Nuclear DNA was stained with DAPI. Coverslips were applied with FluorSave reagent (Calbiochem). Fluorescence microscopy Images were obtained with an Axioplan2 microscope (Carl Zeiss). Fiji-ImageJ software was used to analyze and merge channels.

ELISA

Total human and mouse IgM, IgG and IgA ,as well as BAFF and IL-10 were detected in M96-Nunc ELISA plates as reported in published studies[1,64]. SLPI (R&D), PGRN (Adipogen), IL-6, IL-8, TNF, IFN-γ (PeproTech), and IL-12p70 (eBiosciences) were

measured using a commercially available kit (**Table 3**). To measure pre-immune and post-immune antigen-specific antibody responses, plates were coated overnight with 10 µg/ml relevant antigen in PBS at 4°C, washed three times with PBS and 0.1% Tween 20 (Thermo Fisher Scientific), blocked for 2h at room temperature with PBS and 1% bovine serum albumin (BSA), and washed three times. To capture antigen-specific antibodies, plates were first incubated overnight at 4°C with appropriately diluted sera and washed five times prior to incubation for 1h at 37°C with horseradish peroxidase (HRP)-conjugated goat anti–human detection antibody (Southern Biotech) or biotin-conjugated goat anti-mouse detection antibody (BD Bioscience) followed by 30 minute-incubation with streptavidin-cojugated HRP. After five additional washes, reactions developed with the HRP substrate TMB were stopped using 2N H_2SO_4. The relative concentration of antigen-specific antibodies in serum from naive and immunized mice was expressed as optical density (OD).
The affinity of TNP-reactive antibodies after immunization with TNP-OVA and Sigma adjuvant system (SAS) was determined by coating the plate with TNP-BSA at two different TNP to BSA molar ratios: 2 (TNP(2)-BSA) and 18 (TNP(18)-BSA). Antibody affinity was calculated as OD TNP(18)-BSA to OD TNP(2)-BSA ratio.

qRT-PCR

RNA was obtained by High Pure RNA Isolation kit as specified by the manufacturer (Roche). DNA was reverse transcribed from total RNA using Superscript III kit (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using SYBR green (Promega) with specific primer pairs (**Table 4, 5**) in 96-well

plates with a LightCycler 480 real-time PCR system (Roche Diagnostics). Gene expression was normalized to that of the gene encoding *β-ACTIN* (human samples) or *Gapdh* (mouse samples). Gene expression profile analysis was performed using the SDS 2.0 software or the QuantStudio™ 12K Flex Software (Appied Biosystems).

Western blotting

Immature DCs were incubated alone or with 1mM PMSF or 0.5 μg SLPI. Then, 0.4 μg recombinant human PGRN were added during 2h at 37°C. Cell extracts were obtained by lysing the cells in presence of proteases inhibitors.
Equal amounts of proteins were fractionated onto a 10% SDS-PAGE and transferred onto PVDF membranes (BioRad). After blocking, membranes were probed with an antibody to PGRN (**Table 1**), washed and incubated with the appropriate secondary antibody. Proteins were detected with an enhanced chemiluminescence detection system (Amersham).

Electrophoretic mobility shift assays (EMSA)

Oligonucleotides encompassing commercially available consensus NF-κB-binding in IL-12p40 promoter region (Santa Cruz) were labeled with $[\alpha\text{-}^{32}P]$ ATP and used at approximately 50,000 c.p.m. in each reaction. Band shifts were resolved on non-denaturing 6% polyacrylamide gel.

Immunizations

Mice were i.p. immunized with 50 μg TNP-Ficoll, or 100 μg TNP-OVA and 200 μl Sigma adjuvant system (SAS, Biosearch Technologies and Sigma).
At day 0 and day 7 after immunization with TNP-Ficoll, serum was collected and spleen removed at day 7. At day 0, 7, 14 after immunization with TNP-OVA and SAS serum was collected and spleen removed at day 21. At day 14 mice with immunized with a second challenge of 100 μg TNP-OVA without SAS.

Statistical Analysis

Statistical significance was assessed with the two-tailed unpaired or paired Student's t-test and linear regression with GraphPad Prism 5 software and p values of less than 0.05 were considered significant. In animal experiments, at least five mice per group were used and all groups were age-matched and sex-matched. No animals were excluded from analysis.

Table 1. Human antibodies

ANTIBODY	CLONE	MANUFACTURER	USE
CD11b PE	ICRF44	Biolegend	FC
CD11c PE	Bu15	Biolegend	FC
CD11c PE-Cy7	3.9	Biolegend	FC
CD14 APC-Cy7	HCD14	Biolegend	FC
CD14-biot	UCHM-1	Southern Biotech	MS
CD19 APC-Cy7	HIB19	Biolegend	FC
CD19 PE-Cy7	HIB19	Biolegend	FC
CD3 APC-Cy7	HIT3a	Biolegend	FC
CD3 PE-Cy5	HIT3a	eBioscience	FC
CD4 APC	A161A1	eBioscience	FC
CD40 PE	5C3	Biolegend	FC
CD45RA FITC	HI100	Biolegend	FC
CD45RO-PE	UCHL1	Southern Biotech	FC
CD56 APC-Cy7	5.1H11	Biolegend	FC
CD56 APC	5.1H11	Biolegend	FC
CD80 PE	L307.4	BD Bioscience	FC
CD86 PE	2331	BD Bioscience	FC
HLA-DR FITC	G46-6	BD Bioscience	FC
IgD-biot	11-26c	Southern Biotech	IFA
PGRN purified		gift from Dr. Ding[252]	IFA/WB
PGRN purified	polyclonal	Santa Cruz	IHC
SLPI purified		gift from Dr. Xu[247]	IFA
SLPI-biot	polyclonal	R&D	IHC
TLR4 PE	HTA125	Biolegend	FC
IL-10 purified	JES3-9D7	BD Bioscience	ELISA
IL-10-biot	JES3-12G8	BD Bioscience	ELISA

FC, flow cytometry

MS, magnetic separation

IFA, immunofluorescence analysis

WB, western blot

IHC, immunohistochemistry

ELISA, enzyme-linked immunosorbent assay

Table 2. Mouse antibodies

ANTIBODY	CLONE	MANUFACTURER	USE
B220 purified	MB4B4	Biolegend	FC
B220 PE-Cy7	RA3-6B2	Biolegend	FC
CD11b FITC	M1/70	Biolegend	FC
CD11b PE	M1/70	Biolegend	FC
CD11c APC-Cy7	N418	Biolegend	FC
CD138 APC	281-2	Biolegend	FC
CD21 APC	7E9	Biolegend	FC
CD23 PE	B3B4	Biolegend	FC
CD3 APC-Cy7	145-2C11	Biolegend	FC
CD4 PE-Cy7	145-2C11	Biolegend	FC
CD43 APC-Cy7	1B11	Biolegend	FC
CD5 FITC	53-7.3	Biolegend	FC
CD8 APC	53-6.7	Biolegend	FC
CXCR5 APC	L138D7	Biolegend	FC
F4/80 APC	BM8	Biolegend	FC/IFA
Foxp3 PE	MF-14	Biolegend	FC
GL-7 FITC	GL7	Biolegend	FC
ICOS PE	15F9	Biolegend	FC
IgG1 purified	A85-3	BD bioscience	ELISA
IgG1-biot	A85-1	BD bioscience	ELISA
IgG2b purified	R9-91	BD bioscience	ELISA
IgG2b-biot	R12-3	BD bioscience	ELISA
IgG2c-HRP	polyclonal	Southern Biotech	ELISA
IgG3 purified	R2-38	BD bioscience	ELISA
IgG3-biot	R40-82	BD bioscience	ELISA
IgM purified	R6-60.2	BD bioscience	ELISA
IgM-biot	II/41	BD bioscience	ELISA
IL-10 PE	JES5-16E3	Biolegend	FC
Ly6g FITC	1A8	Biolegend	FC/IFA
MOMA-1-biot	MOMA-1	Abcam	IFA
PD-1 FITC	29F.1A12	Biolegend	FC

FC, flow cytometry

IFA, immunofluorescence analysis

Table 3. Commercial Kits

KIT	MANUFACTURER	USE
Annexin/PI	BD Bioscience	FC
BAFF	Adipogen	ELISA
CellTrace CFSE Cell Proliferation Kit	Invitrogen	FC
IL-12p70	eBiocience	ELISA
IL-6	Peprotech	ELISA
IL-8	Peprotech	ELISA
PGRN	R&D	ELISA
SLPI	R&D	ELISA

FC, flow cytometry

ELISA, enzyme-linked immunosorbent assay

Table 4. Human primers

PRIMER	SEQUENCE	USE
ACTBas	CGATCCACACGGAGTACTTG	qRT-PCR
ACTBs	GGATGCAGAAGGAGATCACT	qRT-PCR
GRNas	CGGGACAGCAGTGTATGTGG	qRT-PCR
GRNs	AAGGAGAACGCTACCACGGA	qRT-PCR
IL10as	TGTCCAGCTGATCCTTCATTTG	qRT-PCR
IL10s	ACCTGCCTAACATGCTTCGAG	qRT-PCR
IL-12p40as	CTCTGGTCCAAGGTCC	qRT-PCR
IL-12p40s	GGGAACTGAAGAAAGATGTTTATG	qRT-PCR
IL-6as	GTGCCTCTTTGCTGCTTTCAC	qRT-PCR
IL-6s	GGTACATCCTCGACGGCATCT	qRT-PCR
IL-8as	ACTTCTCCACAACCCT	qRT-PCR
IL-8s	CCAAACCTTTCCACCC	qRT-PCR
SLPIas	CACTTCCCAGGCTTCCTCCT	qRT-PCR
SLPIs	CCTGGATCCTGTTGACACCC	qRT-PCR
TNFas	AGCTGCCCCTCAGCTTGA	qRT-PCR
TNFs	CCCAGGCAGTCAGATCATCTTC	qRT-PCR
IL-12p40as	AATCCTGGTTCTTCCCAAGTCAG	EMSA
IL-12p40s	CTGACTTGGGAAGAACCAGGATT	EMSA

qRT-PCR, quantitative real time polymerase chain reaction

EMSA, electrophoretic mobility gel shift assay

Table 5. Mouse primers

PRIMER	SEQUENCE	USE
Gapdhas	CCTGCTTCACCACCTTCTTGAT	qRT-PCR
Gapdhs	TGTGTCCGTCGTGGATCTGA	qRT-PCR
Il-10as	CCACTGCCTTGCTCTTATTTTCA	qRT-PCR
Il-10s	AGACCCTCAGGATGCGGC	qRT-PCR
Il-12p40as	TCCAGTGTGACCTTCTCTGCA	qRT-PCR
Il-12p40s	GAGCAGTAGCAGTTCCCTGA	qRT-PCR
Il-6as	TGAATTGGATGGTCTTGGTCC	qRT-PCR
IL-6s	CCCAATTTCCAATGCTCTCC	qRT-PCR
Tnfas	CCACACTGGTTTGCGAAT	qRT-PCR
Tnfs	CGTCGTAGCAAACCACCAAG	qRT-PCR

qRT-PCR, quantitative real time polymerase chain reaction

CHAPTER IV

RESULTS

PGRN and SLPI are highly expressed in human and mouse spleen

We recently found a subset of neutrophils in the spleen, named B cell-helper neutrophils (N_{BH} cells), that contributes to the induction of steadystate TI antibody responses in the MZ. Compared to circulating neutrophils (N_C cells), N_{BH} cells have a distinct gene expression profile and phenotype and express more B cell-activating and immunoregulatory factors, including BAFF and APRIL. Moreover, they also present transcripts encoding for PGRN and SLPI, although the role of these two proteins in splenic immune responses is still completely unknown[1].

To determine whether the expression of PGRN and SLPI is limited only to splenic tissue, circulating Nc cells from healthy donors (HD) were also analyzed. qRT-PCR indicated that in homeostasis N_{BH} cells expressed similar levels of *GRN* but significantly higher amounts of *SLPI* transcripts than Nc cells (**Fig. 1**).

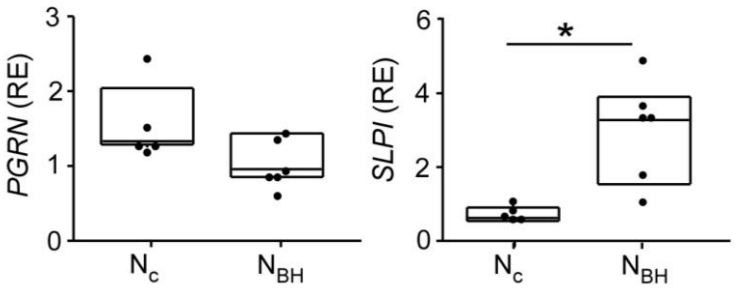

Figure 1. N_{BH} cells express similar levels of *PGRN* but higher levels of *SLPI* transcripts compared to N_C cells. qRT-PCR of PGRN and SLPI in lineage$^-$CD15$^+$CD16$^+$ neutrophils purified from spleen (N_{BH} cells) or peripheral blood (N_c cells) from healthy donors. Results are normalized to ACTB (β-actin) mRNA and presented as relative expression (RE)

compared with that of fresh Nc cells. Data display values from five donors (blood and spleen). Error bars, s.e.m.; *P < 0.05 (two-tailed unpaired Student's *t* test).

Immununohistochemical (IHC) staining of human healthy spleen showed the presence of PGRN and SLPI not only in the perifollicular areas, where N_{BH} cells localize, but also in the red pulp (**Fig.2**)

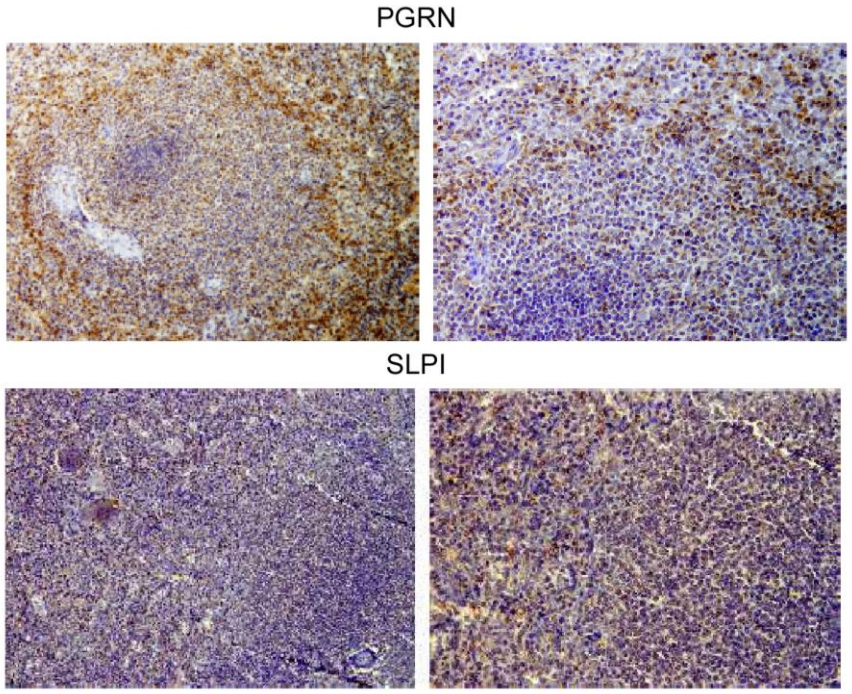

Figure 2. PGRN and SLPI occupy the follicular area, the perifollicular areas, and the red pulp of human spleen. IHC of healthy spleen stained for PGRN or SLPI (brown). Original magnification, ×10 (left panels) or ×20 (right panels). Data show one of two experiments with similar results.

Co-staining with an antibody to neutrophil elastase confirmed that human N_{BH} expressed both SLPI and PGRN (**Fig.3**).

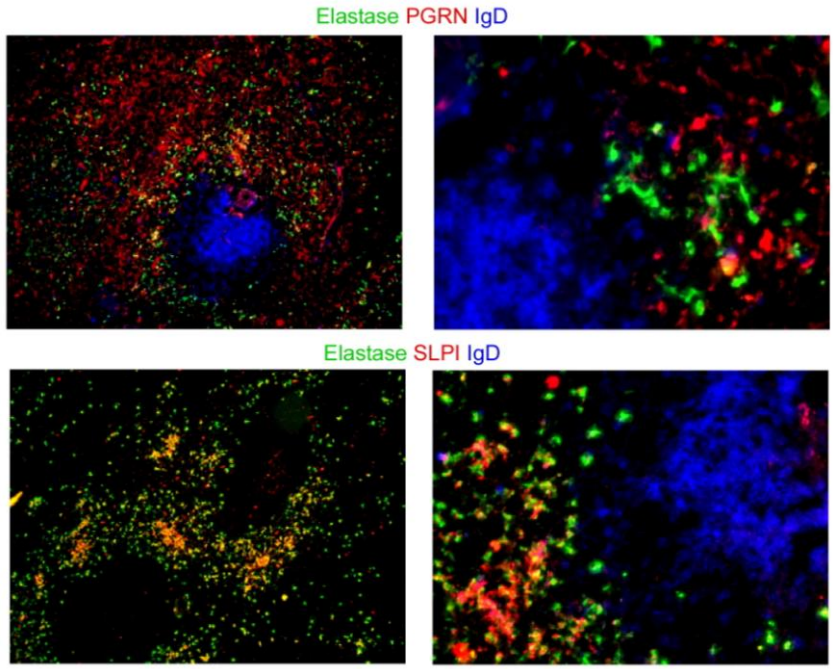

Figure 3. SLPI and PGRN colocalize with N_{BH} cells. Indirect fluorescent antibody (IFA) staining of human spleen for elastase (green), PGRN or SLPI (purple), and IgD (blue). Original magnification, ×10 (left panels) or ×20 (right panels). Data show one of two experiments with similar results.

In mice, we identified PGRN in the perifollicular areas of the white pulp (**Fig. 4**). Splenic macrophages ($F4/80^+$ cells) and neutrophils ($Ly6G^+$ cells) were positively stained with an antibody against PGRN, suggesting that they were able to secrete or bind PGRN. Moreover, double staining of PGRN and CD11c indicated that DCs are in close contact with $PGRN^+$ cells (**Fig. 4**). Having confirmed the presence of PGRN also in mouse spleen, we took advantage of

mice lacking PGRN (*Grn*$^{-/-}$ mice) to unravel the functional role of PGRN both in homeostasis and during immune responses.

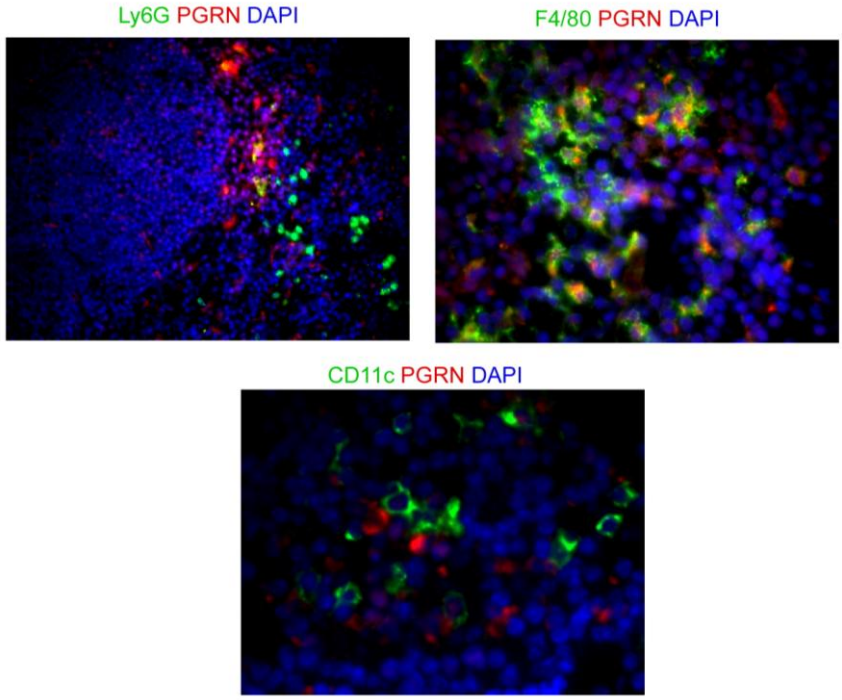

Figure 4. PGRN colocalizes with N_{BH} cells, macrophages, and DCs. Indirect fluorescent-antibody (IFA) staining of mouse spleen using F4/80 or CD11c or Ly6G (green), PGRN (purple), and DNA-binding 4'-6-diamidine-2'-phenylindole (DAPI; blue). Original magnification, ×10 (upper left panels) or ×40 (upper right panel and lower panel). Data show one of two experiments with similar results.

The indirect fluorescent-antibody (IFA) staining of both human and mouse spleen revealed that, in addition to neutrophils and macrophages, also Elastase^{neg} , F4/80^{neg} and Ly6G^{neg} cells were able to secrete or bind these two proteins **(Fig. 3, 4).** For this reason, we determined PGRN and SLPI expression in splenic human B and T cells, NK cells and macrophages by qRT-PCR. We found that both PGRN and SLPI mRNAs were highly expressed by

macrophages and B cells (**Fig. 5**), as confirmed also by the IHC positive staining of the B cell follicles (**Fig. 2**).

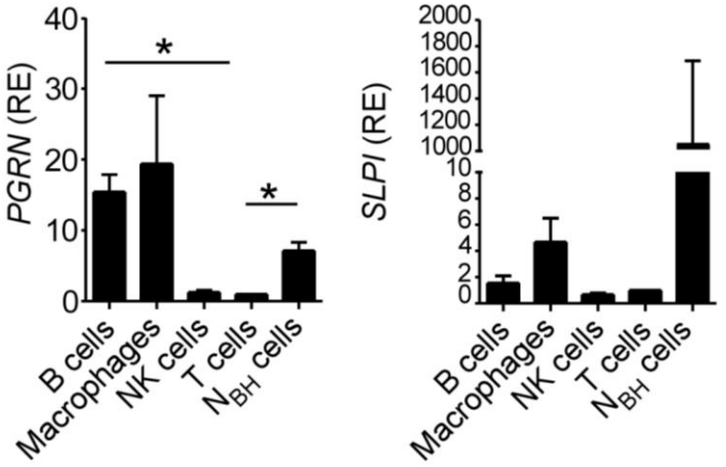

Figure 5. PGRN and SLPI are highly expressed by splenic B cells and macrophages. qRT-PCR of *PGRN* and *SLPI* mRNAs from FACS sorted B cells, macrophages, NK cells, T cells and N_{BH} cells. Results are normalized to ACTB (β-actin) mRNA and presented as relative expression (RE) compared with that of fresh T cells. Data summarize three pooled experiments with 1 donor in each. Error bars, s.e.m.; $P < 0.05$ (two-tailed paired Student's *t* test).

We can conclude from these results that several cell types, including N_{BH} cells, highly express PGRN and SLPI in both human and mouse spleen.

PGRN modulates neutrophil activation

SLPI, the natural inhibitor of neutrophil elastase, can regulate neutrophil differentiation in the bone marrow, since inhibition of SLPI in $CD34^+$ bone marrow hematopoietic progenitors markedly reduces granulocyte colony-stimulating factor (G-CSF)-induced myeloid differentiation *in vitro*. In addition, patients with severe congenital neutropenia (SCN) have strongly reduced SLPI levels in their serum as compared to healthy donors[243]. Similarly, we observed by ELISA that patients with neutrophil disorders (ND) also presented less PGRN, confirming that neutrophils constitute a major source of these two factors (**Fig. 6**).

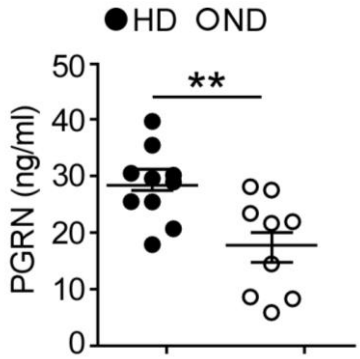

Figure 6. Neutropenia is associated with decreased PGRN levels. ELISA of soluble PGRN in serum from healthy donors (HD) or age-matched patients with neutrophil disorders (ND). Data display values from nine donors (HD and ND). Error bars, s.e.m.; $P < 0.05$ (two-tailed unpaired Student's *t* test).

From these data we could speculate that SLPI and PGRN may regulate each other. Indeed, patients affected by frontotemporal dementia (FTD) due to deleterious mutations at the PGRN gene,

presented significantly less PGRN but also SLPI proteins in their serum (**Fig. 7**).

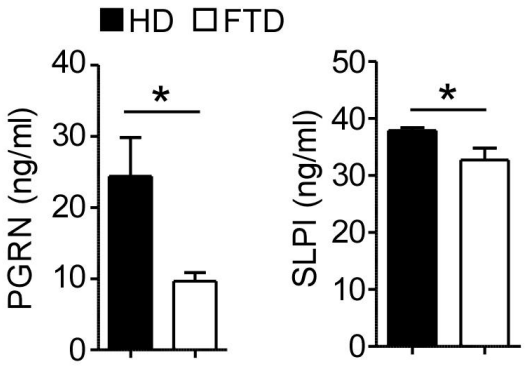

Figure 7. PGRN and SLPI levels are reduced in patients affected by FTD caused by a mutation in *GRN* gene. ELISA of soluble PGRN or SLPI in serum from healthy donors (HD) or age-matched patients with frontotemporal dementia (FTD). Data summarize values from four donors (HD and FTD). Error bars, s.e.m.; $P < 0.05$ (two-tailed unpaired Student's *t* test).

The possible involvement of splenic SLPI and PGRN in neutrophil-mediated immune responses was consistent with the observation that they could be induced by inflammatory stimuli. Human Nc cells were activated with TNF for 4h (**Fig. 8A**) or LPS during 1, 4 and 18h (**Fig. 8B**). qRT-PCR analysis showed that *GRN* mRNA expression increased upon stimulation with LPS or TNF. By contrast, SLPI-encoding mRNA was upregulated only in response to LPS, after 4h and 18h.

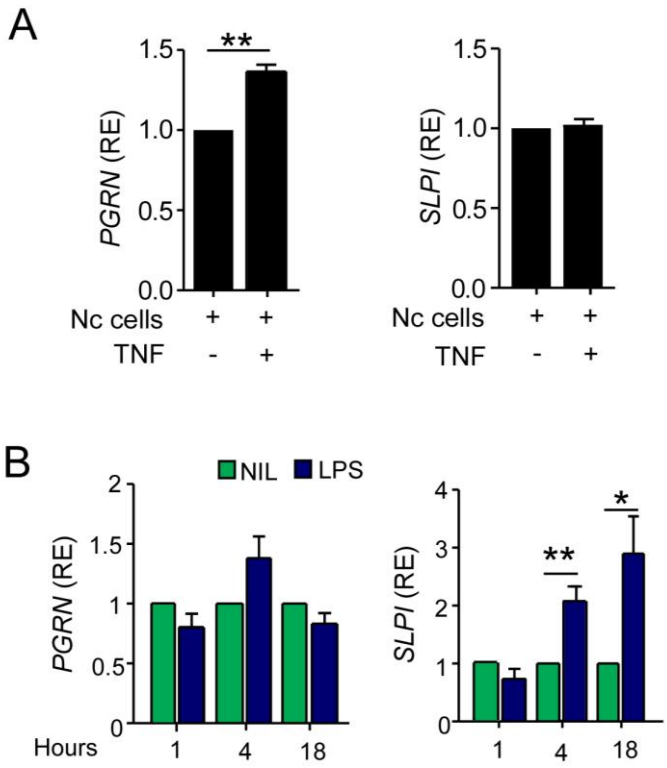

Figure 8. Neutrophils express PGRN and SLPI upon activation. qRT-PCR of *GRN* and *SLPI* mRNAs from Nc cells cultured alone or with 50 ng/ml TNF during 4h (A) or LPS during 1h, 4h or 18h (B). Results are normalized to ACTB (β-actin) mRNA and presented as relative expression (RE) compared with that of Nc cells cultured alone. Data summarize three measurements with 1 donor in each. Error bars, s.e.m.; $P < 0.05$ (two-tailed paired Student's *t* test).

PGRN is maintained intact by SLPI that binds to both PGRN and elastase and thus impedes its cleavage into inflammatory granulins. In addition, PGRN, but not granulins, reduces TNF-induced respiratory burst and degranulation in neutrophils[203]. In

order to better understand the functional properties of PGRN, we aimed to investigate the effect of PGRN on neutrophil activation.

Human Nc cells were stimulated with LPS during 4h with or without PGRN and SLPI. The addition of PGRN and SLPI to the cultures reduced the transcription of *IL-12p40* mRNA, a typical pro-inflammatory cytokine produced by neutrophils (**Fig. 9A**).

This result was confirmed in Nc cells isolated from FTD patients, which expressed increased levels of *IL-12p40* and *TNF* transcripts in response to LPS (**Fig. 9B**). Similarly to what we observed in humans, splenic Ly6G^{+} neutrophils from *Grn*$^{-/-}$ mice had increased expression of *Tnf* in comparison to WT neutrophils under steady state conditions (**Fig. 9C**).

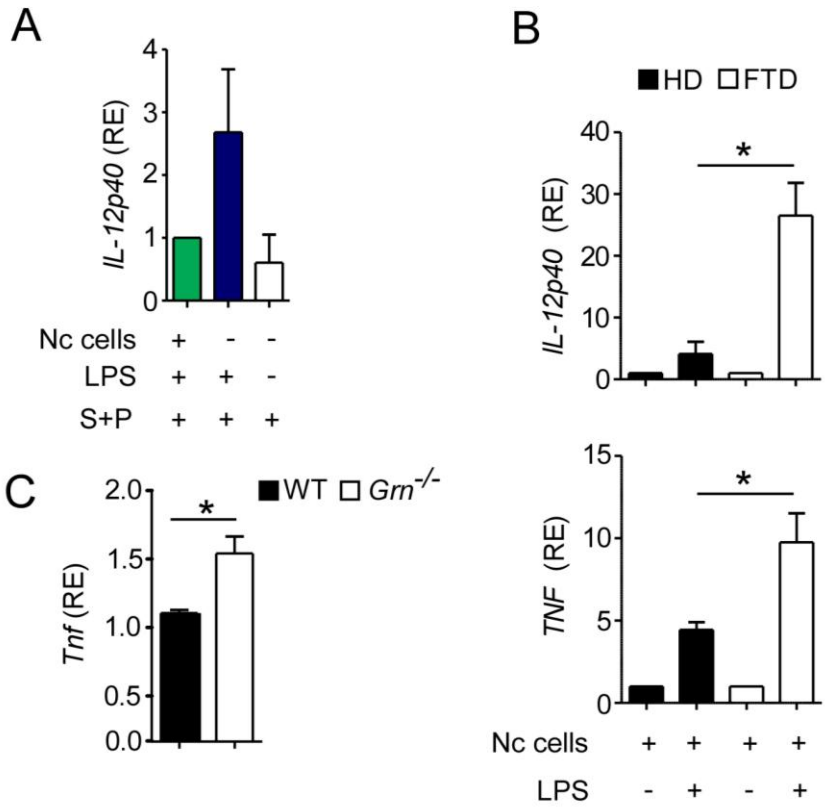

Figure 9. PGRN controls neutrophil activation. (A) qRT-PCR of *IL-12p40* mRNAs from Nc cells cultured alone or with 10 ng/ml LPS during 4h, with or without SLPI (1 μg/ml) and PGRN (10 μM). Results are normalized to ACTB (β-actin) mRNA and presented as relative expression (RE) compared with that of Nc cells cultured alone. (B) qRT-PCR of *IL-12p40 and TNF* mRNAs from Nc cells of healthy donors (HD) or patients with frontotemporal dementia (FTD) cultured alone or with 10 ng/ml LPS during 4h. Results are normalized to ACTB (β-actin) mRNA and presented as relative expression (RE) compared with that of Nc cells cultured alone. (C) qRT-PCR of *Tnf* mRNAs from splenic $Ly6G^+CD11b^+$ neutrophils of WT and $Grn^{-/-}$ mice. Results are normalized to *Gapdh* mRNA and presented as relative expression (RE) compared with that of WT splenic neutrophils. Data summarize values from four donors (HD and FTD, A and B) or three measurements with 1 donor in each (C). Error bars, s.e.m.; $P < 0.05$ (two-tailed paired (A, B) or unpaired (C) Student's test).

Finally, flow cytometric analysis indicated that $Grn^{-/-}$ mice also had reduced frequency and total number of granulocytes in the blood (**Fig. 10A**) and $Ly6G^+CD11b^+$ neutrophils in their bone marrow and spleen (**Fig. 10B**).

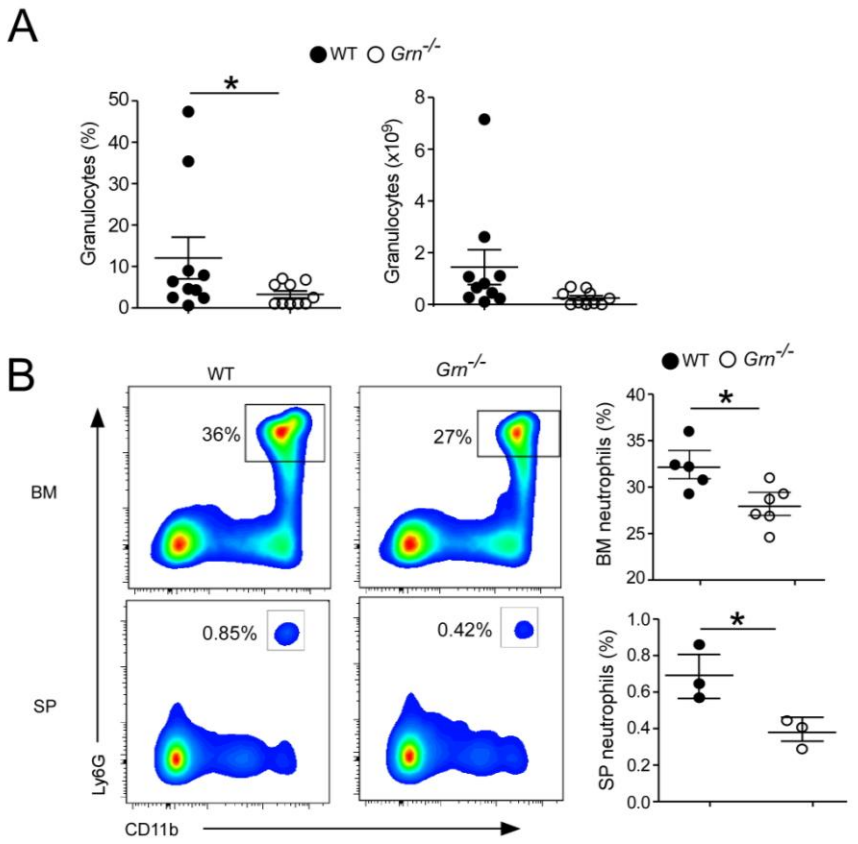

Figure 10. PGRN controls the frequency of granulocytes in blood, spleen and bone marrow. (A) Hemogram of WT and *Grn*$^{-/-}$ mice. (B) Flow cytometric analysis showing frequency of Ly6g^{+}CD11b^{+} neutrophils in bone marrow (BM) or spleen (SP) of WT or *Grn*$^{-/-}$ mice. Numbers in plots indicate the percentage of neutrophils. Data display values from ten (A) or at least three (B, right panels) WT or *Grn*$^{-/-}$ mice, or show analysis in representative mice (WT and *Grn*$^{-/-}$,B, left panels). Error bars, s.e.m.; $P < 0.05$ (two-tailed impaired Student's *t* test).

All these data indicate that PGRN is not only produced in high amounts by neutrophils, but also modulates their activation and might affect their generation and/or survival.

PGRN enhances pre-immune antibody responses to TI antigens

We recently demonstrated the involvement of NBH cells in TI immunoglobulin-mediated responses driven by MZ B cells, both in mice and humans. Indeed, neutrophil depletion in WT mice results in reduced splenic plasmablasts (PBs) and plasma cells (PCs) and TNP-specific IgG2b, IgG2c, IgG3 and IgM antibodies following immunization with TNP-Ficoll[253]. In addition, patients with severe congenital neutropenia (SCN) have reduced serum IgM and IgG to TI antigens under steady-state conditions[1].

Having shown not only the presence of PGRN in the MZ of the spleen but also the lower frequency of neutrophils in *Grn*$^{-/-}$mice, we hypothesized a possible involvement of this protein in TI antibody responses. In accordance with this hypothesis, ELISA assays revealed that pre-immune *Grn*$^{-/-}$ mice had reduced total serum IgM (**Fig. 11A**) and also reduced IgM specific to TI antigens such as phosphorylcholine (PC) and lipopolysaccharides (LPS) (**Fig. 11B**). By contrast, pre-immune total serum IgG3 and IgG2b were comparable in WT and *Grn*$^{-/-}$ mice.

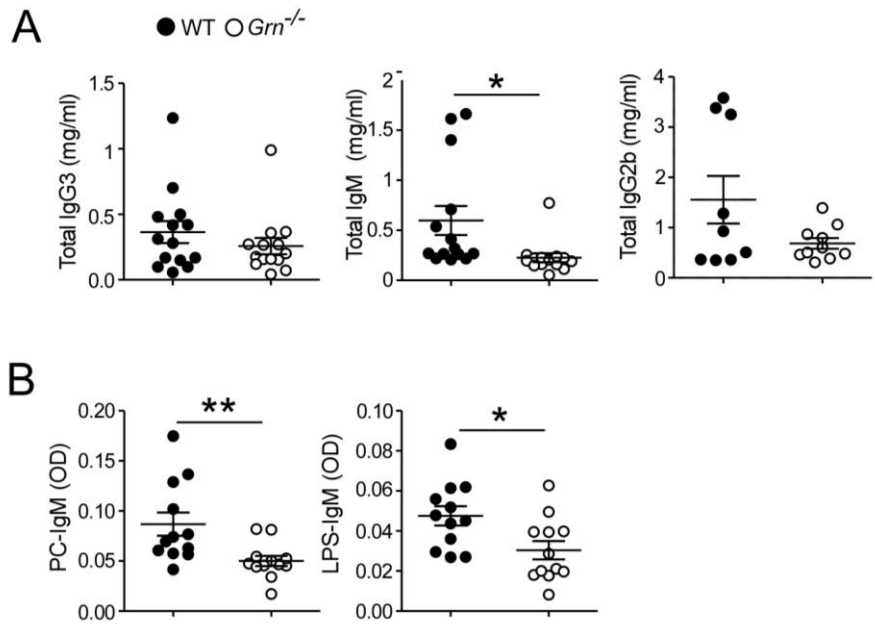

Figure 11. PGRN enhances TI pre-immune antibody responses. (A) Serum IgG3, IgM and IgG2b from WT or *Grn*$^{-/-}$ mice. (B) Serum IgM from WT or *Grn*$^{-/-}$ mice to phosphorylcoline (PC) or lipopolysaccharides (LPS). Data display values from twelve WT or *Grn*$^{-/-}$ mice (A, B). Error bars, s.e.m.; **P* <0.05 (two-tailed unpaired Student's *t* test).

Preimmune humoral alteration in *Grn*$^{-/-}$ mice did not correlate with variation in the MZ B cell compartment, nor in B-1 B cells, CD11b^{+} and MOMA-1^{+} macrophages or CD11c^{+} DCs, as shown by flow cytometry (**Fig. 12A**) and IFA staining of spleen cells (**Fig 12B**).

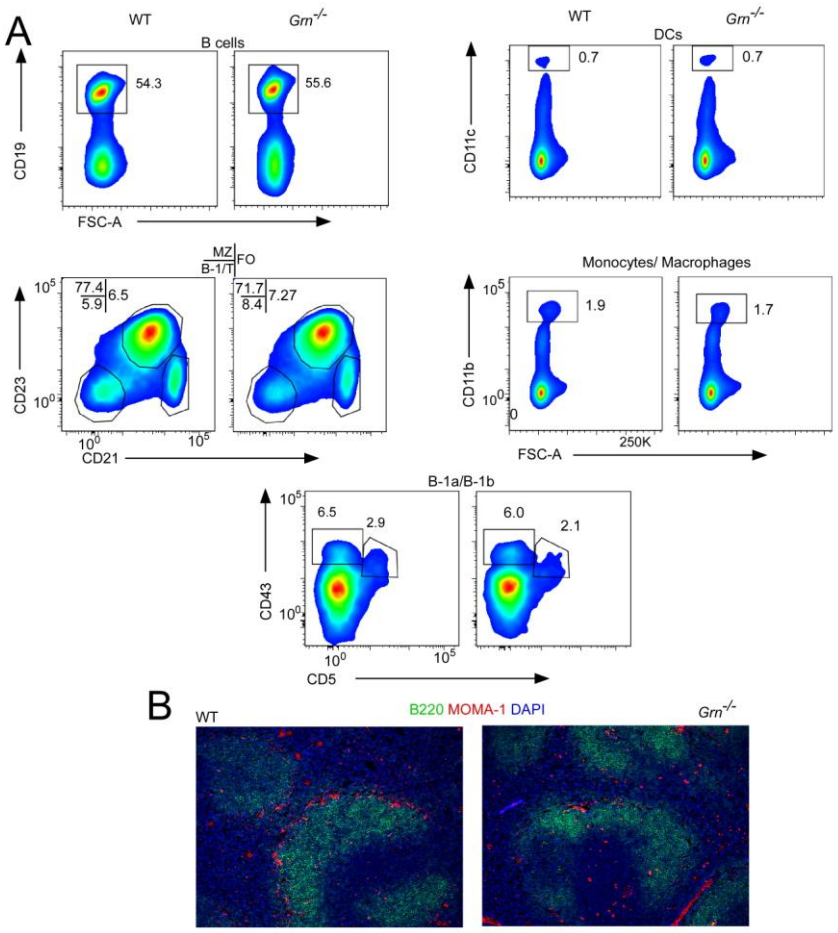

Figure 12. PGRN deficiency does not affect B cell, DC monocyte/macrophage compartments. (A) Flow cytometric analysis showing the frequency of splenic total B cells, MZ B cells, B-1/T cells, Fo B cells, DCs, monocytes/macrophages, B-1a and B-1b in WT or *Grn*$^{-/-}$ mice. Numbers in plots indicate percentage of the different subsets analyzed. (B) IFA staining for B220 (green), MOMA-1 (red) and DAPI (blue) of WT or *Grn*$^{-/-}$ spleens. Data show one of five (A) or two (B) WT or *Grn*$^{-/-}$ mice with similar results.

Accordingly, FTD patients presented decreased total IgM (**Fig. 13A**), and antigen-specific IgG and IgM reactive to *Streptococcus Pneumoniae* (*S. Pneumoniae*) and to LPS from *E. Coli,*

respectively. (**Fig. 13B**). However, these patients showed a conserved frequency of circulating granulocytes (%: 50.8-71).

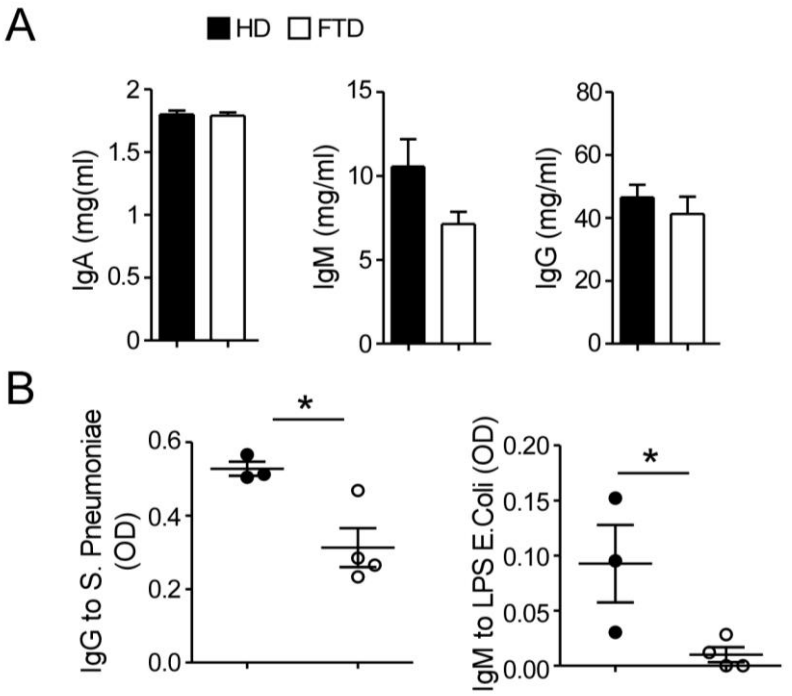

Figure 13. Low PGRN levels in serum are associated with reduced antibodies to TI antigens. ELISA of total (A) and TI antigen specific (B) IgG and IgM in sera from healthy donors (HD) or patients with frontotemporal dementia (FTD). Data summarize three measurements with 1 donor in each (A) or display values from at least four sera from HD or FTD patients (B). Error bars, s.e.m.; *P <0.05 (two-tailed unpaired Student's *t* test).

PGRN does not modulate the magnitude of post-immune TI antibody responses

To analyze TI antibody responses after immunization, WT and *Grn*$^{-/-}$mice were injected with 50 μg of TNP-Ficoll i.p (**Fig. 14A**). Spleen size (not shown) and cell count was similar in WT and *Grn*$^{-/-}$ mice (14B). TNP-reactive antibody responses were analyzed by ELISA and the analysis revealed that TNP-specific IgM and IgG3 serum levels were comparable between WT and *Grn*$^{-/-}$ mice (**Fig 14C**).

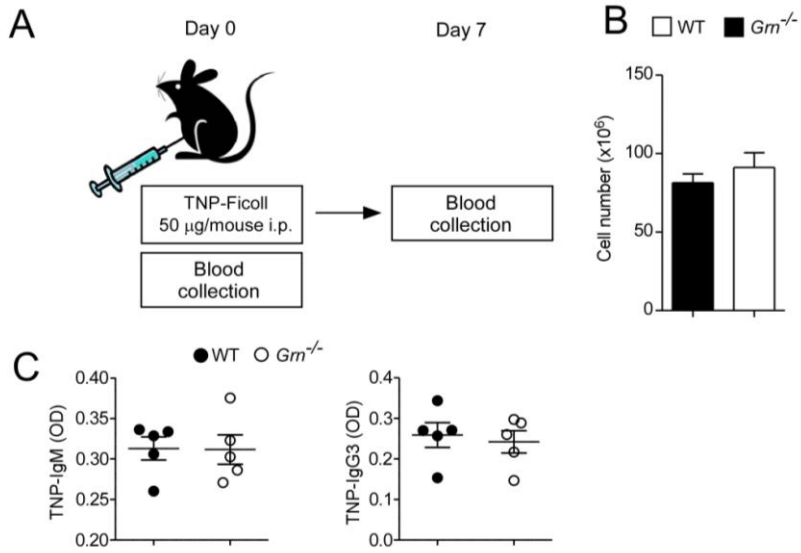

Figure 14. PGRN deficiency does not alter post-immune antibody responses to TI antigen. (A) Immunization protocol: WT and *Grn*$^{-/-}$ mice were immunized with 50 μg of TNP-Ficoll i.p. At day 7, mice were sacrificed, serum and spleen were collected. (B) Absolute number of cells from spleens of WT and *Grn*$^{-/-}$ mice after injection of TNP-Ficoll, day 7. C) ELISA of serum TNP-reactive IgM and IgG from WT and *Grn*$^{-/-}$ immunized with TNP-Ficoll, day 7. Data summarize (B) or display values

(C) from one of two similar experiments with five mice in each (WT and *Grn*$^{-/-}$). Error bars, s.e.m.

The frequency of MZ B cells, B1 B cells, neutrophils, CD11b^{+} DCs, CD8^{+} DCs and CD11b^{+} monocytes/macrophages, all known to be involved in TI immune responses, was determined by flow cytometry. *Grn*$^{-/-}$ mice presented a reduction in the percentage of Ly6G^{+}CD11b^{+} neutrophils and B-1b B cells, but also an increase in CD11b^{+} DCs, corresponding to MZ DCs (Fig. **15A**). By contrast, the frequency of MZ B cells, B-1a B cells, CD8^{+} DCs and CD11b^{+} monocytes/macrophages was similar in both mouse genotypes (**Fig. 15B**).

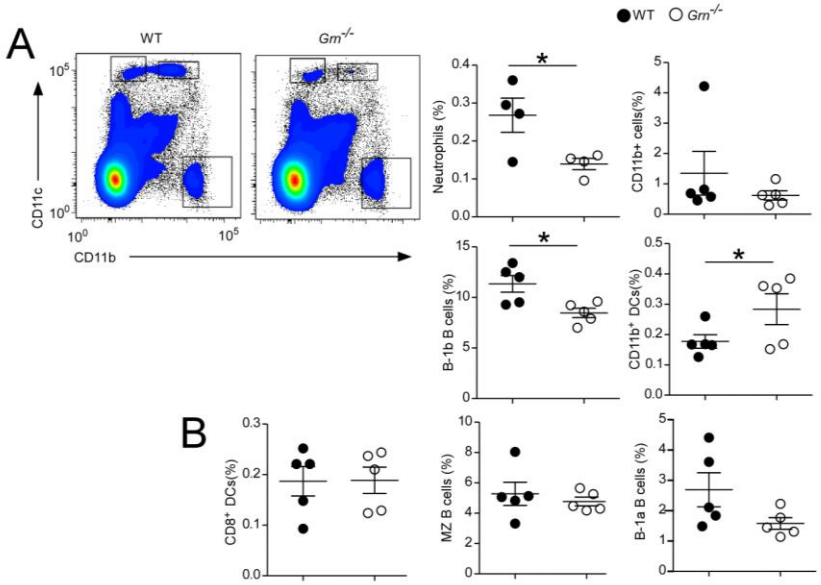

Figure 15. PGRN deficiency alters the composition of myeloid and lymphoid compartments in response to TI antigen. (A) Flow cytometry of neutrophils, CD11b^{+} macrophages/monocytes, B-1b B cells and CD11b^{+} DCs in WT or *Grn*$^{-/-}$ mice immunized with TNP-Ficoll, day 7. (B) Flow cytometry of CD8^{+} DCs, MZ B cells and B-1a B cells in WT or *Grn*$^{-/-}$ mice immunized with TNP-Ficoll, day 7. Data display values from one of two similar experiments with at least four mice in each (A, B, WT and *Grn*$^{-/-}$). Error bars, s.e.m.; *P <0.05 (two-tailed unpaired Student's *t* test).

In summary, PGRN deficiency alters the composition of myeloid and lymphoid compartments but not the magnitude of post-immune IgM and IgG3 antibody responses to TI antigen.

PGRN regulates dendritic cell function

Given that PGRN deficiency modulated $CD11b^+$ DC numbers and that DCs are adjacent to PGRN-producing cells, the effect of PGRN and SLPI on DC phenotype and function was determined.
In human, different splenic DC populations have been characterized according to the expression of specific cell-surface markers including CD11c, CD1b/c, CD16 and CD141. In mice, they can be subdivided into two prominent subsets, including $CD11b^+$ DCs and $CD8^+$ DCs. One of the main differences between these two species is that in human a population of DCs that exclusively populates the MZ has not been described so far, and $CD11c^+$ can be found both in T and B cell zones[173].

$CD14^+$ monocytes were isolated from human blood and differentiated to immature $CD11c^+$ DCs by addition of IL-4 and GM-CSF. To determine whether human DCs were able to promote the cleavage of PGRN into granulins (GRNs), cell extracts from immature DCs (defined by low/null expression of costimulatory molecules including CD40, CD80, CD86, and MHC class II molecules) were incubated with recombinant PGRN in the presence or absence of PMSF (a serine protease inhibitor) or SLPI for 30 minutes. Western Blot assay using an antibody that recognizes the unprocessed form of the protein showed that proteases secreted by immature DCs processed PGRN into GRNs,

and that SLPI efficiently inhibited this conversion as much as PMSF did (**Fig. 16**).

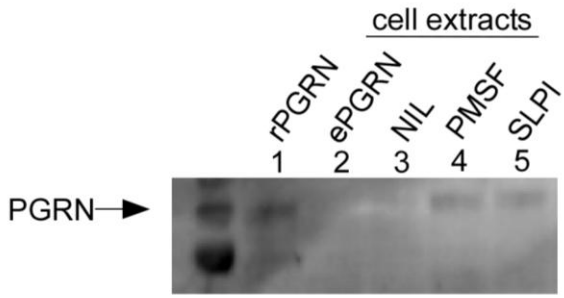

Figure 16. Human DCs can process PGRN into GRNs. Immature DCs were incubated alone (lane 3) or with 1mM PMSF (lane 4) or 0.5 μg SLPI (lane 5). Then, 0.4 μg recombinant human PGRN (rPGRN) were added during 2h at 37°C (lane 1,3-5). Cell extracts and endogenous PGRN (ePGRN, lane 2) were analyzed by Western blot. Data are from one of three experiments giving similar results.

For this reason, subsequent experiments evaluating the function of full-length PGRN in DCs were carried out in the presence of its processing inhibitor SLPI.

As discussed earlier, PGRN is abundantly expressed in the MZ of human spleen and co-localizes with LPS, the microbial ligand for TLR4[1]. Compared to circulating DCs (cDCs), splenic DC (spDCs) had higher expression of HLA-DR, CD40, CD80 and CD86, all markers of a fully mature state (**Fig. 17**). This phenotype could correlate with the presence in the spleen of LPS and other bacterial products signalling through TLR receptors. In agreement with this hypothesis, spDCs expressed high levels of TLR4 on their surface (**Fig. 17**).

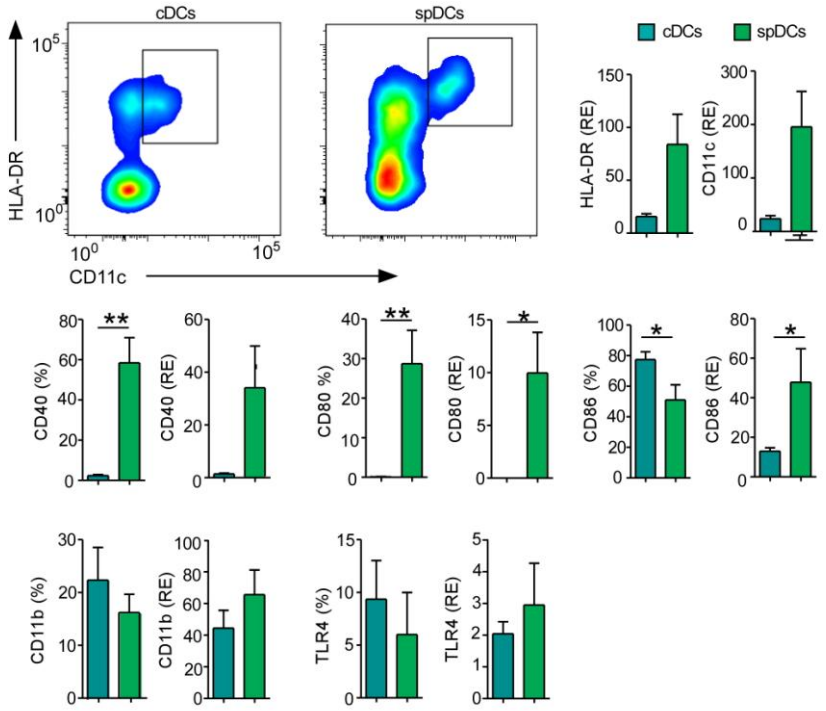

Figure 17. Splenic DCs have a more activated phenotype comparing to circulating DCs. Flow cytometric analysis of the frequency and mean fluorescence intensity (MFI) of HLA-DR, CD11c, CD40, CD80, CD86, CD11b, TLR4 in FACS sorted lineage$^-$CD11c$^+$HLA-DR$^+$ DCs from peripheral blood (circulating DCs, cDCs) or spleen (spDCs) of healthy donors. RE=MFI antigen-specific antibody staining/ MFI isotype control antibody staining. Data summarize six measurements with 1 donor in each (cDCs and spDCs). Error bars, s.e.m.;*P <0.05 (two-tailed unpaired Student's t test).

To mimic *in vivo* conditions, immature DCs were stimulated *in vitro* with low concentration of LPS, in the presence or absence of PGRN, for 24h. LPS-activated DCs are thus defined as "mature DCs".

The maturation state of DCs was analyzed by flow cytometry, using as readout the percentage of positive cells and the mean

fluorescence intensity (MFI) for typical DC maturation markers. Upon LPS stimulation, untreated or PGRN-treated DCs presented the same expression levels of HLA-I, HLA-II, CD40, CD80, CD83, and CD86 maturation molecules on their surface (**Fig. 18**).

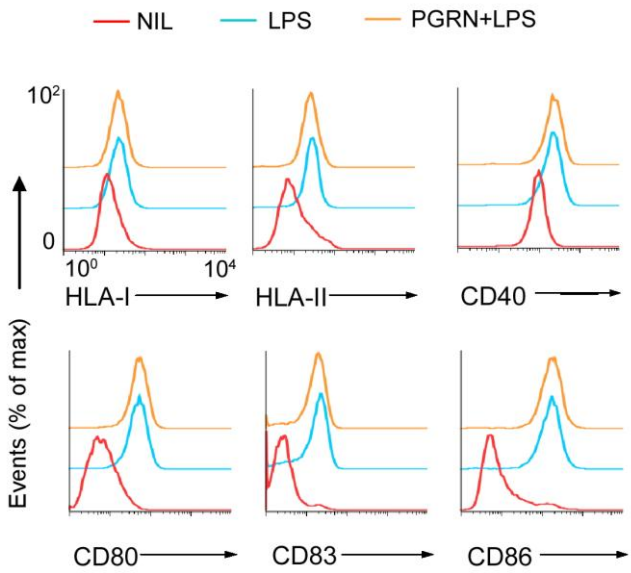

Figure 18. PGRN does not modify DC phenotype upon LPS-induced maturation. Flow cytometry of DCs treated or not with 10μM PGRN and 1 μg/ml SLPI, and activated during 24h with 10 ng/ml LPS. Data show one of ten experiments with similar results.

To further extend this analysis, qRT-PCR and ELISA assays were performed to evaluate the effect of PGRN on DC activation. Consistent with the described anti-inflammatory role of PGRN[210], DCs treated with PGRN expressed less transcripts for the pro-inflammatory cytokines IL-12p40, TNF, and IL-8 (**Fig. 19A**) and secreted less IL-12p70, IL-6, TNF, and IL-8 in the supernatant (**Fig.**

19B) in response to LPS. Secretion of BAFF was comparable in untreated and PGRN-treated DCs.

In contrast, mRNA expression and release of the anti-inflammatory cytokine IL-10 were increased in PGRN-treated DCs.

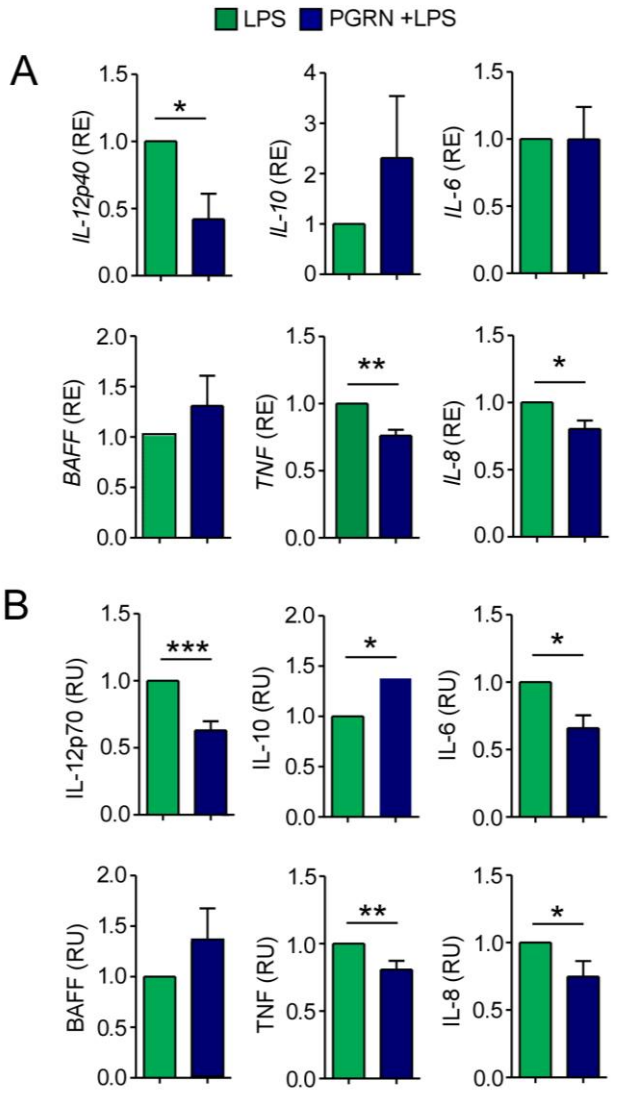

Figure 19. PGRN regulates DC activation. (A) qRT-PCR of *IL-12p40*, *IL-10, IL-6*, *BAFF*, *TNF* and *IL-8* in DCs cultured with 10 μM PGRN and 1

μg/ml SLPI with or without 10 ng/ml LPS during 5 hours. Results are presented as relative expression (RE) compared with that of DCs cultured with LPS alone. (B) ELISA of IL-12p70, IL-10, IL-6, BAFF, TNF and IL-8 from the supernatant of DCs cultured with 10 μM PGRN and 1 μg/ ml SLPI with or without 10 ng/ml LPS during 24 hours. Results are presented as relative units (RU) compared with that of DCs cultured with LPS alone. Data summarize at least three (A) or seven (B) pooled experiments with one donor in each. Error bars, s.e.m.; *P <0.05 (two-tailed paired Student's *t* test).

The viability of DCs was not affected by PGRN addition, nor when left unstimulated (not shown), nor upon LPS stimulation (**Fig. 20**).

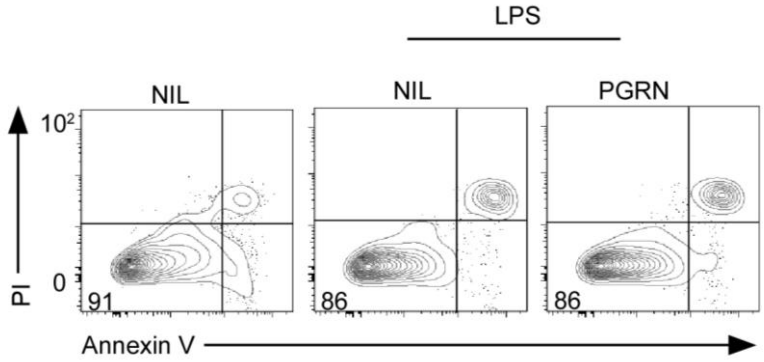

Figure 20. PGRN does not affect DC viability. Flow cytometry of propidium iodide (PI) and annexin V in DCs cultured with 10 μM PGRN and 1 μg/ml SLPI with or without 10 ng/ml LPS during 24 hours. Numbers in plots indicate percentage of live cells. Data show one of three experiments with similar results.

Collectively, these experiments indicate that human DCs can convert PGRN into GRNs unless SLPI is also present and that PGRN can affect the cytokine secretion profile but not the phenotype of DCs in response to LPS.

PGRN attenuates NF-κB signalling in dendritic cells

While pathways downstream of PGRN have been investigated in cancer and dementia to identify potential therapeutic targets, very few information is currently available about PGRN-dependent signalling in immune responses[198,254,255]. Published data in bone marrow-deived macrophages indicate that PGRN inhibits TNF-induced activation of p38, JNK and ERK1/2, all mitogen activated protein kinase (MAPK) family members[226]. Data from the same authors show that PGRN also inhibits NF-κB signalling induced by TNF[226].

LPS binds to TLR4 and triggers two distinct sequential signalling pathways, the first requiring Myd88 and the second controlled by the adaptor protein TRAF and TRIM that lead to a second phase of MAPK and NF-κB signaling[256,257].
Due to the modulatory effect of PGRN on IL-12p70 secretion and since IL-12 expression is induced by LPS and regulated through NF-κB, subsequent experiments were designed to ascertain the role of this transcription factor in PGRN-mediated DC function.

An Electromobility Shift Assay (EMSA) was performed to determine the activation of NF-κB proteins in nuclear extracts from immature and mature DCs, in the presence or absence of PGRN. The binding of this transcription factor to a consensus sequence belonging to the promoter of IL-12p40 gene was analyzed. After 90 minutes from the addition of LPS, we could detect only a modest reduction in the binding of NF-κB to its consensus sequence (**Fig. 21**).

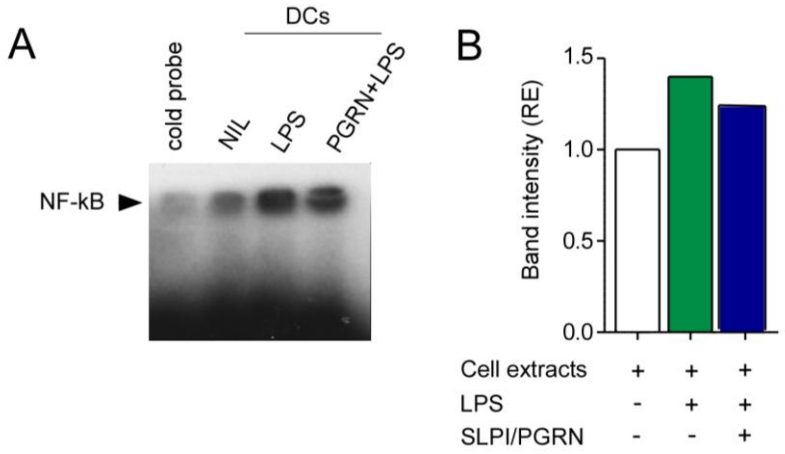

Figure 21. PGRN attenuates NF-κB signalling in DCs. (A) EMSA assays of nuclear protein extracts from DCs treated or not with 10μM PGRN and 1 μg/ml SLPI, and activated during 90 minutes with 10 ng/ml LPS. Samples were incubated with a radioactive probe encompassing a NF-κB binding sequence belonging to IL-12p40 gene promoter. Unlabeled (cold) probe were used to test the specificity of the reaction. (B) Band intensity krelative to NF-κB in DCs cultured alone (NIL). Data are from two experiments giving similar results (A,B).

This result suggest that PGRN might modulate the expression of IL-12 by eventually signaling through other transcription factors finally converging or not into the NF-κB pathway.

PGRN influences dendritic cell capacity to prime $CD4^+$ T cells

Having observed that PGRN reduced mature DC secretion of pro-inflammatory cytokines including IL-12p70 and increased the production of IL-10, all key factors in DC-mediated T cell activation, we analyzed the capacity of PGRN-treated DCs to stimulate $CD4^+$ T cells.

Firstly, naive $CD45RA^+CD45RO^-CD4^+$ T cells were purified from the peripheral blood of HD and cultured with untreated or PGRN-treated mature DCs. Cell proliferation was evaluated. Although no difference was observed in the proliferation rate of naive $CD4^+$ T cells cultured with both DC populations (**Fig. 22A**), PGRN-treated DCs induced less IFN-γ secretion in T cells than untreated DCs (**Fig. 22B**).

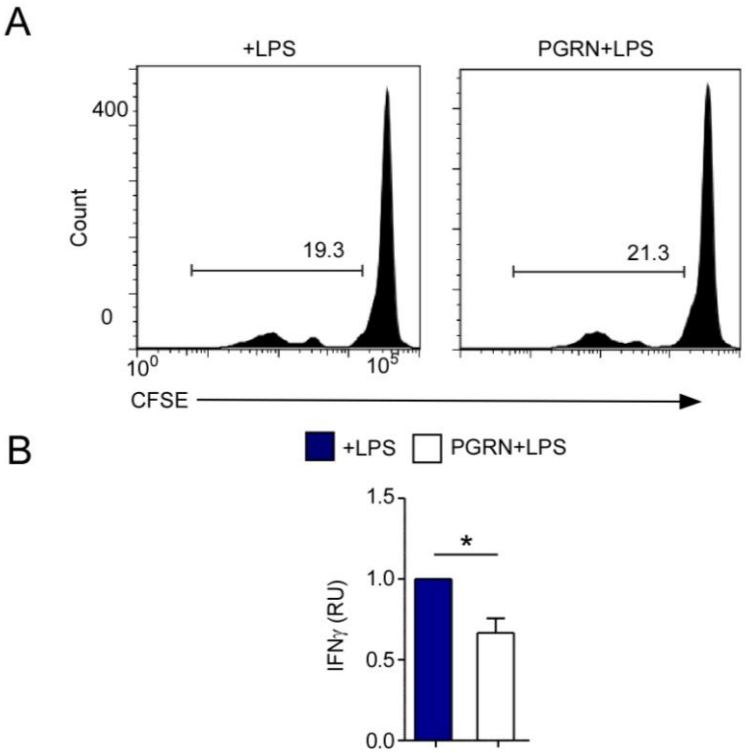

Figure 22. PGRN-treated DCs efficiently induce proliferation of naïve CD4$^+$ T cell but reduce their IFN-γ secretion. (A) Flow cytometry of CFSE-labelled CD3$^+$CD4$^+$CD45RA$^+$CD45RO$^-$ naïve CD4$^+$ T cells cultured with DCs left alone or treated with 10μM PGRN and 1 μg/ml SLPI, before addition of 10 ng/ml LPS, during 5 days. Numbers in plots indicate percentage of proliferating T cells. (B) ELISA of IFNγ from the supernatant of CD3$^+$CD4$^+$CD45RA$^+$CD45RO$^-$ naïve CD4$^+$ T cells cultured with DCs left alone or treated with 10μM PGRN and 1 μg/ml SLPI, before addition of 10 ng/ml LPS, during 5 days. Data show one of three experiments with similar results (A) or summarize measurements from three pooled experiments with one donor in each (B). Error bars, s.e.m.; *P <0.05 (two-tailed paired Student's t test).

In human spleen, most of the CD4$^+$ T cells are CD45RA$^-$CD45RO$^+$ and thus belong to the memory subset **(Fig. 23A)**. The percentage of memory compared to naïve CD4$^+$ T cells increased with age (**Fig. 23B**).

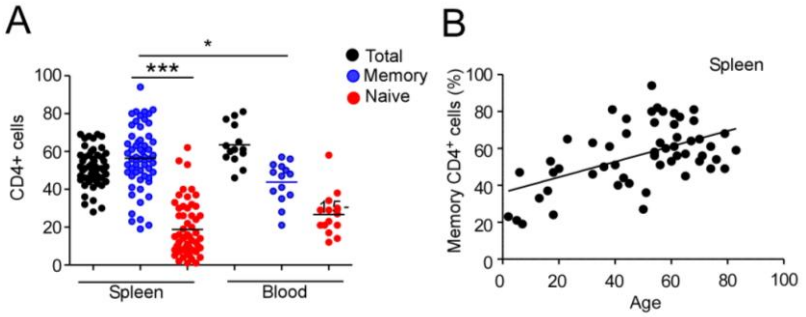

Figure 23. Most of $CD4^+$ T cells in human spleen belong to the memory subset. (A) Flow cytometric analysis of frequency of $CD3^+CD4^+$ (total), $CD3^+CD4^+CD45RA^-CD45RO^+$ (memory), $CD3^+CD4^+CD45RA^+CD45RO^-$ (naïve) T cells from spleen and peripheral blood of healthy donors. (B) Flow cytometric analysis of frequency of $CD3^+CD4^+CD45RA^-CD45RO^+$ memory T cells from spleen of healthy donors with ages ranging from 2 to 83 years old. Data display values from fifty (spleen, A and B) or fourteen donors (blood, A). Error bars, s.e.m.; *P < 0.05 two-tailed paired Student's *t* test (A); P<0.0001, r^2: 0.2778, linear regression (B).

For this reason, the stimulatory capacity of mature PGRN-treated DCs was analyzed also when co-cultured with $CD4^+$ memory T cells. Of note, we isolated both naive and memory T cells from peripheral blood and not from spleen to avoid any possible influence of pro-inflammatory or anti-inflammatory factors present in the tissue that could condition T cell activation.

After three days, T cell proliferation and cytokine secretion were analyzed.

Even though the proliferation was comparable (**Fig. 24A**), memory T cells cultured with PGRN-treated DCs secreted more IL-10 and less IFN-γ (**Fig. 24B**).

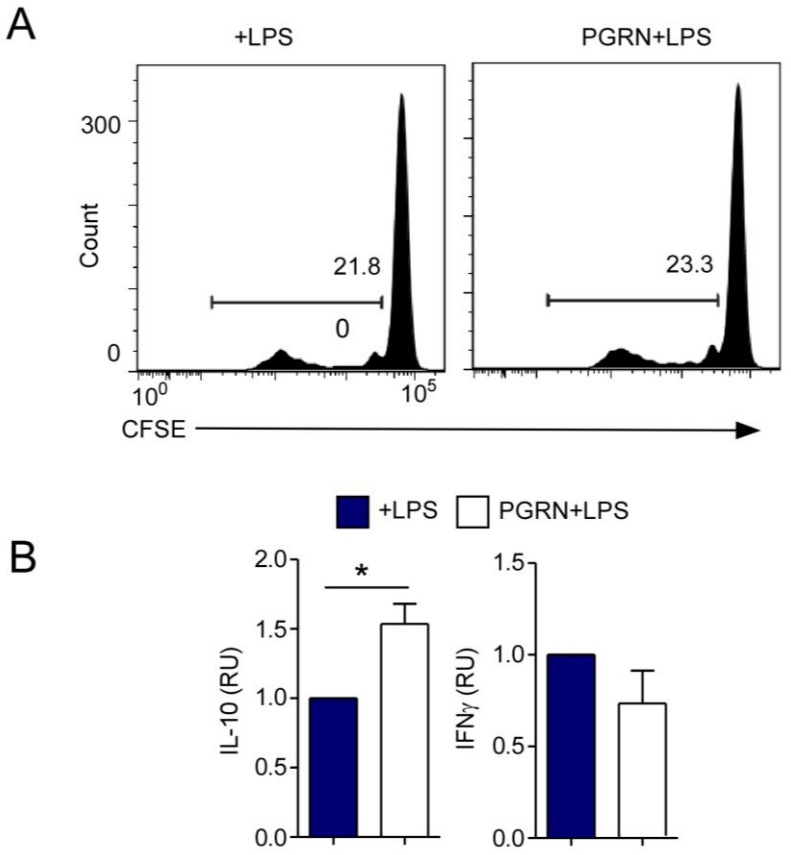

Figure 24. PGRN-treated DCs efficiently induce proliferation of memory CD4$^+$ cell, increase their IL-10 production and reduce their IFN-γ secretion. (A) Flow cytometry of CFSE-labeled CD3$^+$CD4$^+$CD45RA$^-$ CD45RO$^+$ memory T cells cultured with DCs left alone or treated with 10μM PGRN and 1 μg/ml SLPI, before addition of 10 ng/ml LPS, during 3 days. Numbers in plots indicate percentage of proliferating cells (B) ELISA of IL-10 and IFN-γ from the supernatant of CD3$^+$CD4$^+$CD45RA$^-$CD45RO$^+$ memory CD4$^+$ T cultured with DCs left alone or treated with 10μM PGRN and 1 μg/ml SLPI, before addition of 10 ng/ml LPS, during 3 days. Data show one of three experiments with similar results (A) or summarize measurements from three (B, left histogram) or two (B, right histogram) pooled experiments with one donor in each. Error bars, s.e.m.; *P <0.05 (two-tailed paired Student's t test).

Interestingly, intracellular staining revealed that splenic $CD4^+$ T cells from *Grn*$^{-/-}$ mice produced less IL-10 than WT $CD4^+$ T cells, suggesting that PGRN might modulate IL-10 secretion in T cells (**Fig. 25**). By contrast, IFN-γ secretion was comparable between WT and *Grn*$^{-/-}$ mice.

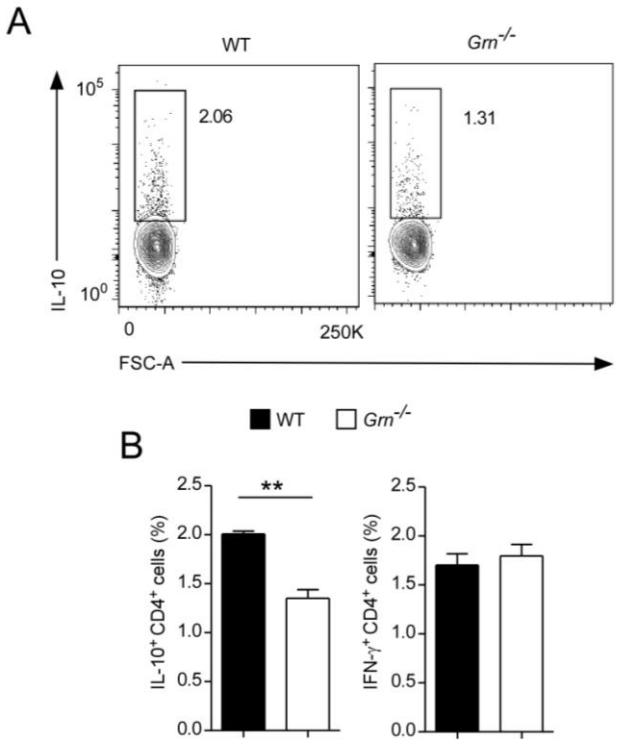

Figure 25. PGRN induced IL-10 production in $CD4^+$ T cells. (A, B) Flow cytometry of intracellular IL-10 and IFN-γ in $CD3^+CD4^+$ T cells from spleens of WT and *Grn*$^{-/-}$ mice. Numbers in plots indicate percentage of IL-10 producing $CD4^+$ cells. Data show one of three experiments with similar results (A) or display values from three WT and *Grn*$^{-/-}$ mice (B). Error bars, s.e.m.; **P* <0.05 (two-tailed unpaired Student's *t* test).

All together the presented results indicate that PGRN modulates the capacity of mature DCs to reduce IFN-γ secretion and increase IL-10 production in $CD4^+$ T cells.

PGRN enhances post-immune TD antibody responses

We then verified whether PGRN could affect *in vivo* DC phenotype and function in mice. For this purpose, we took advantage of $Grn^{-/-}$ mice.

Flow cytometry analysis of the two predominant DC subsets indicated that the percentage of $CD11b^+$ DCs and $CD8^+$ DCs was comparable in WT and $Grn^{-/-}$ mice (**Fig. 26**).

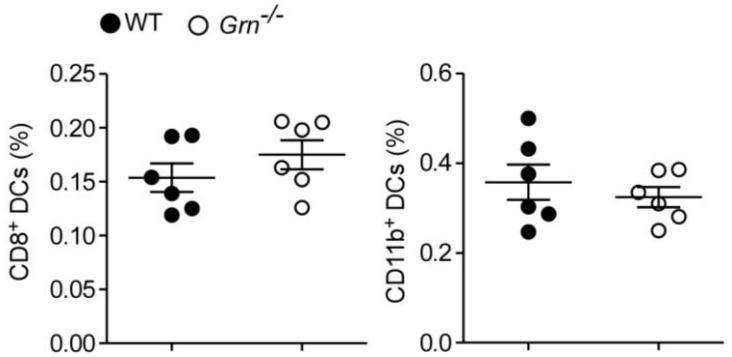

Figure 26. PGRN does not determine DC frequency in spleen. Flow cytometric analysis of frequency of $CD11c^+CD8^+$ and $CD11c^+CD11b^+$ DCs from spleen of WT and $Grn^{-/-}$ mice. Data display values from six WT and $Grn^{-/-}$ mice.

Splenic $CD11c^+$ from both mouse genotypes were purified and their transcriptional profile was analyzed by qRT-PCR. No significant

differences were observed between WT and *Grn*$^{-/-}$ DCs in their *Tnf*, *Il-6*, *Il-10* and *Il-12p40* transcripts (**Fig. 27**).

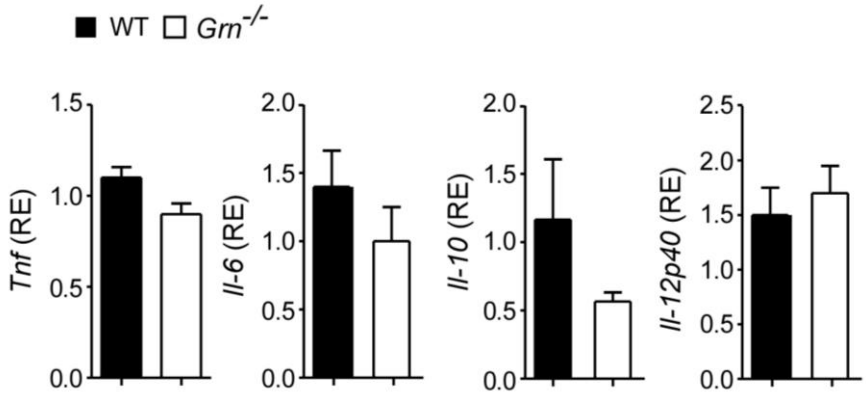

Figure 27. PGRN does not affect DC activation in steady state. qRT-PCR of *Tnf*, *Il-6*, *Il-10* and *Il-12p40* in freshly FACS sorted CD11c^{+} DCs from spleen of WT or *Grn*$^{-/-}$ mice. Results are normalized to *gapdh* mRNA and presented as relative expression (RE) compared with that WT CD11c^{+} DCs. Data display values from three WT and *Grn*$^{-/-}$ mice. Error bars, s.e.m.; *P < 0.05 (two-tailed unpaired Student's *t* test). Error bars, s.e.m.

Based on the effect that human PGRN-conditioned mature DCs had on CD4^{+} T cell priming, we hypothesized that PGRN could affect T cell responses during inflammation. To assess this hypothesis, WT and *Grn*$^{-/-}$ mice were immunized with TNP-OVA, a TD antigen, together with Sigma Adjuvant System (SAS). Mobilization of white blood cells was analyzed at day 0, 7 and 14. At day 14, mice were boosted with a second challenge of the same antigen, without adjuvant. At day 21 mice were sacrificed, the serum was collected and analysis of the different cell populations was performed **(Fig. 28)**.

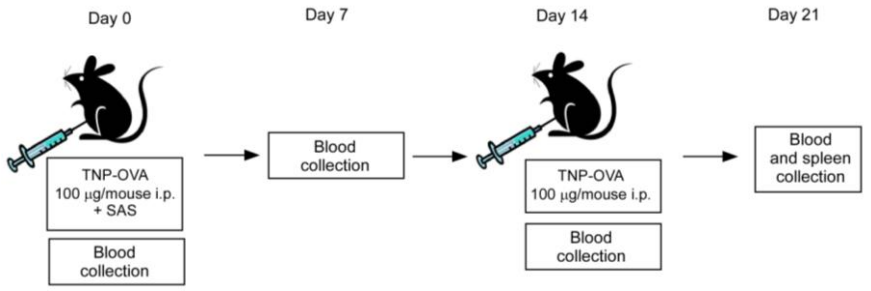

Figure 28. TD immunization protocol. WT and *Grn*$^{-/-}$ mice were immunized with 100 μg/mouse TNP-Ficoll i.p. with addition of SAS as adjuvant. At day 14 mice received a second challenge with 100 μg/mouse TNP-Ficoll i.p without SAS. At day 21 mice were sacrificed and spleen was collected. Blood was isolated for hemogram and ELISA at day 0,7,14, and day 21 (only for ELISA assay).

Spleen size was comparable in immunized WT and *Grn*$^{-/-}$ mice, as well as the total cell numbers (**Fig. 29**).

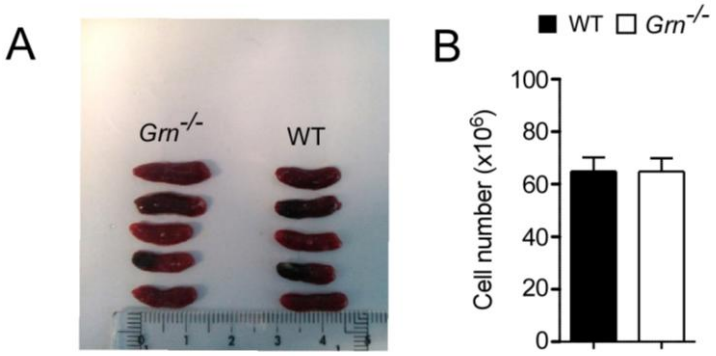

Figure 29. Spleen size and absolute cell number are preserved in the absence of PGRN in response to TD antigen. (A) Spleens from WT and *Grn*$^{-/-}$ mice after injection of TNP-OVA, day 21. (B) Absolute number of cells from spleens of WT and *Grn*$^{-/-}$ mice after injection of TNP-OVA and SAS, day 21. Data display spleens (A) or summarize results from one of

two experiments with similar results (B) with five WT and *Grn*$^{-/-}$ mice in each. Error bars, s.e.m.

A drastic reduction in the percentage and number of circulating (**Fig. 30A**) and splenic granulocytes (**Fig. 30B**) was observed at day 7 and day 21, respectively, indicating that PGRN was able to modulate the generation and/or survival of granulocytes in response to antigen. By contrast, the number and frequency of circulating lymphocytes and monocytes were not altered by the absence of PGRN (**Fig. 30A**).

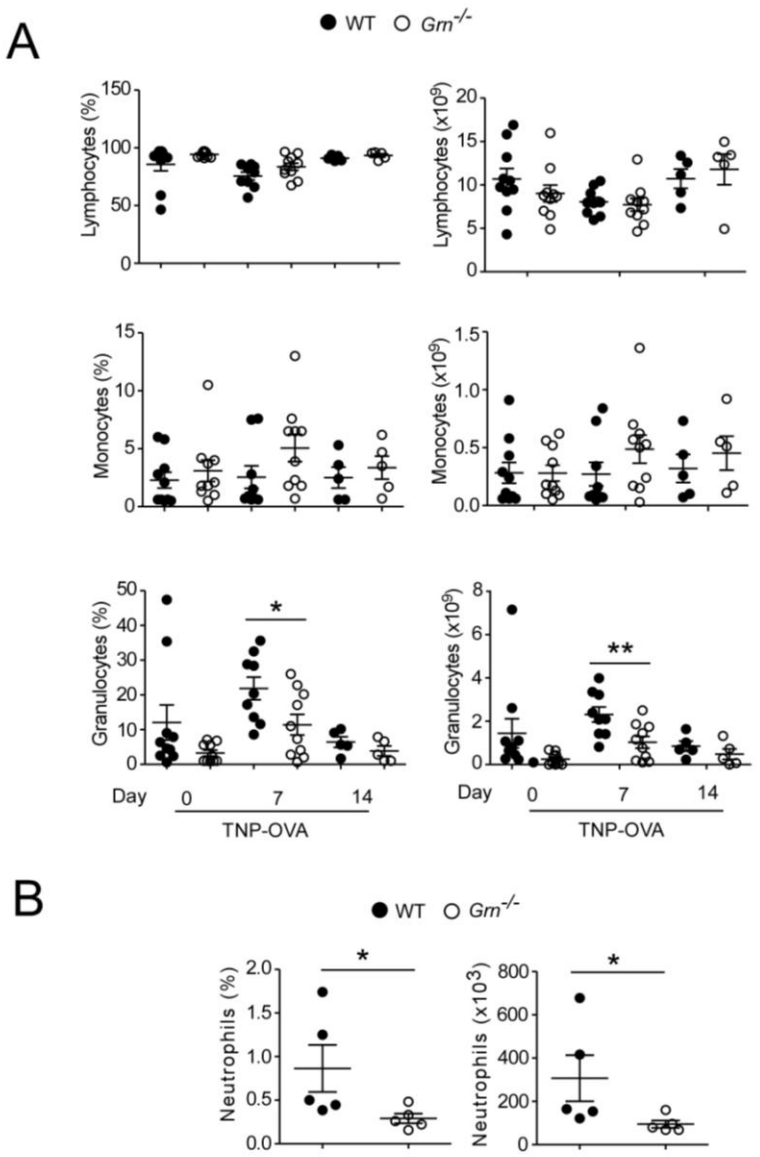

Figure 30. PGRN determines the frequency of neutrophils in response to TD antigens. (A) Hemogram of blood from WT and *Grn*$^{-/-}$ mice after injection of TNP-OVA, at day 0, 7, and 14. (B) Flow cytometric analysis of the frequency of CD11b^{+}Ly6G^{+} neutrophils in spleens of WT and *Grn*$^{-/-}$ mice. Data display values from ten (A) or five (A, B) WT and *Grn*$^{-/-}$ mice. Error bars, s.e.m.; *P < 0.05 two-tailed unpaired Student's test.

Next, we measured by ELISA TNP-specific IgM, IgG1, IgG2b, IgG2c, and IgG3 antibodies in sera from immunized mice. *Grn*$^{-/-}$ mice presented higher levels of TNP-IgM compared to WT mice at day 21. By contrast, TNP-specific IgG1 and IgG2c antibodies were reduced at day 14 and at day 21 (**Fig. 31**).

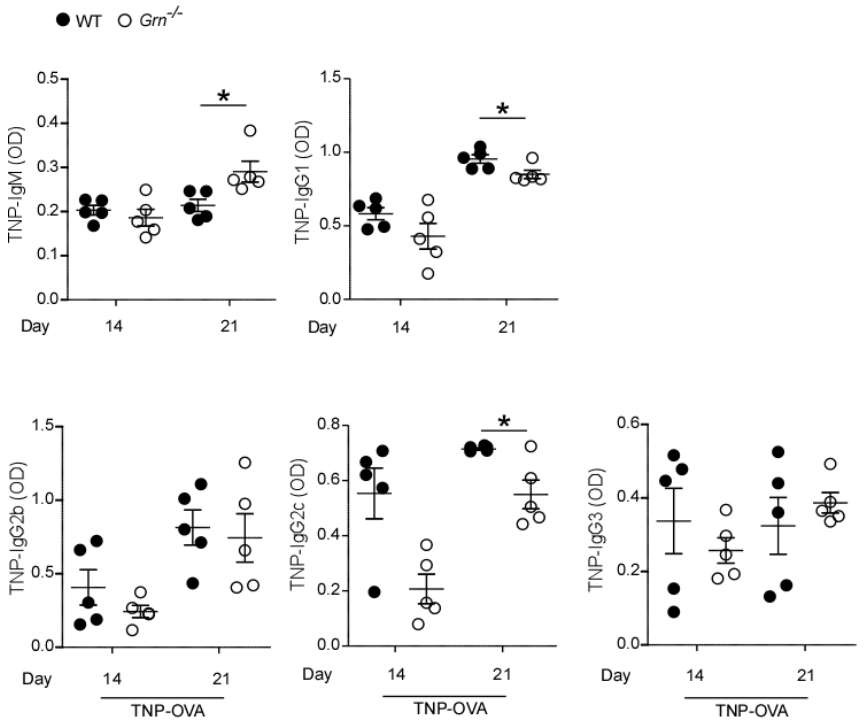

Figure 31. PGRN enhances post-immune antibody responses to TD antigen. ELISA at day 14, and 21 of TNP-reactive IgM, IgG1, IgG2b, IgG2c, and IgG3 in serum from WT and *Grn*$^{-/-}$ mice after injection of TNP-OVA. Data display values from one of two similar experiments with five WT and *Grn*$^{-/-}$ mice in each. Error bars, s.e.m.; *P < 0.05 two-tailed unpaired Student's *t* test.

Next, the affinity, i.e. the strength of interaction between the antibody and the antigen, of the TNP-reactive antibodies generated during the immune response was evaluated. ELISA assay show that PGRN deficiency reduced also the affinity of TNP-reactive IgG2b, IgG2c and IgG3 antibodies (**fig. 32**).

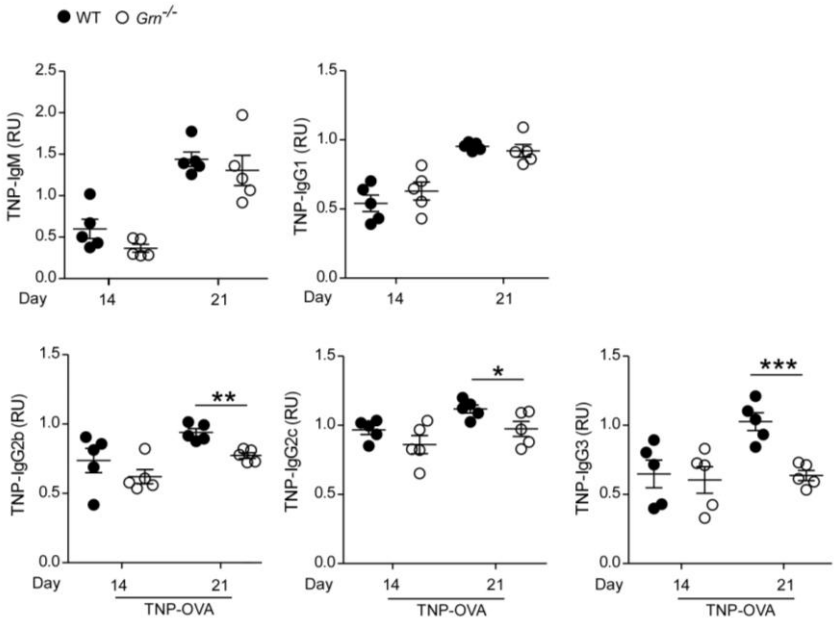

Figure 32. PGRN enhances post-immune generation of high affinity antibodies. ELISA at day 14 and 21 of TNP-reactive IgM, IgG1, IgG2b, IgG2c, and IgG3 in serum from WT and *Grn*$^{-/-}$ mice after injection of TNP-OVA. Results are presented as ratio (RU) of OD TNP(18)-BSA/OD TNP(2)-BSA. Data display values from five WT and *Grn*$^{-/-}$ mice. Error bars, s.e.m.; *P < 0.05 two-tailed unpaired Student's *t* test.

T cell-dependent production of high affinity antibodies involves interaction between T cells and B cells and the generation of Tfh cells. Tfh cell generation is a multi-step process involving initial interaction with DCs within the T cell zone followed by interactions with B cells at the T-B border and within the follicle[258].

While antigen presentation by activated B cells is important for the maintenance of Tfh cell responses, antigen presentation by DCs is necessary and sufficient to induce the initial expression of Bcl6, CXCR5 and the inducible T-cell co-stimulator (ICOS) and to launch the Tfh cell differentiation program[258].

Based on the above considerations, the frequency of Tfh cells upon antigen challenge was analyzed. WT and *Grn*$^{-/-}$ mice had comparable frequency and number of Tfh cells (**Fig. 33**).

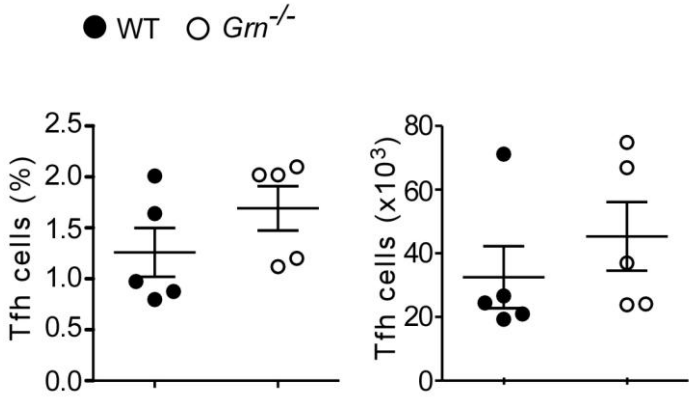

Figure 33. PGRN does not alter the frequency of Tfh cells in response to TD antigens. Flow cytometric analysis of the frequency of CD4$^+$PD-1$^+$CXCR5$^+$ICOS$^+$ T follicular helper (Tfh) cells in spleens from WT and *Grn*$^{-/-}$ mice. Data display values from five WT and *Grn*$^{-/-}$ mice. Error bars, s.e.m.; *P < 0.05 two-tailed unpaired Student's *t* test.

Despite the reduction in serum class switched immunoglobulins, *Grn*$^{-/-}$ mice presented higher percentage and number of GL7$^+$ GC B cells and total B220$^+$CD138$^+$ antibody secreting cells (ASCs) (**Fig. 34**).

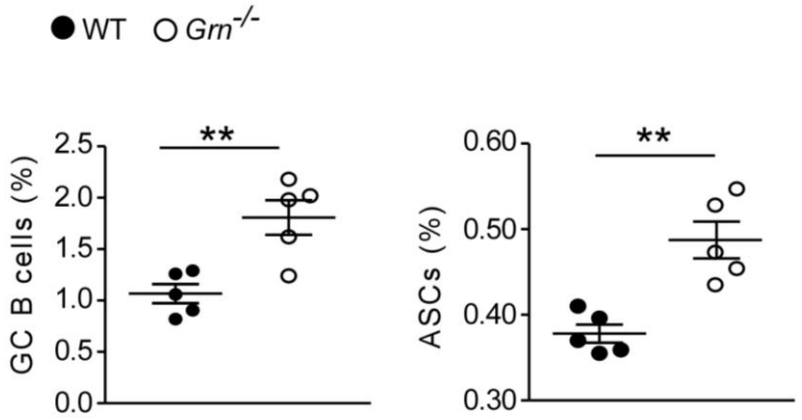

Figure 34. PGRN regulates the frequency of germinal center B cells and antibody secreting cells in response to TD antigens. Flow cytometric analysis of the frequency of $B220^{+}GL^{-}7^{+}$ germinal center (GC) B cells and $B220^{-}CD138^{+}$ antibody secreting cells (ASCs) in spleens from WT and *Grn*$^{-/-}$ mice. Data display values from five WT and *Grn*$^{-/-}$ mice. Error bars, s.e.m.; *P < 0.05 two-tailed unpaired Student's *t* test.

Increase in GC B cells and ASCs in *Grn*$^{-/-}$ spleen was accompanied by higher numbers of both $CD11c^{+}CD8^{+}$ DCs and $CD11c^{+}CD11b^{+}$ DCs that populated the spleen of the immunized mice (**Fig. 35**).

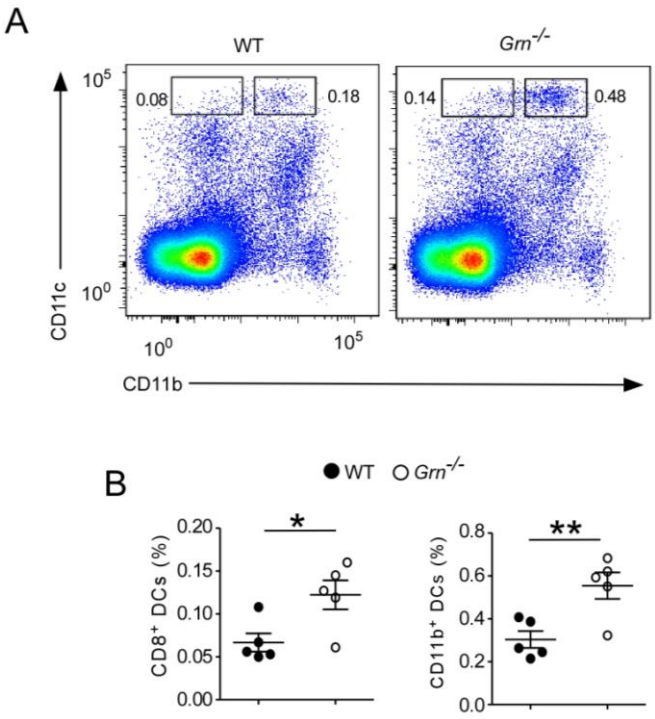

Figure 35. PGRN regulates the frequency of CD8$^+$ DCs and CD11b$^+$ DCs in response to TD antigens. (A,B) Flow cytometric analysis of the frequency of CD11c$^+$CD8$^+$ and CD11c$^+$CD11b$^+$ DCs in spleens from WT and *Grn*$^{-/-}$ mice. Numbers in plots indicate the percentage of DCs. Data show analysis in representative mice (WT and *Grn*$^{-/-}$, A) or display values from five WT and *Grn*$^{-/-}$ mice (B). Error bars, s.e.m.; *P < 0.05 two-tailed unpaired Student's *t* test.

Since Treg cells are key regulators of immune responses and several studies support a role of PGRN in the generation and function of Treg cells[43,226,259] we also analyzed the percentage of CD4$^+$Foxp3$^+$ cells and we observed that they were reduced in *Grn*$^{-/-}$ mice compared to WT mice (**Fig. 36**).

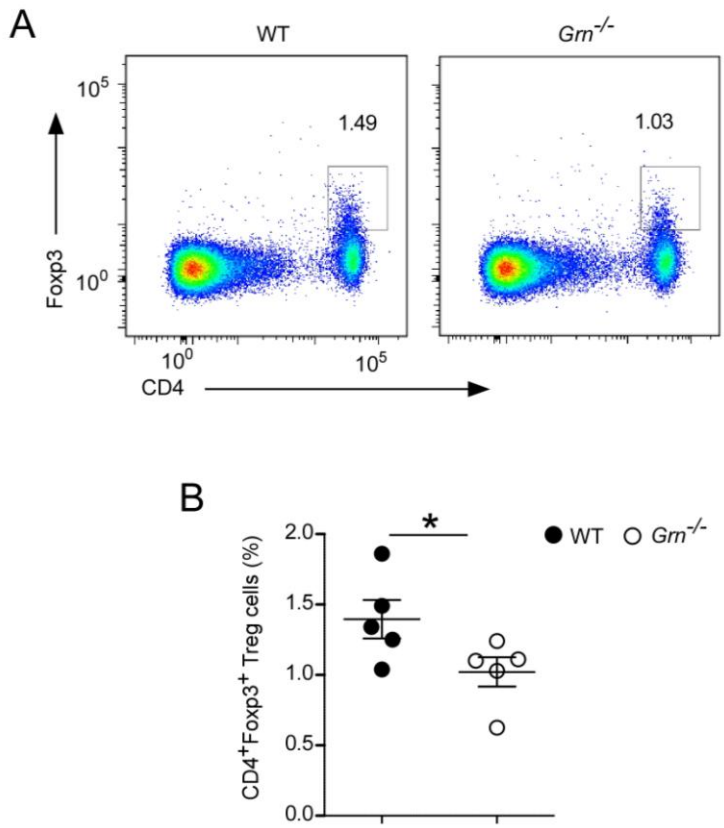

Figure 36. PGRN upregulates the frequency CD4$^+$Foxp3$^+$ Treg cells in response to TD antigens. (A,B) Flow cytometric analysis of the frequency of CD4$^+$Foxp3$^+$ cells in spleens from WT and *Grn*$^{-/-}$ mice. Numbers in plots indicate the percentage of Treg cells. Data show analysis in representative mice (WT and *Grn*$^{-/-}$, A) or display values from five WT and *Grn*$^{-/-}$ mice (B). Error bars, s.e.m.; *P < 0.05 two-tailed unpaired Student's *t* test.

In conclusion, our data reveal a complex scenario where PGRN enhances the generation of high affinity antibodies to TD antigen. Indeed, PGRN deficiency leads to a suboptimal Ig response. The complex mechanisms used by PGRN to participate in TD humoral responses are still partially unclear, but our data suggest that

PGRN might regulate the frequency and the effector function B lymphocyte-activating cells such as neutrophils, DCs and $CD4^{+}Foxp3^{+}$ Treg cells. Further experiments will clarify the connections between all these different compartments.

CHAPTER V

DISCUSSION

We have shown that PGRN and SLPI were abundantly present in human spleen. In addition to splenic macrophages and B cells, also NBH cells were important producers of both proteins. In neutrophils, the secretion could be further increased in response to inflammatory stimuli such as TNF and LPS.
On the other hand, PGRN was able to modulate not only the frequency, but also the function of neutrophils, by reducing TNF expression upon activation.

PGRN enhanced pre-immune antibody responses to TI antigens, both in humans and mice. By contrast, PGRN did not affected post-immune reactions to TNP-Ficoll, a classical TI antigen, although an alteration in the composition of myeloid (including DC subset) and lymphoid compartments could be observed.

Moreover, PGRN modulated the function of mature DCs, by reducing the release of pro-inflammatory cytokines such as IL-12 and TNF and increasing the secretion of IL-10 in response to LPS, through a mechanism only partially dependent on NF-κB. In addition, PGRN-treated DCs promoted the production of IL-10 and reduced the release of IFN-γ in $CD4^+$ T cells.

Finally, PGRN enhanced antibody responses against TNP-OVA, a TD antigen. Augmented high-affinity immunoglobulin production was accompanied by an increase in the frequency of neutrophils.

We conclude that PGRN is an immunoregulatory factor able to directly or indirectly regulate the frequency and/or function of neutrophils, DCs, T and B cells. This capability might affect T- and

B-cell responses that take place in the spleen, both in homeostasis and during inflammation.

PGRN and SLPI are highly expressed in human and mouse spleen

The precise structure of human spleen is still partially unknown, as well as its cellular composition. Lack of information is principally due to the paucity of available human samples. Our group recently identified in the MZ of the spleen a population of neutrophils (N_{BH} cells) capable of eliciting B cell responses against TI antigens. They are characterized by a unique phenotypical and genic profile that distinguishes them from circulating neutrophils (Nc)[1]. For the first time SLPI and PGRN were detected in human N_{BH} cells.

Granulins were originally isolated from equine neutrophils[260], and SLPI was shown to be synthesized during neutrophil differentiation and stored at low amounts in secondary granules[261]. Our data showed that PGRN mRNA levels were comparable in N_{BH} and Nc cells, while SLPI transcripts were higher expressed in N_{BH} cells. Higher levels of SLPI could reflect the need to re-establish homeostasis in N_{BH} cell-occupied perifollicular areas containing microbial products such as LPS. Indeed, SLPI is a potent serine protease inhibitor that exerts substantial antibacterial activity by reducing serine protease-mediated tissue damage. It is also critical for neutrophil functions such as migration and degradation of phagocytosed microorganism[240].

PGRN was previously described only in rat spleen, where it localizes mainly in the MZ and sporadically in the red pulp, as we observed in mouse spleen[262]. By contrast, even though PGRN was more abundant in perifollicular areas of human spleen, it was also expressed by cells in the red pulp. This can be explained by the fact that in normal adult human spleens, single B lymphocytes and macrophages are regularly distributed all over the red pulp, together with sinusoid-lining cells (endothelial cells lining the venous sinusoids) and plasma PCs[36,263,264]. Indeed, we found that B cells and macrophages, together with N_{BH} cells, expressed PGRN and SLPI transcripts at higher levels compared to other cell types.

Of note, patients with neutrophil disorders presented less PGRN and SLPI in their serum. On the other hand, patients with FTD due to a mutation in *GRN* gene and known to have less PGRN in plasma[265], also had less SLPI. All these data suggest that these two proteins may regulate each other. Since SLPI prevents PGRN cleavage by binding to PGRN cleavage sites or to serine proteases that process PGRN[203], one hypothesis is that *de novo* synthesis of SLPI is reduced when PGRN levels are reduced, to avoid an over-inhibition of the activity of proteases or GRNs. Another possibility is that in the absence of SLPI, PGRN levels are regulated to impede exacerbate GRN-mediated inflammatory responses. It has been recently shown that elastase induces the expression of SLPI, which subsequently regulated neutrophil differentiation and survival[243]. Our results indicated that also PGRN might modulate differentiation, survival or recruitment of neutrophils, which were reduced in blood, spleen and bone marrow of *Grn*$^{-/-}$ mice. On the other hand, the proteases proteinase 3 and elastase, directly

involved in early neutrophil activation events, controls PGRN levels, since *Prtn3*$^{-/-}$*Ela2*$^{-/-}$ mice presented accumulation of PGRN[213]. Although a clear mechanism has not been described so far, these results strongly indicate a complex interplay between proteases, SLPI and PGRN that finally regulates neutrophil differentiation and function.

PGRN modulates neutrophil activation

We found that PGRN could reduce activation in human neutrophils, by downregulating *TNF* and *IL-12p40* mRNA expression. These results were confirmed both in patients with FTD and *Grn*$^{-/-}$ mice, whose neutrophils showed a more activated profile.

All these data are in accordance with previously published studies investigating the effect of PGRN on neutrophil activation. PGRN, but not GRNs, potently and specifically inhibit TNF-induced signal transduction in human neutrophils, thereby blocking cell spreading, respiratory burst and degranulation[203]. Similarly, PGRN significantly reduces reactive oxygen species (ROS) production of neutrophils activated by antigen-antibody immune complexes causing a variety of human diseases, like autoimmune disorders and infections[213].

By contrast, divergent data are provided about the effect of PGRN on neutrophil recruitment in vivo. In a model of renal ischemia/reperfusion injury, *Grn*$^{-/-}$ mice show significantly aggravated renal injury as evidenced by higher tubulointerstitial neutrophil and macrophage infiltration[266]. Analogously, PGRN ameliorates cerebral ischemia/reperfusion-induced inflammation, by inhibiting neutrophil recruitment into the brain[267]. Conversely,

when applied to a cutaneous wound, progranulin increased the accumulation of neutrophils, macrophages, blood vessels and fibroblasts[223]. In these settings, the generation of GRN peptides can stimulate epithelial cell lines to secrete the neutrophil chemoattractant IL-8[203]. We observed that in steady state and after immunization with both TI and TD antigens, the absence of PGRN dramatically reduced the frequency of splenic neutrophils in mice, indicating that this factor might be a potent regulator of neutrophil migration.

Since we observed a decrease in the frequency of neutrophils in bone marrow and granulocytes in blood, we could speculate that their reduction in spleen could also be due to either a lessening in granulopoiesis or in cell survival. In vitro experiments with human Nc cells did not indicate a direct effect of PGRN on neutrophil survival (data no shown), pointing out the possibility that other mechanisms could account for the observed results. However, it is reported that low doses of TNF induce survival in human neutrophils, while a dominant pro-apoptotic effect was observed at higher doses[268]. Since PGRN competes with TNF, its deficiency in *Grn*$^{-/-}$ mice could favor the binding of TNF to TNFRs expressed by neutrophils. Cytokines reported to enhance neutrophil survival, such as IL-1α, IL-1β, IL-6, and GM-CSF should be compared in sera from WT and *Grn*$^{-/-}$ mice.

PGRN modulates post-immune but not pre-immune antibody responses to TI antigens

Our group demonstrated that neutrophil deficiency leads to impaired TI antibody responses, both in human and mice[1,253]. Having shown that the presence of PGRN correlated with the frequency of neutrophils, we wondered whether in the absence of PGRN TI antibody responses could be altered. The effect of PGRN on humoral immune responses has never been investigated before. We observed that PGRN deficiency affected the generation of total IgM natural antibodies, both in humans and mice.
Under homeostatic conditions, MZ B cells and B1 B cells produce antibodies that recognize molecular signatures shared by both foreign and autologous cells, thus facilitating the clearance of both intruding microorganisms and host apoptotic cells[4,269].

We showed that PGRN was involved in the production of antibodies against LPS and PC. PC is one of the main components of the eukaryotic membrane and is expressed by several bacterial species, both commensal and pathogenic, which acquire it from the host. Most humans have a substantial immune response to PC and natural PC-specific antibodies (anti-PC) have been reported to constitute between 5–10% of the total IgM pool[270]. In mice, IgM antibodies against PC are known to protect against *Streptococcus pneumoniae* infection[271]. In humans, low levels of IgM antibodies against PC correlate with myocardial infarction and stroke[272]. More recently, decreased levels of anti-PC IgM antibodies were also associated to dementia development[272]. Several groups are currently identifying a role of anti-PC antibodies in the protection

against infection and clearance of apoptotic cells, and our results suggest that PGRN could be involved in these events[273].

MZ B cells, after interacting with antigens exposed on macrophages, DCs or neutrophils, rapidly differentiate into PBs that produce large amounts of IgM immunoglobulins[1,64,96,269,274].

Since we did not find any alteration in DC and monocyte/macrophage (including MZ-associated MOMA-1$^+$ MMMs) compartments in *Grn*$^{-/-}$ mice, we argue that neutrophil reduction might explain the diminished pre-immune TI antibody responses. Adoptive transfer of PGRN-sufficient N_{BH} cells to *Grn*$^{-/-}$ mice would clarify whether other cell populations are eventually involved.

A quite different scenario was observed after immunization with TNP-Ficoll. Indeed, although the magnitude of post-immune IgM and IgG3 antibody responses was similar in WT and *Grn*$^{-/-}$ mice, we could detect differences in the frequency of splenic neutrophils, CD11b$^+$ MZ DCs and B1b cells.

Blood-derived CD11c+ DCs were reported to transport killed bacteria to B cells and initiate and support TI responses through the secretion of BAFF and APRIL[96].
MZ DCs are also important for the uptake of blood-borne particulate antigens and the promotion of TI antibody responses[269]. *Cd47*$^{-/-}$ mice, deficient in SIRPα^+ CD11c^{high}CD11b^{high}CD8α^- DCs, corresponding to MZ DCs, show an impairment in IgG responses to TI antigens[275]. Because of their shared B-cell helper function, reduction in N_{BH} cells due to PGRN deficiency could eventually be bypassed by an increase in MZ DCs. Indeed, both cell type secrete B cell stimulating factors such as BAFF and APRIL[1,189,276] and

neutrophils can acquire unique properties that are typically reserved for professional APCs, such as the expression of several DC markers (e.g., MHC II and costimulatory molecules) and the capacity to present exogenous antigens[277]. This mechanism could explain why, although less B1 B cells and N_{BH} cells colonized the spleen, the amount of TNP-reactive antibodies was similar in *Grn*$^{-/-}$ and WT mice.

PGRN regulates dendritic cell function

We determined that human DCs were able to cleave full-length PGRN into GRNs and that SLPI impeded the cleavage. So far, only neutrophils and microglias have been shown to be able to process PGRN. Neutrophils use principally elastase and chymotrypsine to hydrolyze PGRN[203], whereas macrophage and microglias use the matrix metalloproteinase (MMP)-12[214]. Monocyte-derived DCs express membrane MMP-1, MMP-9 and MMP-12, all candidate serine proteases[278,279]. New experiments are required to determine which proteases are responsible for PGRN cleavage in human DCs.

We observed that in unstimulated mouse CD11c^{+} DCs, PGRN did not alter cell activation, since *Tnf*, *Il-6*, *Il-10* and *Il-12p40* mRNA expression was comparable in WT and *Grn*$^{-/-}$ mice. In accordance, *in vitro* addition of PGRN to human DCs left unstimulated did not have any effect on their phenotype or cytokine secretion (data not shown).

These observations in mice suggest that in the absence of PGRN either other mechanisms or the simultaneous deficiency of both PGRN and GRNs might guarantee the preservation of intact

immune responses. Indeed, *Grn*$^{-/-}$ mice have a normal phenotype, do not show morphological, hematologic (except for neutrophil compartment), or biochemical abnormalities[224].

Human spleen is continuously exposed to antigens from commensal or pathogenic microbes, even in the absence of infection. Macrophages, DCs and neutrophils of the innate immune system are the first cells that encounter such type of antigens and are required to provide an efficient immunosurveillance of the circulatory system without inducing inflammation[4].
Human DCs treated with PGRN and then exposed to low doses of LPS are capable of upregulating maturation markers such as CD40, CD80, CD86 at the same extent than untreated DCs. By contrast, the gene expression and the secretion pattern of pro-inflammatory cytokines including TNF and IL-12p70 resulted highly compromised. Our results were comparable to data obtained in microglial cultures exposed to LPS, where PGRN siRNA reduces the amount of cytokines including TNF, IP-10, IL-6, and possibly IL-1β[214]. Similarly, mouse bone marrow-derived macrophages from *Grn*$^{-/-}$ mice stimulated with different TLR agonists had similar cell-surface marker expression and phagocytic capacity than WT, but produce higher levels of transcripts and proteins for pro-inflammatory chemokines and cytokines[224]. In those settings, authors argued that PGRN synergizes with LPS in inducing IL-10 expression and that IL-10 serves as a negative regulator of inflammatory responses. We also observed that PGRN increased IL-10 secretion in human DCs matured with low doses of LPS.

On the other hand, PGRN was shown to compete with TNF for the binding to TNF receptors, both TNFR1 and TNFR2[226]. Production

of TNF is a hallmark of the inflammatory response to pathogen exposure that coordinates innate and adaptive immune responses by functioning in an autocrine or paracrine manner[280]. The presence of PGRN could impede the binding of TNF to the receptors, thus hampering the amplification of the inflammatory reaction.

The modulation of DC activation mediated by PGRN is not due to a decrease in cell viability, that is comparable in PGRN-treated and untreated DCs. However, several works report that PGRN possesses pro-survival and pro-proliferative properties, since it increases the viability of cultured neurons[281], is required for tumor growth in some breast cancer lines[282], stimulates the proliferation and migration of endothelial cells[223] and favors angiogenic processes in vivo[283].

We observed that PGRN interfered with LPS-induced cytokine production in human DCs. Moreover, LPS induced TNF and the binding of TNF to either TNFR1 or TNFR2 can ultimately activate NF-κB. Based on these observations, PGRN could be involved in both TLR4- and TNF-induced signaling pathways.
PGRN and the antagonist of TNF/TNFR signaling via targeting to TNF receptors (Atsttrin, a recombinant mutant form of PGRN that cannot be cleaved) blocked TNF-induced phosphorylation of IKK and IκBα, degradation of IκBα, NF-κB nuclear translocation, phosphorylation of p38, JNK (c-Jun N-terminal kinase), and ERK1/2 (extracellular signal–regulated kinase 1/2) in bone marrow-derived macrophages[226]. In human DCs, we could detect only a partial PGRN-mediated inhibition of NF-κB translocation induced by LPS. This reduction cannot account for the high difference we

found in the secretion of NF-κB-dependent cytokines such as IL-12, comparing PGRN-treated and untreated DCs. Our analysis was done at early time point (90 minutes) and an explanation could be that higher inhibition of NF-κB translocation could occur later, once IL-10 reach enough regulatory levels, or TNF is not more allowed to bind to its receptors. EMSA assay performed at later time points could help to understand the rate of PGRN-mediated inhibition.

Alternatively, PGRN might activate other transcription factors, such as CREB. CREB promotes anti-inflammatory immune responses, by inhibiting NF-κB activity and inducing the secretion of IL-10 and the generation of Tregs cells[284,285].
That PGRN was able to induce CREB was suggested in a light-induced retinal-damage model in mice, where PGRN secreted by adipose-derived stem cells enhanced phosphorylation of the hepatocyte growth factor (HGF) receptor, and activated CREB via the PKC pathway[286]. Activation of CREB by PGRN during immune responses has still to be investigated.

PGRN influences dendritic cell capacity to prime $CD4^+$ T cells

IL-12 from TLR-activated DCs induces the transcription factor STAT4, which enhances IFN-γ production by Th1 cells[287]. Moreover, DC secretion of IL-10 can act in autocrine and paracrine manner and not only inhibits pro-inflammatory Th1 activity[288] but can also induce both suppressive[289] and Th2 T cell phenotype[179].
Both naive and memory $CD4^+$ T cells cultured with PGRN-treated DCs secreted less IFN-γ than T cells cultured with untreated DCs,

reflecting the decrease secretion of IL-12 and increased production of IL-10 by PGRN-treated DCs. In addition, memory $CD4^+$ T cells also produced more IL-10 when cultured with DCs pre-exposed to PGRN. Similarly, $CD4^+$ T cells from *Grn*$^{-/-}$ mice produced less IL-10 than WT $CD4^+$ T cells. IL-10 is a cytokine produced at different levels by several Th-cell subsets including Th1, Th2, Th17, and Treg cells and is a critical component in preventing excessive inflammation in response to commensal and pathogenic bacteria. WT but not IL-10$^{-/-}$ $CD45RB^{low}CD4^+$ T cells were able to prevent $CD45RB^{high}$ $CD4^+$ T cell-induced colitis[290,291]. Moreover, T-cell–specific deletion of IL-10 leads to enhanced pro-inflammatory cytokine secretion and decrease survival after *T. gondi*[292] and *Plasmodium chabaudi*[293] infection.

Recent studies analyze the direct effect of PGRN on T cell activation and function. Lack of PGRN signaling in $CD4^+$ T cells exacerbates experimental colitis[294]. In addition, PGRN protected human Treg from a negative regulation by TNF and promoted the differentiation of Treg from naïve T cells. Furthermore, PGRN down-regulated IFN-γ secretion in TNF-activated effector T cells[226]. PGRN is required also for the proliferation[259] and immunosuppressive function of mouse Tregs[295]. Of note, PGRN is important for the Tregs formation under inflammatory conditions, and does not influence their development under normal immune homeostasis[295] (and data not shown). Our results provide a new indirect mechanism through which PGRN can modulate T cell activation. Additional experiments are required to ascertain whether DC-treated DCs are able to induce Treg cells.

PGRN enhances post-immune TD antibody responses

When we immunized *Grn*$^{-/-}$ mice with the TD antigen TNP-OVA and the adjuvant SAS, we observed that the production of TNP-specific IgG1 and IgG2c was lower compared to WT mice. By contrast, *Grn*$^{-/-}$ mice generated more TNP-specific IgM than WT. The affinity of the IgG antibodies generated during the immune response was also reduced in the absence of PGRN.

These results suggest that PGRN might promotes IgG class switching, affinity maturation and antigen-specific antibody production.

Although the frequency of Tfh cells was comparable in both strains, PGRN deficiency led to an increase in the percentage of DCs (both CD11b^{+} DCs and CD8^{+} DCs), GC B cells and PCs. These data reflect the anti-inflammatory role of PGRN. Indeed, they support the hypothesis that PGRN is important to avoid the outgrowth of non-antigen-specific B cells in GC that would lead to fewer antigen-specific cells.

Either T or B cell-derived TNF is necessary for the formation of primary B cell follicles, FDC networks and GC[296,297]. In this context, the absence of PGRN could exacerbate TNF signalling and generate an overexpansion of GC B cells and PCs.

Although studies providing direct evidence for DC–B cell interactions in vivo exist, the contribution of individual DC subsets to B cell activation and subsequent antibody responses have never been investigated in depth. MZ-associated DCs, expressing the DC-inhibitory receptor 2 (DCIR2), are capable to prime B cells and initiate extrafollicular Ab responses to TD antigen[298].

On the other hand, Linterman and collegues identified in the GC a subset of Tfh cells, called follicular regulatory T (Tfr) cells that are

generated in response to TD antigens. They control Tfh cell and GC B cell expansion and inhibit the selection of non-antigen-specific B cells including those carrying self-reactive receptors[299]. Since we observed that the percentage of $Foxp3^+$ Treg cells was reduced in *Grn*$^{-/-}$ after immunization, and based on the data already existing on the involvement of PGRN in Treg cell differentiation and function, we postulate that PGRN may also affect the generation of Tfr cells. This hypothesis would explain the decrease generation of antigen-specific antibodies and would suggest that PGRN is fundamental for the antibody-mediated responses against infection.

We need to specify that in the described animal model we cannot discriminate between PGRN and GRN functions, since both components are supposed to participate in TD antibody reactions. We can speculate that immune responses are regulated in a manner similar to that previously described for wound repair by Zhu et al.[203] According to this hypothesis, GRN peptides might be involved in the initiation of the immune reaction in reponse to pathogens, by inducing the formation of a GC and the production of antibodies with high affinity for the antigen. In contrast, PGRN might regulate the humoral reponse by avoiding the outgrowth of non-antigen-specific B cells in GC through the induction of Tfr cells or by modulating IL-6 and TNF secretion in immune cells like DCs or neutrophils[296,300]. The balance between PGRN and GRN peptides might depend on the level of splenic SLPI. The discrete contribution of PGRN and GRNs could be clarified by exogenous administration of Atsttrin to immunized PGRN-deficient mice.

Absence of PGRN was previously shown to lead to a decreased ability to clear *Listeria monocytogenes* infection, with prolongation

of inflammation in the brain[224]. However, the authors did note investigate the link between PGRN and the humoral component of the immune response. Moreover, PGRN expression was induced significantly in gastric epithelial cells after infection with *Helicobacter pylori*[301], a condition where GCs and PCs are detected in the lamina propria.

$Grn^{-/-}$ mice also presented less percentage and number of neutrophils in their spleen. However, the role of neutrophils in TD antibody responses is still largely unknown. Neutrophils can influence DC capacity to prime T cells through the secretion of maturation-inducing cytokines such as TNF[59]. Neutrophils also release IL-12, which promotes the polarization of naive T cells into IFN-γ releasing inflammatory Th1 cells[302].
Neutrophil depletion in congenic lupus-prone mice decreases the frequency of IFN-γ producing T cells, GC B cells, and autoantibody production. The same authors found that neutrophils localize within T-cell zones and contribute to T cell responses through a BAFF-dependent mechanism[303]. Neutrophils can also induce adaptive reponses by migrating from inflamed skin to the draining lymph nodes where they induce T cell proliferation[304]. Reduction in neutrophils could thus be an additional mechanism to explain the observed decrease of antigen-specific antibodies. However, further studies are required to support this hypothesis.

In conclusion, we propose a scenario where PGRN may function as an endogenous adjuvant that bridges the humoral arms of the innate and adaptive immune systems. This property could be harnessed to enhance vaccine-induced TI and TD antibody responses against pathogenic determinants.

CHAPTER VI

CONCLUSIONS

We can conclude from this study that:

- PGRN and SLPI are highly expressed by several cell types that populate human and mouse spleen, including B cells, macrophages and neutrophils.

- PGRN is important for neutrophil activation, since it controls the secretion of pro-inflammatory citokines such as TNF and IL-12.

- PGRN controls the frequency of bone marrow, peripheral (N_C cells), and splenic (N_{BH} cells) neutrophils, by possibly regulating their generation, survival or recruitment.

- PGRN favors pre-immune IgM antibody responses against TI antigens.

- PGRN does not alter post-immune IgM and IgG3 antibody responses against TI antigens, although it modulates the composition of myeloid (neutrophils and DCs) and lymphoid (B1 B cells) compartments upon immunization.

- PGRN has an anti-inflammatory effect on the cytokine release and function of DCs stimulated by LPS, partially due to NF-κB inhibition.

- PGRN-treated DCs, compared to untreated DCs, induce lower IFN-γ secretion and higher IL-10 production in $CD4^+$ T cells.

- PGRN enhances post-immune IgG antibody responses and alters the composition of myeloid and lymphoid compartments in response to TD antigen, by regulating GC B cell, PC, neutrophil, DC and Treg cell frequency.

Based on the above considerations, we propose the following model:

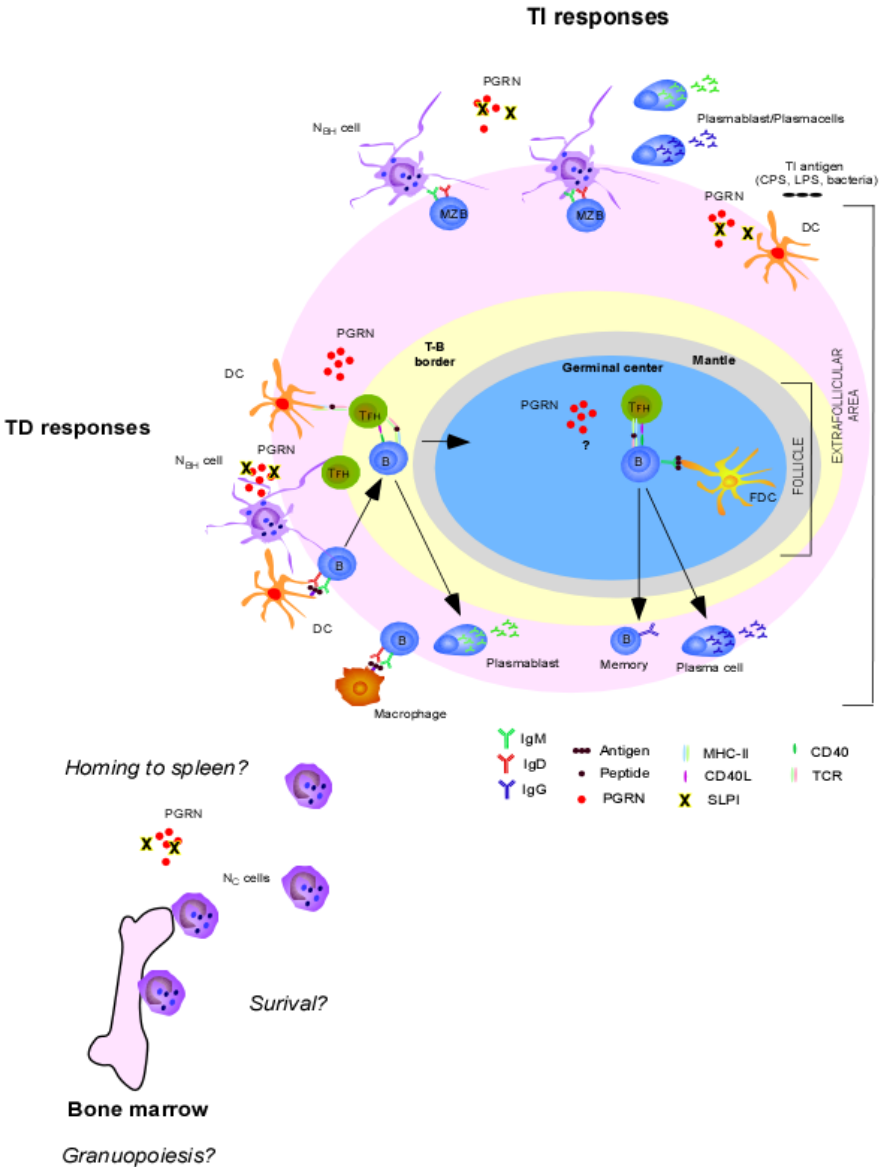

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Figure 1. Model describing the proposed functions of PGRN in humoral immune responses in spleen. PGRN is expressed in the follicles, in the MZ and in the red pulp of human spleen, where it modulates both TI and TD responses.

(1) TI responses. In the MZ, a site continually exposed to antigen, splenic neutrophils (N_{BH} cells) induce homeostatic MZ B cell responses to maintain a noninflammatory environment[1,64,253].

PGRN increases neutrophil frequency both in steady state and after immunization with TI and TD antigens and may do it by enhancing bone marrow granulopoiesis, both directly or indirectly through SLPI. Alternatively, PGRN could increase the survival of circulating neutrophils (N_C) or N_{BH} cells or recruit neutrophils to spleen by increasing the secretion of chemokines such as IL-8 in DCs, SLCs or macrophages. As a consequence, N_{BH} cells may cooperate with ILCs and stromal cells to create multi-component niches for the activation, differentiation and survival of both MZ B cells and PCs, in order to generate pre-immune (or natural) antibodies to circulating commensal and self-antigens under homeostatic conditions[1,64]. On the other hand, PGRN also regulates the frequency of MZ $CD11b^+CD11c^+$ DCs that are essential to support the expansion of MZ B cells and to promote early immune responses against T cell-independent particulate antigens[84]. PGRN may thus maintain a balance among MZ B cell helper subsets, including MZ DCs and N_{BH} cells, in order to provide an efficient but tightly regulated antibody response.

(2) TD responses. TD responses recruit either follicular or, less frequently, MZ B cells and involve the activation of a GC reaction that selects high-affinity memory B cells through a complex pathway requiring Tfh cells and FDCs. PGRN may regulate the expansion of Tfh, GC B cells and PCs to control the strength and the magnitude of the humoral response and to induce the production of antigen-specific antibodies in response to pathogens. PGRN may controls these responses in several manners. PGRN may dampen DC and T

cell activation by reducing secretion of pro-inflammatory cytokines and increasing the release of the immunoregulatory IL-10 in DCs and DC-primed T cells, or by inducing the generation of Tfr cells. Alternatively, PGRN may regulate TD responses by increasing granulopoiesis, neutrophil survival or migration to secondary lymphoid organs, where neutrophils can directly or indirectly induce T cell activation.

ANNEX I

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ANNEX II

Abbreviations

AD	Alzheimer's disease
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ASC	Antibody secreting cell
BAFF	B-cell activating factor
BCL-6	B cell lymphoma 6
BCMA	B cell maturation antigen
BCR	B cell receptor
BLIMP1	B limphocyte-induced maturation protein 1
CD40L	CD40 ligand
cDC	Classical DC
CLR	C-type lectin receptor
CpG	Deoxyribocytidinephosphateguanosine
CRD	Carbohydrate-recognition domain
CRD	cysteine-rich domain
CSR	Class switching recombination
CTL	Cytotoxic T lymphocyte
CVID	Common variable immunodeficiencies
DC	Dendritic cell
DLL1	Notch ligand Delta-like 1
dsRNA	Double-strand RNA

ESAM	Endothelial cell-specific adhesion molecule
FasL	Fas ligand
FDC	Follicular dendritic cell
FTD	Frontotemporal dementia
GC	Germinal center
GEP	Granulin-epithelin precursor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRN	Granulin
H	Heavy
HDL	High density lipoprotein
ICOS	Inducible T-cell co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
iNKT	Invariant NKT
IRA	Innate response activator
LLR	Leucine-rich repeat
LPS	Lipopolysaccharide
LT-DC	Lymphoid tissue-resident DC
LTi	Lymphoid tissue-inducer
MALT	Mucosal -associated lymphoid tissue
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MHC	Major histocompatibility complex
Mig-DC	Migratory DC

MMM	Metallophilic macrophage
MMP	Matrix metalloproteinase
MMR	Macrophage mannose receptor
MPO	Myeloperoxidase
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
Myd88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
MZM	Marginal zone macrophage
N_{BH}	B cell-helper neutrophils
NET	Neutrophil extracellular trap
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	(NOD)-like receptor
NOD	Nucleotide oligomerization domain
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen associated molecular pattern
PB	Plasmablast
PC	Plasma cell
PCDGF	PC cell-derived growth factor
pDC	Plasmacytoid DC
PD-1	Programmed cell death-1
PEPI	Proepithelin
PGRN	Proganulin
PI3	Phosphatidyl inositol-3

PIK3	Phosphatidylinositol 3-kinase
PRR	Pattern recognition receptor
RA	Retinoic acid
RLR	Retinoic-acid-inducible gene 1-like receptors
ROS	Reactive oxygen species
SAS	Sigma Adjuvant System
SCN	Severe congenital neutropenia
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
SLPI	Secretory leukocyte protease inhibitor
ssRNA	Single-strand RNA
TACI	Transmembrane activator and CAML interactor
TCR	T cell receptor
TD	T cell dependent
Tfh	T follicular helper
Th	T helper
TI	T cell independent
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF receptor associated factor
Treg	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
V(D)J	Variable diversity joining segments

ANNEX III

List of publications

Original articles

1. G. Magri, M. Miyajima, S. Bascones, A. Mortha, I. Puga, L. Cassis, C. M. Barra, L. Comerma, A. Chudnovskiy, M. Gentile, D. Llige, M. Cols, S. Serrano, J. I. Aróstegui, M. Juan, J. Yagüe, M. Merad, S. Fagarasan, and A. Cerutti, “Innate lymphoid cells integrate stromal and immunological signals to enhance antibody production by splenic marginal zone B cells.,” Nat. Immunol., vol. 15, no. 4, pp. 354–64, Apr. 2014.

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6. E. Ripoll, R. Pluvinet, J. Torras, R. Olivar, A. Vidal, M. Franquesa, L. Cassis, J. M. Cruzado, O. Bestard, J. M. Grinyó, J. M. Aran, and I. Herrero-Fresneda, “In vivo therapeutic efficacy of intra-renal CD40 silencing in a model of humoral acute rejection,” Gene Ther., vol. 18, no. 10, pp. 945–952, Apr. 2011.

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Reviews

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Book chapter

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