Purinergic Regulation of Adaptive Immune System

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Table of Contents

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Abstract

The importance of extracellular ATP for cell-to-cell communication in the nervous and vascular systems has been deeply studied for years, while less characterized is its role in regulating immune responses. Extracellular ATP is sensed by a class of ubiquitously expressed plasma membrane receptors termed P2 purinergic receptors (P2Rs), whose activation elicits different responses. Furthermore, extracellular ATP is rapidly degraded by ectonucleotidases such as CD39 and CD73 into adenosine, which in turn exerts additional regulatory effects through its own P1 receptors. In the first part of my PhD I addressed the role of ATP-gated ion channel P2X7 receptor in regulating T follicular helper (Tfh) cells functions. Tfh cells support affinity maturation of B cells in germinal centers (GCs) and differentiation to IgA-secreting plasma cells in gut-associated lymphoid tissues (GALTs). This function is critical in maintaining intestinal homeostasis and efficient mucosal defense. We have shown that P2X7 receptor is selectively and robustly upregulated in Tfh cells isolated from chronically stimulated secondary lymphoid organs, such as Peyer's patches (PPs) and mesenteric lymph nodes. Deletion of *P2rx7* in Tfh cells resulted in resistance to cell death, enhanced GC reaction in PPs, increased IgA binding to commensals, and reduction of mucosal bacteria. Our results unravel P2X7 as a regulator of the adaptive IgA response in the small intestine to allow commensalism and systemic stimulation of the immune system.

In the second part of my PhD I addressed the role of the ectonucleotidases CD73 in the human B cell memory generation. It was previously shown from my host lab that peripheral human B cells co-expressing CD73 and CD39 have a more prompt capacity to expand and differentiate into antibody secreting cells *in vitro*. This property has been linked to the hydrolyzing enzymatic function of CD39 and CD73, which in tandem generates adenosine starting from extracellular ATP. We developed a method to functionally dissect antibody repertoire in different human B cell subsets differentiated according to CD73 expression and identified a novel subset of antigen-experienced B cells. We have shown for the first time that human peripheral $IgM^+IgD^+CD27^-$ B cells lacking expression of CD73 carry somatically mutated variable region genes, in contrast to $CD73^+$ counterpart. Furthermore, $IgM^+IgD^+CD27^-CD73^-$ B cells were less prone to perform class switch recombination *in vitro* and showed comparable functional properties with GC-independent memory B cells. Collectively, these results reveal CD73 as a novel human B cell marker that identifies functional distinct populations with different genetic hallmarks and a distinct differentiation fate.

Acronyms and abbreviations

ADA – adenosine deaminase

ADP – adenosine diphosphate

AID – activation-induced cytidine deaminase

AIRE – autoimmune regulator gene

AK – adenosine kinase

AMP – adenosine monophosphate

APC – antigen presenting cell

ATP – adenosine triphosphate

Bcl6 – B cell lymphoma 6

BCR – B cell receptor

Blimp-1 – B-lymphocyte-induced maturation protein 1

BM – bone marrow

CIA – collagen-induced arthritis

CLP – common lymphoid progenitor

CLR – C-type lectin receptor

CSR – class switch recombination cTECs

– cortical thymic epithelial cells

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

DAG – diacylglycerol

DAMP – danger associated molecular pattern

DC – dendritic cell

DN – double negative

DP – double positive

DZ – dark zone

EAE – experimental autoimmune encephalomyelitis

FDC –follicular dendritic cell

FO – follicular B cell

GALT – gut-associated lymphoid tissue

GC – germinal center

HEV – high endothelial venule
HIF – hypoxia-inducible factor

HIGM – hyper-IgM syndrome

HSC – hematopoietic stem cell

IgH – immunoglobulin heavy chain

IgL – immunoglobulin light chain

IP3 – inositol trisphosphate

ITAM – immunoreceptor tyrosine-based activation motif

JIA – juvenile idiopathic arthritis

LLPC – long-lived plasma cell

LZ – light zone

MHC – major histocompatibility complex mTECs

– medullary thymic epithelial cells

MZ – marginal zone

NFAT – nuclear factor of activated T cells

NFkB – nuclear factor kB

NLR – NOD-like receptors

PAMP – pathogen-associated molecular pattern

PC – plasma cell

PIP2 – phosphatidylinositol 4,5-bisphosphate

PKC – protein kinase C

PP – Peyer's patch

PRR – pattern recognition receptors

SFK – Src-family kinase

SFMC – synovial fluid mononuclear cell

SLAM – signaling lymphocytic activation molecule

SLE – systemic lupus erythematosus

SP – single positive

STAT – signaling transducer and activator of transcription

T1D – type-1 diabetes

TCM – T central memory cell

TCR – T cell receptor

TD – thymus dependent

TdT – terminal deoxynucleotidyl transferase

TEM – T effector memory
Tfh – follicular helper T cell

Tfr – follicular regulatory T cell

Th – T helper cell

TI – thymus independent

TLR – toll-like receptor

TNF – tumor necrosis factor

Tph – peripheral helper T cell

TRA – tissue restricted antigen

Treg – regulatory T cell

TRM – tissue-resident memory T cell

TSSP – thymus-specific serine protease

WT – wild-type

1. Introduction

1.1 Overview of innate and adaptive immune systems and their interplay

The immune system is composed of a complex network of cells and molecules that are responsible for generating a response against any type of foreign substances. Historically immunity meant protection from infectious diseases, but research over the last several years has shed light on the additional roles of the immune system in patrolling cancer cells and, in some cases, generating a detrimental response against self-antigens which could finally lead to emergency of autoimmune diseases. To exploit this complex function the immune system has evolved two main cellular and molecular components: the innate and adaptive immunity (Figure 1). The innate immune system, which is in place before infection and is poised to respond rapidly, relies on cells expressing a limited number of germline-encoded receptors to recognize the molecular patterns of common pathogens. The basic protective strategy of the innate immune system is to constitutively produce generic receptors that recognize conserved patterns on different classes of pathogens in order to trigger an inflammatory response that limits pathogen invasion (Janeway and Medzhitov 2002, Hoffmann 2003, Akira *et al.* 2006). Twenty years ago, Janeway proposed a simple and revolutionary hypothesis that the innate immune system senses microbial infection using receptors that are predominantly expressed on sentinel cells referred to as 'pattern recognition receptors' (PRRs) that recognize the molecular signature known as 'pathogen-associated molecular patterns' (PAMPs) (Janeway 1989). Despite the fast and specific pathogen response, the early innate systems of defense do not lead to immunological memory making crucial the contribution of the adaptive immunity (Ausubel 2005). There are two types of adaptive immune responses, called humoral immunity, mediated by B lymphocytes and cell-mediated immunity, which is mainly regulated by T lymphocytes. These cellular subsets display on the surface an antigen receptor, which is clonally restricted and generated through the somatic diversification of antigen-receptor genes to generate a vast repertoire of cells, each of which expresses a different antigen receptor (Cooper and Alder 2006). The adaptive immune response is initiated when the innate immune system fails to eliminate the infection; therefore, activated antigen-presenting cells and the antigen are delivered to the draining lymphoid tissues. When a recirculating lymphocyte encounters its specific foreign antigen in peripheral lymphoid tissues, it is induced to proliferate and its progeny then differentiate into effector cells that can eliminate the infectious agent. A subset of these proliferating lymphocytes differentiates into memory

cells, ready to respond rapidly to a second exposure to the same pathogen. The effective development of the overall immune response depends on careful interplay and regulation between innate and adaptive immunity (Abbas *et al.* 2012, Murphy and Weaver 2016).

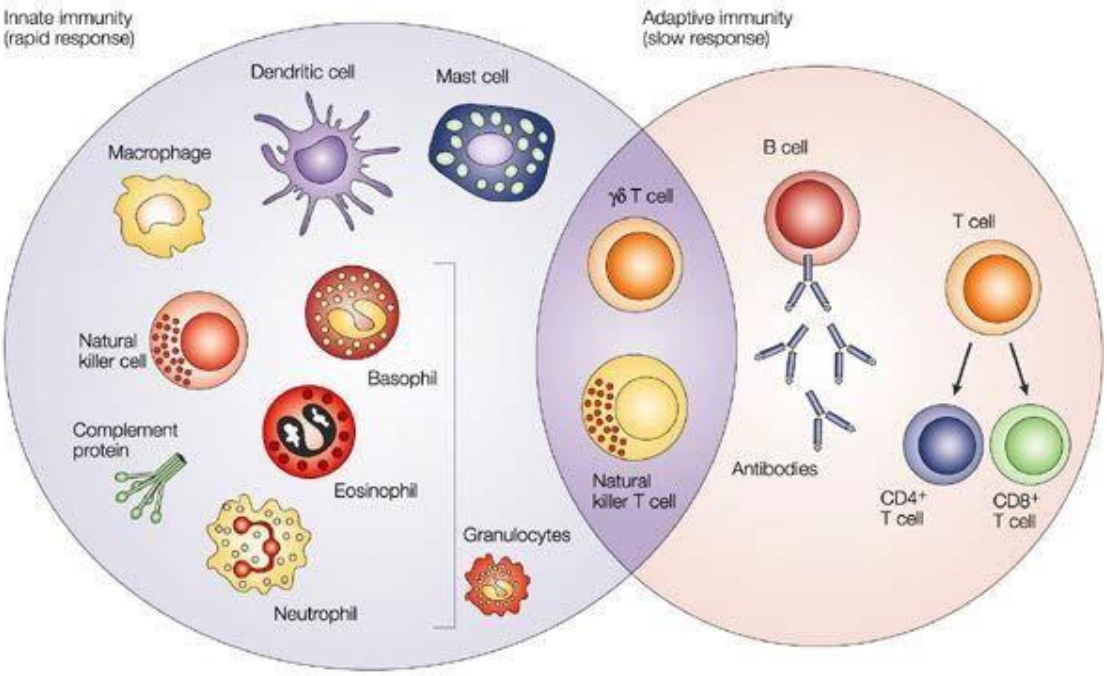

Figure 1. Overview of innate and adaptive immune system. *The innate immune response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity* (Dranoff 2004).

The clonal selection of lymphocytes provides a theoretical framework for understanding all the key features of adaptive immunity, like the ability to recognize all pathogens specifically and to provide enhanced protection against reinfection. Each lymphocyte carries cell-surface receptors of a single specificity, generated by the random recombination of variable receptor gene segments and the pairing of different variable chains. This molecular process ensures that the total repertoire of receptors can recognize virtually any antigen. T and B lymphocytes use their cell surface receptor to recognize antigenic configurations of specific pathogens and respond to antigen triggering by clonal amplification, cellular differentiation and production of antibodies with the same antigen binding specificity (Burnet 1959).

Despite the division of labor existing between innate and adaptive immune system, the tight interplay between the two systems is now emerging. Specifically, PRRs have been shown to be expressed by B and T lymphocytes and intriguingly generate complementary responses (Bernasconi *et al.* 2003, Imanishi *et al.* 2007).

1.2 T lymphocytes

Generation of T lymphocytes

T lymphocyte development is a process occurring early in life in the thymus, a mediastinal organ that enlarges during childhood and undergoes atrophy at puberty. The thymus has a bi- lobar structure, each lobe being composed by multiple lobules organized in an outer cortical and an inner medullary region (Abbas *et al.* 2012, Murphy and Weaver 2016). Multipotent lymphoid progenitors are generated from hematopoietic stem cell precursors in the bone marrow (BM) from which they continuously emigrate to reach the thymus. Within the thymus a complex sequence of events occurs that leads to the generation of a large pool of mature T lymphocytes each expressing a unique T cell receptor (TCR) that can recognize a specific peptide bound to major histocompatibility complex (MHC) molecules displayed on the surface of antigen presenting cells (APCs) (Takahama 2006). The stages of development of the major lineages of T cells are delineated by the surface expression of the co-receptor molecules CD4 and CD8. The earliest precursors, $CD4^{-}CD8^{-}$ double negative (DN) cells proliferate extensively and become

$CD4^{+}CD8^{+}$ double positive (DP) cells, which are still immature and are the targets for TCRbased selection events. The *positive selection* process takes place in the cortex of the thymus and is mediated by specialized cortical thymic epithelial cells (cTECs) which express high amounts of MHC class II molecules on their surface and are endowed with distinguishing antigen processing

machinery (Jameson *et al.* 1995). In fact, they possess a unique catalytic subunit of the proteasome, called β5t, and lysosomal proteases (cathepsin L and thymus-specific serine protease, TSSP) that determine their unique array of MHC-displayed peptides. Moreover, cTECs display an high rate of constitutive macroautophagy, through which self-proteins from endogenous cell compartments can be presented on MHC class II molecules (Klein *et al.* 2014). Positively selected T cells then migrate to the medulla, where approximately 5% die by apoptosis because of a very strong interaction between the TCR and self-peptide-MHC complexes. This process, known as *negative selection*, is essential to purge the T cell repertoire from potentially autoreactive clonotypes (Baldwin *et al.* 1999). Negative selection is primarily carried out by medullary thymic epithelial cells (mTECs); through the autoimmune regulator gene (AIRE) mTECs can ectopically express a broad set of tissue restricted antigens (TRAs), and therefore present self-antigens which would not otherwise be available for screening of auto-reactive T cells (Anderson *et al.* 2002, Liston *et al.* 2003). Low-affinity interactions between the newly expressed TCRs and self–peptide–MHC molecules select a broad repertoire that will be refined to react strongly to pathogen-derived peptides bound to the same MHC in the periphery. Thymocytes at the single positive (SP) stage are finally ready to leave the medulla and reach the peripheral lymphoid sites. $CD8^+$ T cells recognize peptides bound to MHC class I molecules which are expressed by virtually all nucleated cells in the body and generally present peptides derived from intracellular proteins. $CD4^+$ T cells express TCRs that instead bind peptides bound on MHC class II molecules, which are expressed on the surface of professional APCs, such as dendritic cells (DCs), B cells, macrophages, and monocytes and generally present peptides derived from extracellular antigens (Figure 2) (Germain 2002, Abbas *et al.* 2012, Murphy and Weaver 2016).

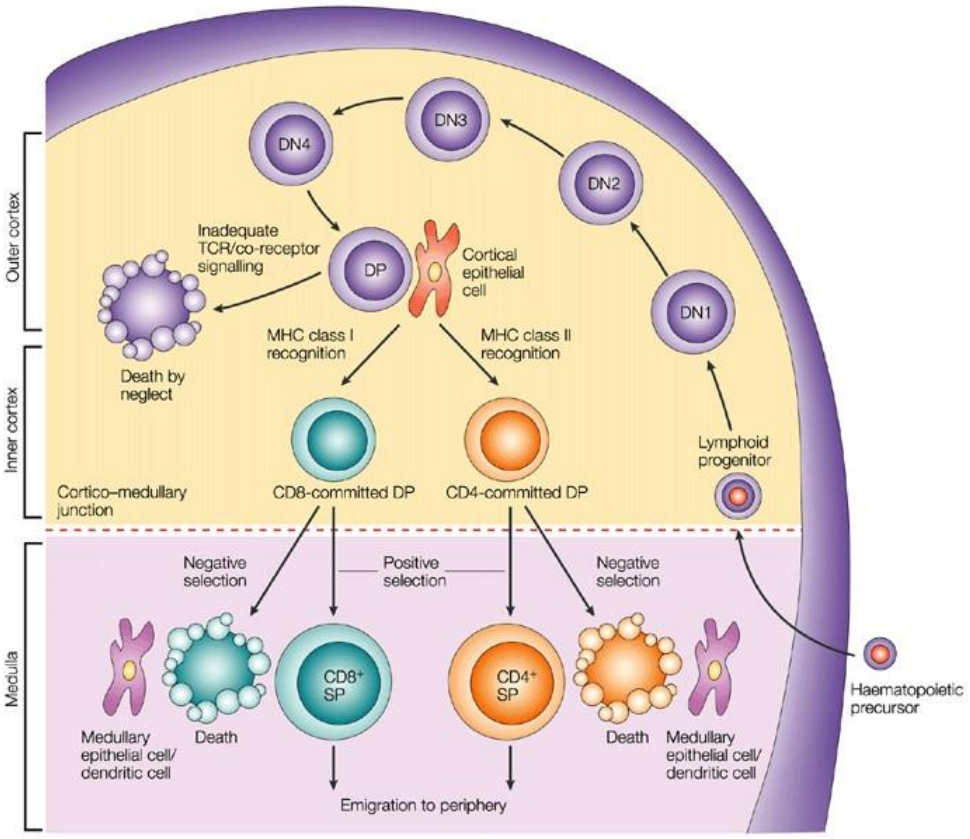

Figure 2. Overall scheme of T-cell development in the thymus. *Committed lymphoid progenitors arise in the BM and migrate to the thymus. Early committed T cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1, CD44$^+$CD25$^-$; DN2, CD44$^+$CD25$^+$; DN3, CD44$^-$CD25$^+$ and DN4, CD44$^-$CD25$^-$). As cells progress through the DN2 to DN4 stages, they express the pre-TCR, which is composed of the non-rearranging pre-Tα chain and a rearranged TCR β-chain. Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) transition and replacement of the pre-TCR α-chain with a newly rearranged TCR α-chain, which yields a complete αβ TCR. The DP thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides. The fate of the DP thymocytes depends on signaling that is mediated by interaction of the TCR with these selfpeptide–MHC ligands. The appropriate, intermediate level of TCR signaling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide–MHC-class-I complexes become CD8$^+$ T cells, whereas those that express TCRs that bindself-peptide–MHC-class-II ligands become CD4$^+$ T cells; these cells are then ready for export from the medulla to peripheral lymphoid sites* (Germain 2002).

Early events of CD4 T cell activation

TCR activation promotes a number of signaling cascades that ultimately determine cell fate through regulating cytokine production, cell survival, proliferation, and differentiation. The αβ TCR has no intrinsic enzymatic activity and transduction of the extracellular event into a cytosolic cascade is mediated by a complex composed of two CD3 molecules, each consisting of two chains, εδ and εγ respectively, and a homodimer of ζ chains. These invariant components are associated with the TCR through electrostatic interactions between oppositely charged residues in the transmembrane region (Call *et al.* 2002). All these invariant dimers possess immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular portion, which contain two tyrosine residues that upon phosphorylation provide a site for recruitment of Src-homology 2 (SH2) domain-bearing enzymes (Irving and Weiss 1991, Letourneur and Klausner 1992). The first event upon antigen recognition and TCR triggering is the activation of the Src-family kinases (SFKs), particularly Lck and Fyn are the first molecules initiating the signaling cascade (Brownlie and Zamoyska 2013). Lck is regulated like other SFKs, via its C-terminal negative regulatory tyrosine, which is normally not phosphorylated, rendering Lck in a so-called 'primed' state ready to be activated. During TCR-MHC/peptide interactions, Lck is recruited to the complex via its noncovalent association with CD4 co-receptor; the interaction with this co-receptor facilitates its participation in the initiation of TCR signal transduction (Turner *et al.* 1990). Active Lck phosphorylates the CD3 and ζ chain ITAMs, resulting in the recruitment of the ζ chain associated protein of 70 kDa (ZAP-70) kinase (Palacios and Weiss 2004). Subsequently, ZAP- 70 phosphorylates T-cell-specific adapters, such as LAT and SLP-76, leading to the recruitment and activation of other enzymes (Brownlie and Zamoyska 2013). The γ1 isoform of the enzyme phospholipase C (PLCγ1) for instance is recruited to the plasma membrane by the adaptor LAT and catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), which results in the production of inositol trisphosphate (IP3) and diacylglycerol (DAG). These second messengers are responsible for rapid and sustained increase in cytoplasmic free calcium and activation of protein kinase C (PKC), respectively (Weiss and Littman 1994). The rapid increase in cytosolic free calcium is thought to influence calcium/calmodulin-dependent events, like the activation of a serine/threonine phosphatase called calcineurin, which in turn activates the nuclear factor of activated T cells (NFAT) transcription factor (Macian 2005). Furthermore, the PKC is involved in the activation and nuclear translocation of the nuclear factor kB (NFkB) transcription factor (Vallabhapurapu and Karin 2009). As a result of this activation process, the T cells proliferate, produce effector

molecules, such as cytokines, and express membrane receptors critical for adhesion to target cells.

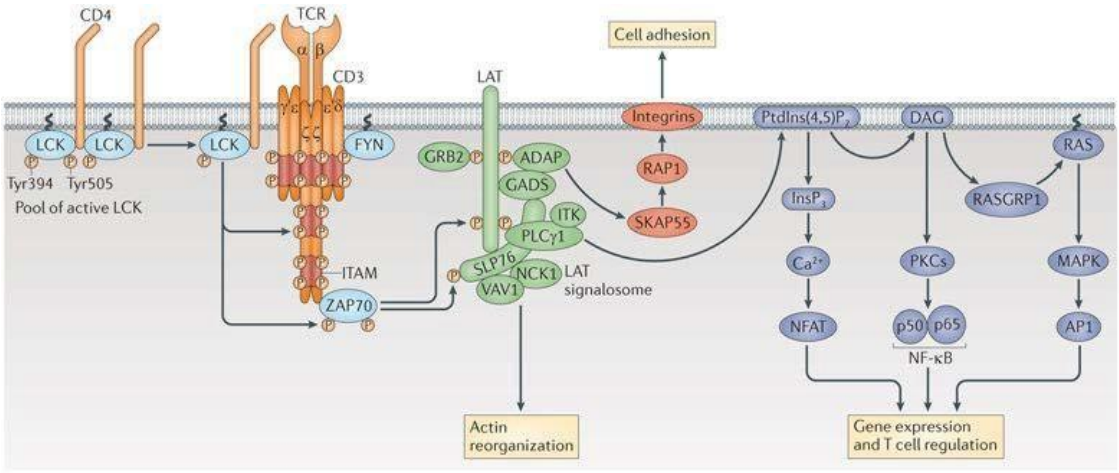

Figure 3. Overview of TCR signaling. *TCR signal transduction is initiated by the recognition of cognate peptide–MHC molecules. The first molecule to be recruited to the TCR–CD3 complex is the SRC family kinase (SFK) member LCK, which phosphorylates ITAMs of the CD3γ chain, CD3δ chain, CD3εchains and the ζ-chains. Phosphorylation of the ITAMs enables the recruitment of ZAP70, its phosphorylation by LCK and its activation. Activated ZAP70 phosphorylates four key tyrosine residues on linker for activation of T cells (LAT), which recruits numerous signaling molecules to form a multiprotein complex, termed the LAT signalosome. Important molecules that constitute this complex include phospholipase Cγ1 (PLCγ1), growth factor receptor-bound protein 2 (GRB2), GRB2-related adaptor protein GADS, SLP76 (SH2 domain-containing leukocyte protein of 76 kDa), adhesion- and degranulation-promoting adaptor protein (ADAP), interleukin-2-inducible T cell kinase (ITK), NCK1 and VAV. The LAT signalosome propagates signal branching to three major signaling pathways, the Ca^{2+}, the mitogen activated protein kinase (MAPK) kinase and the nuclear factor-κB (NF-κB) signaling pathways, leading to the mobilization of transcription factors that are critical for gene expression and essential for T cell growth and differentiation. Signals initiated from the TCR also result in actin reorganization and the activation of integrins by inside-out signaling* (Brownlie and Zamoyska 2013).

Immune responses are initiated in the T cell area of secondary lymphoid organs where DCs present antigens, taken up locally or in peripheral tissues, to naïve T cells. For the antigen recognition, the establishment of highly regulated contact between T cells and APCs is crucial. This is accomplished through the formation of the immunological synapse, a cellular structure that forms at the interface between a T cell and an APC cell that expresses the appropriate peptide–MHCII complexes. Within the synapse, a ring of adhesion molecules surrounds TCRs and costimulatory molecules (Dustin and Cooper 2000). During this interaction, DCs deliver

three different types of signals to the T cells: they present the antigen displaying peptides loaded on their surface MHC molecules (1st signal), provide co-stimulation through surface receptors (2nd signal), and produce cytokines (3rd signal). The strength and type of such signals, together with other environmental cues, instruct proliferating T cells toward specific effector and memory fates, acquiring functions that are believed to be optimal for the clearance of the eliciting pathogen (Lanzavecchia 1999). The first signal is delivered through the TCR when it engages an appropriate peptide–MHC complex. Upon TCR-peptide– MHC complexes ligation naïve T cells leave the quiescent G0 phase and enter the G1 phase, start producing IL-2 and synthesizing CD25, the α subunit of the IL-2 receptor, which greatly increases their sensitivity to the cytokine (Acuto and Michel 2003). The second signal is generally referred to as co-stimuli and is conferred by counter-receptors expressed by APCs or by soluble factors such as cytokines and chemokines. This signal is necessary for the functional expansion of naïve T cell clones: antigen presentation in the absence of costimulation leads either to an unresponsive state, defined as anergy, in which the T cell is refractory to any further stimulation, or to switch to a regulatory (tolerogenic) phenotype (Schwartz 2003). It is now accepted that a major T cell co-stimulatory pathway involves the CD28 molecule, which is constitutively expressed on naïve T cells, and its ligands B7.1 (CD80) and B7.2 (CD86) expressed at high levels by activated professional APCs. Other crucial pathways are regulated by co-stimulator ligand (ICOSL), whose receptor on T cells is ICOS and CD40 on B cells and DCs whose receptor on T cells is CD40 ligand (CD40L) (Figure 4). Both ICOS and CD40L are not expressed by naïve T cells and are upregulated upon TCR stimulation. CD28 triggering by B7.1 and B7.2 molecules increases the production of IL-2 which binds to the IL-2 receptor, promoting survival and expansion of T cells through the induction of anti-apoptotic factors, such as Bcl-xL (Boise et al. 1995). ICOS enhances proliferation and effector functions on T cells in an IL-2-independent fashion (Greenwald et al. 2005). The CD40-CD40L interaction is important for enhancing the antigen presentation properties of B cells and DCs and therefore indirectly promoting T cell proliferation (Grewal et al. 1997).

One important feature of the immune system is that each time an immune reaction is initiated regulatory mechanisms are in place to switch off and avoid a chronic inflammation. For this purpose, after activation T cells start to express inhibitory molecules, which dictate the termination of the immune response. One of the first inhibitory molecules discovered is cytotoxic T-lymphocyte-associated protein 4 (CTLA4), also known as CD152, it is expressed on activated T cells, especially regulatory T cells (Tregs), displaying high sequence similarity to CD28 (Walker 2013). This molecule functions as an immune checkpoint, down-regulating T cell

activation, possibly through competition with CD28 for binding to B7 molecules (of note, CTLA-4 affinity for those receptors is about 20 times higher). The CD28/CTLA-4 pathway is considered an internal mechanism, which controls the balance between T cell activation and self-tolerance, in a process that is influenced by the expression of two shared ligands (Lenschow *et al.* 1996, Greenwald *et al.*2005, Gardner *et al.* 2014).

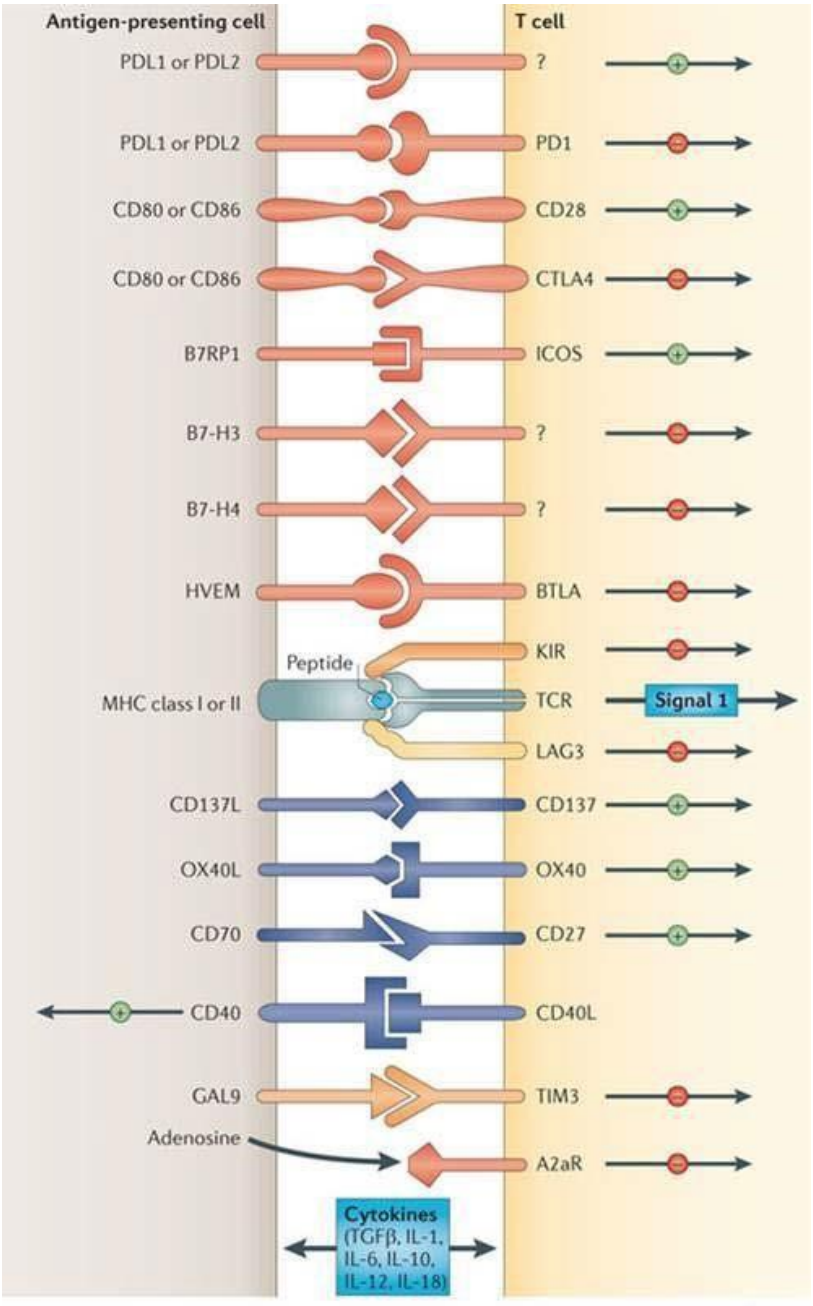

Figure 4. Ligand–receptor interactions between T cells and APCs that regulate the T cell response to antigen. *These responses can occur at the initiation of T cell responses in lymph nodes (where the major APCs are dendritic cells) or in peripheral tissues or tumors (where effector responses are regulated). In*

general, T cells do not respond to these ligand–receptor interactions unless they first recognize their cognate antigen through the TCR. Many of the ligands bind to multiple receptors, some of which deliver co-stimulatory signals and others deliver inhibitory signals. In general, pairs of co- stimulatory–inhibitory receptors that bind the same ligand or ligands — such as CD28 and CTLA4— display distinct kinetics of expression with the co-stimulatory receptor expressed on naive and resting T cells, but the inhibitory receptor is commonly upregulated after T cell activation. One important family of membrane-bound ligands that bind both co-stimulatory and inhibitory receptors is the B7 family. All of the B7 family members and their known ligands belong to the immunoglobulin superfamily. Many of the receptors for more recently identified B7 family members have not yet been identified. Tumor necrosis factor (TNF) family members that bind to cognate TNF receptor family molecules represent a second family of regulatory ligand–receptor pairs. These receptors predominantly deliver co- stimulatory signals when engaged by their cognate ligands. Another major category of signals that regulate the activation of T cells comes from soluble cytokines in the microenvironment. Communication between T cells and APCs is bidirectional. In some cases, this occurs when ligands themselves signal to the APC. In other cases, activated T cells upregulate ligands, such as CD40L, that engage cognate receptors on APCs (Pardoll 2012).

Dynamics of Naïve CD4 T cell priming

Once being released from primary lymphoid organs in the bloodstream, the majority of lymphocytes continuously recirculate through almost all compartments of the body to bring the entire range of antigen-specific immune cells into close contact with potential invading pathogens. In this context, the principal function of secondary lymphoid organs is to facilitate the physical contact between APCs and rare antigen-specific lymphocytes. Lymph nodes are encapsulated bean-shaped lymphoid organs located along the lymphatic system. Structurally they are subdivided into three main regions: the cortex, the paracortex and the medulla (Abbas *et al.* 2012, Murphy and Weaver 2016). The cortex contains densely packed B cells and follicular dendritic cells (FDCs) arranged into discrete B cell follicles. FDCs cluster in the centre of the follicle and form a dense network in which B cells search for antigens. By contrast, T cells accumulate in the T cell zone of the paracortex and continuously recirculate through lymphoid organs thanks to the expression of surface molecules such as CCR7 and CD62L, among others, which mediate extravasation upon binding of their ligands expressed on specialized afferent vessels, called high endothelial venules (HEVs) (Cyster 1999, Girard *et al.* 2012). The recognition of the appropriate antigen by incoming T cells leads to their expansion and differentiation into effector cells. Once inside the lymph node, in fact, naïve T cells are driven and kept in the T cell zone by a gradient of CCL18-CCL19-CCL21, produced by stromal cells as well as by DCs. If not activated, T cells leave the lymph node following upregulation of sphingosine 1-phosphate receptor-1 (S1P1), re-enter the circulation and resume their screening activity elsewhere (Rot and von Andrian 2004). By continuously moving, every single naïve T cell can screen in a few hours thousands of antigens, thus greatly increasing the chance to encounter its cognate one.

The main APCs involved in the T cell activation process are DCs and a critical event in the initiation of most immune responses is their migration from the infected peripheral tissue to the draining lymphoid organ, bearing antigens derived from the infecting agent. Discovered in 1973 by Steinman and Cohn, DCs are usually defined as cells with stellate morphology characterized by numerous dendrites, which allow efficient antigen sampling and phagocytosis from the extracellular space (Steinman and Cohn 1973). In the peripheral tissues, resident DCs can sense and take up pathogens as well as soluble antigens, as they can efficiently exert phagocytosis (Reis e Sousa *et al.* 1993), fluid phase and receptor mediated pinocytosis and macropinocytosis (Sallusto and Lanzavecchia 1994, Sallusto *et al.* 1995). The sensing and uptake are made possible by the impressive variety of phagocytic and pathogen-sensing receptors these cells are endowed with, such as C-type lectin receptors (CLRs), toll-like receptors (TLRs), NOD-like receptors (NLR) and scavenger receptors (Osorio and Reis e Sousa 2011). Following antigen recognition and uptake, DCs undergo a process known as activation or maturation, which fully enables them for stimulation of naïve T cells, as they undergo major changes in their biological activities (Reis e Sousa 2006). To ensure the stimulation of naive T cells, DCs require efficient directional migration toward T cell zones either within their respective lymphoid organ of residence or toward remote tissue-draining lymph nodes. Peripheral DCs migration via afferent lymphatics is CCR7 dependent (Förster *et al.* 1999) and the upregulation of this chemokine receptor selectively drives DCs through a gradient of chemo-attractant factor produced in the T cell area, mainly CCL19 and CCL21 (Sallusto *et al.* 1998). In this way, tissue resident DCs transport antigen to the draining lymph nodes and while migrating, they also upregulate costimulatory molecules such as CD80 and CD86, and initiate production of cytokines. T cell encounter of MHC-peptide on DCs that lack costimulatory molecules contributes to peripheral tolerance (Hawiger *et al.* 2001, Probst *et al.* 2003). Alternatively, the antigen can be delivered to the lymph node directly via the lymph, where it is taken up by resident DCs that become activated *in situ*. Lymph node-resident DCs can also present antigens handed over by tissue-derived migratory DCs (Allan *et al.* 2006). The process through which naive $CD4^+$ T cells are firstly stimulated in the lymph node by their cognate antigen is known as “priming” (Mempel *et al.* 2004). During this process firstly, DCs and T cells display high motility, and perform a great number of interactions in search for a high affinity peptide-MHC complex recognition. As a second step, when the naïve T cell TCR matches its cognate peptide-MHC complex on a DC, both cells arrest and stably interact for 12-24h, during which the T cell starts producing IL-2. Finally, the T cell dissociates from the DC, the latter moving away in search of other potential interaction candidates, the former launching an intensive proliferative activity (Mempel *et al.* 2004). The clonal expansion phase that follows the initial priming can bring a single

naïve T cell through more than 15 cell divisions, generating a population of more than 50,000 daughter cells (Zehn *et al.* 2012).While proliferating, these cells differentiate and acquire specific functional properties. This process generates both effector T cells, that are short lived and readily deal with the invading agent, and memory T cells that confer efficient protection in case of pathogen reattack. The effector phase of a T cell response is followed by a 1-2 weeks long contraction during which most of the clonally expanded cells undergo apoptosis. Only about 5% of the progeny enter the memory compartment and remain in a quiescent state to confer protection to a second encounter with the same pathogen (Seder and Ahmed 2003, Pepper and Jenkins 2011).

Memory CD4 T cell generation

One evolutionary step of the adaptive immune system over the innate arm is the ability to remember the past and generate a more prompt and robust response after a second exposure to antigen. Immunological memory is purely determined by a change in frequency and properties of antigen specific B and T lymphocytes, which persist also after the antigen is eliminated. Memory T cells exhibit considerable heterogeneity in particular in their surface-receptor expression, effector function and trafficking properties and they can be classified in three main subsets: central memory T cells (TCM cells), effector memory T cells (TEM cells), tissueresident memory T cells (TRM cells) and follicular helper memory T cells (Tfh) (Figure 5) (Hamann *et al.* 1997, Sallusto *et al.* 2004, Jaigirdar and MacLeod 2015).

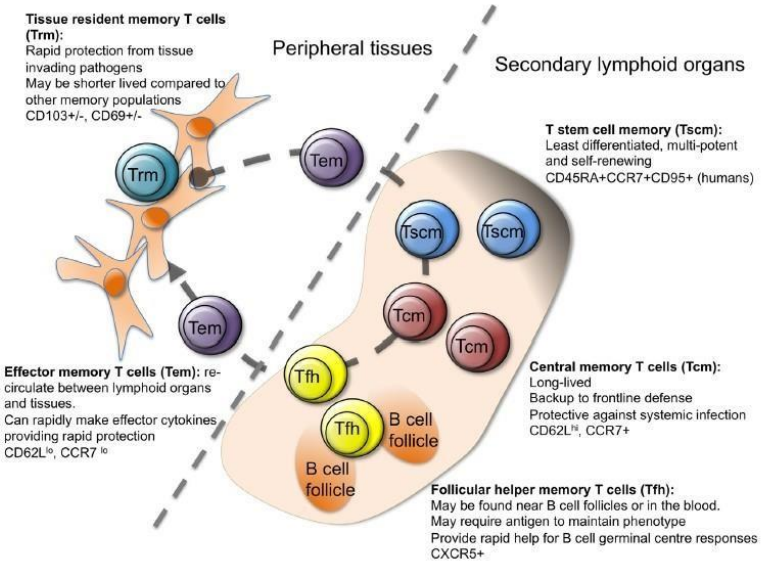

Figure 5. Heterogeneity in memory CD4 T cells. *Memory CD4 T cells can be found in lymphoid organs, blood, and at tissue sites. Stem cell memory T cells (Tscm) and central memory T (Tcm) cells are found in lymphoid organs and in the blood. Both populations are relatively undifferentiated compared to other memory CD4 T cell subsets, and are long lived. Follicular helper memory T (Tfh) cells can also be found in the blood and lymphoid organs. They express the B cell follicle homing receptor, CXCR5, which can position them near B cell follicles to provide rapid B cell help upon reactivation. Effector memory (Tem) and tissue resident memory T (Trm) cells can both be found in peripheral tissues and are more differentiated than Tcm and Tscm. Tem are migratory, passing through tissues and the blood, while Trm are restricted to tissues. Both populations can respond rapidly to tissue invading pathogens* (Jaigirdar and MacLeod 2015).

Nearly 15 years ago, Lanzavecchia and colleagues first identified heterogeneity in human peripheral blood based on the expression of the lymph node homing CC-chemokine receptor CCR7. TEM cells are $CCR7^-$ and express homing receptors that facilitate migration to nonlymphoid sites of inflammation and produce a variety of microbicidal cytokines, including IFN-γ, IL-4, and IL-5, within several hours of TCR stimulation. TCM constitutively express CD62L and CCR7, which are involved in migration through lymph nodes and mucosal lymphoid organs and positioned in the T cell areas of these organs. Following TCR triggering, TCM produce mainly IL-2, but after proliferation, they efficiently differentiate to effector cells and produce large amounts of IFN-γ, or IL-4 (Sallusto and Lanzavecchia 1994). Much of the understanding about the gene expression programs that govern the formation and function of memory populations has been achieved studying recirculating populations of TEM cells and TCM cells. However, subsequent studies have highlighted the presence of tissue-resident memory T cells (TRM cells) at sites of potential reinfection, such as the intestinal, genital and respiratory mucosa and skin. The unique location of these memory cells allow them to coordinate the initial response to pathogen by directly recognizing antigen, recruiting circulating memory cells and diminishing pathogen load in the earliest phase of infection (Gebhardt *et al.* 2009, Schenkel *et al.* 2014).

An important aspect in the definition of immunological memory is the self-renewal capacity of the cells. According to the stem cell model of immunologic memory a single memory lymphocyte re-encountering antigen gives rise to one set of progeny capable of terminal differentiation and another with self-renewing capability. If so, then the memory lymphocyte not only protects against future reinfection, but also maintains the long-term production of effector lymphocytes in chronic, persistent infections (Fearon *et al.* 2001, Turtle *et al.* 2009).

CD4 T helper cells plasticity and subset

It is well appreciated that in addition to the heterogeneity of memory T cells, diversity of cell fate among the progeny of activated T lymphocytes also exists in the effector T cell population.

$CD4^+$ T cells play central roles in shaping the immune response by regulating the function of many other immune and non-immune cell types (Abbas *et al.* 2012, Murphy and Weaver 2016). They help B cells to produce antibody, regulate the activity of phagocytes and APCs, sustain and enhance $CD8^+$ T cell response and memory formation, induce a responsive state in peripheral tissues. Importantly, they also regulate the magnitude and persistence of such responses to prevent tissue damage. To achieve such a multiplicity of functions, $CD4^+$ T cells are endowed with a surprising capacity of differentiation toward phenotypically and functionally distinct effector lineages. The functional specialization of the distinct lineages is coordinated by genetic programs controlled by lineage-specifying transcription factors, whose activation induces expression of distinct soluble mediators and surface molecules that support interactions with other immune cells (Sallusto and Lanzavecchia 2009). Many different factors influence the outcome of the priming process; however, the main driver of T cell differentiation is considered to be the cytokine milieu produced during activation, which is mostly determined by DCs (Pulendran 2005). Cytokines, acting on T cell cytokine receptors, modulate activation of intracellular cascades that eventually lead to the phosphorylation of a given signaling transducer and activator of transcription (STAT) protein. Once phosphorylated, STAT proteins dimerize and translocate into the nucleus, where they promote transcription of a series of lineage-specifying molecules, among which are "master" regulator transcription factors. STATs and master regulators together orchestrate the coordinate expression of a variety of molecules, including effector cytokines and tissue homing chemokine receptors, which define the fate of the proliferating T cells. The principal subsets for $CD4^+$ T cells are Th1, Th2, Th17, Th22, Th9, Tfh, and Tregs (Figure 6) (O'Shea and Paul 2010, Zhu and Paul
2010, Zhu *et al.* 2010). DC-derived IL-12 acts on the IL-12R to activate expression of STAT4, determining acquisition of a Th1 phenotype (Hsieh *et al.* 1993) concomitant with upregulation of T-bet as transcriptional factor. Th1 cells are characterized by production of IFN-γ (together with TNF-α and other inflammatory cytokines) and expression of inflamed tissue homing receptors, such as CXCR3 and CCR5. On a functional level Th1 are the main players in response to viruses and intracellular bacteria, as they efficiently activate macrophages and cytotoxic $CD8^+$ T cells, and act on non-immune cells by inducing an anti- viral state and upregulation of MHC

molecules (Abbas *et al.* 2012, Murphy and Weaver 2016). IL-4 induces instead activation of STAT-6 and consequently of GATA3. GATA3 is the master regulator of Th2 cells, characterized by expression of chemokine receptors such as CCR4 and CCR3 and by production of IL-4, IL-5, and IL-13. All these cytokines act synergistically with IL-4 in the induction of STAT5 activation (Le Gros *et al.* 1990, Yamane and Paul 2012). Th2 cells are endowed with effector mechanisms required to counteract invasion from multicellular parasites, such as helminths, protect from venom, but also provoke onset of allergic diseases. Th2 effector responses are characterized by eosinophils and basophils infiltration, mucus production, smooth muscle contraction, and production of mastcell-activating IgE (Allen and Sutherland 2014). TGF-β, IL-6 and IL-1β induce STAT-3 (Bettelli *et al.* 2006, Acosta-Rodriguez *et al.* 2007a, Chung *et al.* 2009) and ultimately RORγt (Ivanov *et al.* 2006), a transcription factor that specifies for the Th17 lineage (Park *et al.* 2005). Th17 produce cytokines like IL-17A/IL-17F, IL-22 which promote the activation of neutrophils on the one hand, and act on epithelial cells to induce anti-microbial peptides, on the other (Acosta-Rodriguez *et al.* 2007b, Annunziato *et al.* 2007). Moreover Th17 are required in case of infection from fungi or extracellular bacteria (Hernández-Santos and Gaffen 2012). A population characterized by the capacity to produce IL-22, but not IL-17, has been identified and defined as Th22 subset (Duhen *et al.* 2009, Trifari *et al.* 2009). Recently Th9 cells, producing exclusively IL-9, have also been identified and associated to several functions, mainly helminth clearance (Goswami *et al.* 2012, Schlapbach *et al.* 2014). Regulatory T cells (Tregs) have a critical role in protecting from excessive tissue damage during inflammation, as well as from autoimmunity (Sakaguchi *et al.* 2008, Wing and Sakaguchi 2010). These cells can be originated through two distinct pathways. Natural Tregs (nTregs) originate in the thymus, where the driving element is thought to be the strength of selfpeptide recognition upon antigen presentation by TECs. In particular such interaction has been postulated to happen with a strength that is below threshold for negative selection, but higher than that for non-self-specific T lymphocytes. This would induce tolerization in potentially autoreactive cells and prevent autoimmunity (Klein *et al.* 2014). Alternatively, Treg phenotype can be induced in the periphery, (iTregs), by antigen-presentation in the absence of costimulatory molecules and inflammatory cytokines (Lan *et al.* 2012). Cytokines such as TGF-β and IL-2 have been shown to play an important role in driving this fate decision by inducing the specific transcription factor Foxp3 (Fontenot *et al.* 2003, Hori *et al.* 2003). Tregs can dampen the immune response acting through both release of soluble factors such as TGF-β, and expression of surface immune-inhibitory receptors such as CTLA4 (Tang *et al.* 2004, Sakaguchi *et al.* 2009).

Last, but not least, follicular helper CD4 T (Tfh) cells are the specialized providers of B cell help and are instrumental for mounting an efficient and high affinity antibody response, as they direct the germinal center reaction (Crotty 2011). Tfh cells differentiation is a multi-stage process which depends on the expression of the master regulator transcription factor B cell lymphoma 6 (Bcl6) (Johnston *et al.* 2009, Nurieva *et al.* 2009). Distinguishing features of Tfh cells are the expression of CXCR5, PD-1 and ICOS, among other molecules, and the production of IL-21 which is crucial in the germinal center formation and Tfh polarization (Crotty 2011). Tfh cell differentiation requires input from several surface receptors (CD28, ICOS and CD40L) and signaling lymphocytic activation molecule (SLAM) family members, as well as from cytokines and their associated signaling pathways (for example STAT3 or SLAM associated protein (SAP; also known as SH2D1A), which all culminate in the induction of Bcl6 (Yu *et al.* 2009). Tfh cell differentiation begins at the priming stage when naïve $CD4^+$ T-helper cells interact with antigen presenting DCs in the T-zone of a secondary lymphoid organ. Activated $CD4^+$ T cells undergo rapid changes in their expression of costimulatory molecules and chemokine receptors. Downregulation of CCR7 expression and concomitant upregulation of the chemokine receptor CXCR5 subsequently allow these activated T cells to migrate to the T-B zone border of secondary lymphoid organs, where they interact with antigen-specific B cells. Some of these early Tfh cells, together with a few antigen-specific B cells enter the follicle to establish a germinal center (GC) reaction in which somatic hypermutation and selection of high-affinity B cells result in the generation of memory B cells and plasma cells (Okada and Cyster 2006, Allen *et al.* 2007, Haynes *et al.* 2007). The major help signals provided by Tfh cells are CD40L, IL-21 and IL-4 which are crucial to keep GC B cells alive and induce their proliferation (Crotty 2011). Furthermore, Tfh cells regulate GC size (Johnston *et al.* 2009, Rolf *et al.* 2010, Hams *et al.* 2011), restrict lowaffinity B cell entry into the GC, support high affinity B cell occupancy of the GC (Schwickert *et al.* 2011), and select high affinity B cells during affinity maturation (Victora *et al.*, Good- Jacobson and Shlomchik 2010). A role of Tfh in driving the differentiation of high affinity germinal center B cells into plasma cells has been proposed (Kräutler *et al.* 2017). Studies of Tfh cells in human remained relatively limited until recently, mainly due to difficulties in assessing the dynamics of this cell population in human lymphoid organs. However, our knowledge on the biology of Tfh cells and their contribution to disease has significantly increased in the past decade, especially since the identification of circulating memory Tfh cells (cTfh). In fact emerging evidences show that blood $CXCR5^+CD4^+$ T cells contain long-lived memory cells which share functional properties with Tfh cells. Accordingly, blood cTfh cells are recognized and defined as $CXCR5^+$ $CD45RA^-$ $CD4^+$ T cells (Morita *et al.* 2011, Schmitt *et*

al. 2014). Due to the essential role of Tfh cells in regulating high-affinity antibody production and B-cell memory formation, deficiency of those cells can lead to immunodeficiency, while excessive Tfh cells might contribute to the development of autoimmunity and lymphoid malignancy (King *et al.* 2008, Tangye *et al.* 2012, Tangye *et al.* 2013a). Many mouse models have demonstrated that aberrant generation and/or activation of Tfh cells contributes to the pathogenesis of autoimmune diseases (Luzina *et al.* 2001, Vinuesa *et al.* 2005, Linterman *et al.* 2009, Hua and Hou 2013). These observations also appear applicable to human diseases.

For instance the detection of $CD4^{+}CXCR5^{+}$ T cells in organs that are affected by autoimmune disorders, such as the salivary glands in Sjögren's syndrome, suggested that aberrant Tfh cell development can drive human autoimmunity (Salomonsson *et al.* 2002). Increased frequency of Tfh cells has also been shown in the peripheral blood of patients with systemic lupus erythematosus (SLE) (Hutloff *et al.* 2004) and myasthenia gravis (Tackenberg *et al.* 2007). Furthermore, memory T cells from type-1 diabetes (T1D) patients showed high expression of Tfh cell markers, including CXCR5, ICOS, BCL6, and IL-21. This observed expansion has been linked with the altered IL2 sensitivity in T1D patients (Kenefeck *et al.* 2015). Several primary immunodeficiencies have also been associated with genetic defects that might affect Tfh cells differentiation and function. For instance Hyper-IgM syndrome (HIGM) is a rare human immunodeficiency characterized by normal or elevated serum IgM levels with absence of IgG, IgA and IgE, resulting in a profound susceptibility to bacterial infections (Notarangelo *et al.* 1992). The molecular basis of the X-linked form (HIGM1) is due to mutations in the gene coding for CD40L (Aruffo *et al.* 1993, Korthauer *et al.* 1993). Besides the Ig switch defect and remarkable paucity in germinal center formation (Facchetti *et al.* 1995) these patients show a considerably decrease in the number of cTFH (Bossaller *et al* 2006, Tangye *et al.* 2013b).

Recently, follicular regulatory T (Tfr) cells were identified as a Treg cell subset that localizes in the germinal center to suppress B-cell response. Sharing certain key features of Tfh cells, like Bcl6 and CXCR5 expression, Tfr cells also express Treg-associated molecules including Foxp3, CD25, GITR, and CD103. Unlike Tfh cells, Tfr cells express Blimp-1, which is required to control the GC. It has been shown that Tfr cells can limit the number of Tfh cells and furthermore the production of antibodies specific for an immunizing self-antigen (Chung *et al.* 2011, Linterman *et al.* 2011).

Using a multidimensional cytometry, transcriptomics and functional assays, Rao et al have recently described a subset of human $CD4^{+}PD\text{-}1^{high}CXCR4^{-}$ T cells, characterized by a strong ability to support B cell differentiation. This subset, which they named peripheral helper T cells

(Tph), is expanded in the synovial fluid and peripheral blood of rheumatoid arthritis patients participating in the autoimmune response (Rao *et al*. 2017).

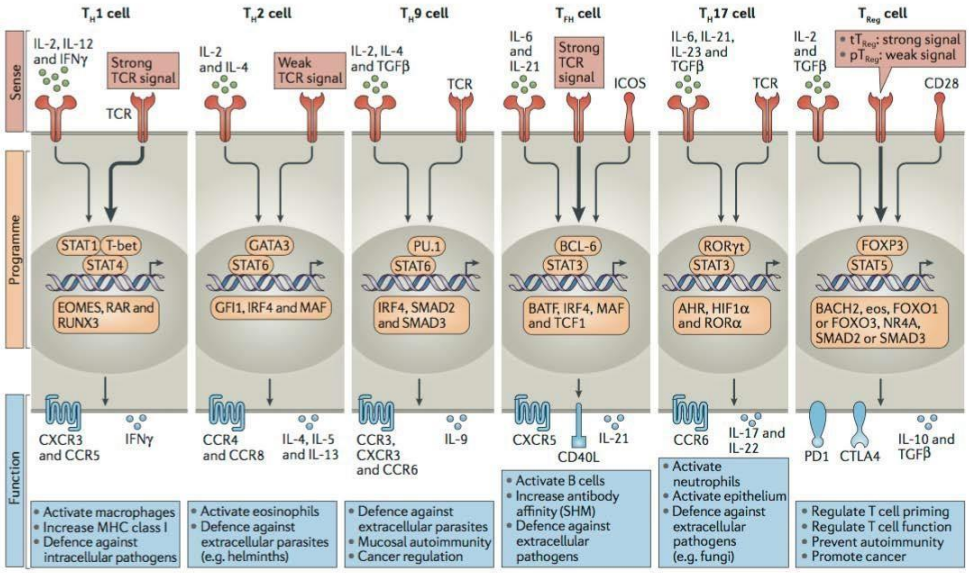

Figure 6. Polarized CD4⁺ T cell subsets. *Each CD4⁺ T cell subset can be defined by their distinct abilities to sense (red), programme (orange) and function (blue) in the control of specific pathogens or immune pathologies. The inductive cytokines, polarizing transcription factors and cytokines or chemokine receptors that are characteristic of each subset are shown, along with their association with specific forms of immune defense. AHR, aryl hydrocarbon receptor; BATF, B cell-activating transcription factor; BCL-6, B cell lymphoma 6; CCR, CC-chemokine receptor; CD40L, CD40 ligand; CTLA4, cytotoxic T lymphocyte antigen 4; CXCR, CXC-chemokine receptor; EOMES, eomesodermin; FOXO, forkhead box O; FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; GFI1, growth- factor independent 1; HIF1α, hypoxia-inducible factor 1α; ICOS, inducible T cell co-stimulator; IFNγ, interferon-γ; IL, interleukin; IRF4, interferon-regulatory factor 4; MAF, macrophage-activating factor; NR4A, nuclear receptor 4A; PD1, programmed cell death 1; pTReg cell, peripherally derived regulatory T cell; RAR, retinoic acid receptor; ROR, retinoic acid receptor-related orphan receptor; RUNX3, runt-related transcription factor 3; SHM, somatic hypermutation; STAT, signal transducer and activator of transcription; TCF1, T cell factor 1; TCR, T cell receptor; TFH, T follicular helper; TGFβ, transforming growth factor-β; TH, T helper; Treg cell, regulatory T cell; tTreg cell, thymus-derived regulatory T cell* (DuPage and Bluestone 2016).

1.3 B lymphocytes

Central and peripheral B cell development

B cell generation occurs throughout life and proceeds through distinct stages that begin in the fetal liver and adult bone marrow (BM) where hematopoietic stem cells (HSC) develop via various B lineage-restricted precursors into immature B cells, which then enter the periphery. The BM, located in the medullary cavities of the bones, contains a dense network of medullary vascular sinuses and it is in the extravascular spaces between the sinuses that blood cells and their precursors are packed (Lichtman 1981). The BM microenvironment is responsible for a proper B cell development, which requires the presence and complex interplay of particular growth factors and cytokines such as stem cell factor (SCF), fms-like tyrosine kinase ligand (FLT3-L), or IL-7, all of which are provided by BM stromal cells (Nagasawa 2006). In addition to these exogenous factors, developing B cells depend on intrinsic signals which promote proliferation and differentiation (Herzog and Jumaa 2012). The different stages in the B cell differentiation process can be identified by the expression of CD antigens and the rearrangement status of immunoglobulin heavy (IgH) and light (IgL) chains. The current consensus is that B lineage-committed cells pass through a $CD34^{+}CD10^{+}CD45RA^{+}$ common lymphoid progenitors (CLPs) stage which mature via $CD34^{+}CD19^{+}CD10^{+}$ pro-B and $CD34^{-}$ $CD19^{+}CD10^{+}$ pre-B into $CD34^{-}CD19^{+}sIgM^{+}$ immature B cells (Galy *et al.* 1995). The pro-B cell stage represents the earliest BM cell committed to the B cell lineage, which is characterized by the expression of lineage-restricted surface molecules like CD19 and CD10 and by the expression of Rag-1 and Rag-2 proteins which are responsible for the first recombination event at the heavy chain locus (Mombaerts *et al.* 1992, Shinkai *et al.* 1992). The D-J recombination brings together one D and one J gene segment, with the deletion of the
DNA segments located in between. Subsequently one of the many 5'V genes is joined to the DJ unit resulting in a rearranged VDJ exon (Jung *et al.* 2006). At the pro-B cell stage no surface expression of heavy chain is detected. At this stage the expression of terminal deoxynucleotidyl transferase (TdT) enzyme is essential since it catalyzes the non-templated addition of junctional nucleotides. Pro-B cell differentiation into pre-B cells is characterized by loss of CD34 and TdT and based on cell-cycle analysis human pre-B cells can be generally subdivided into large proliferating pre-BI cells, large proliferating pre-BII cells, and small resting pre-BII cells. The pre-BI cells, which express CD19, complete IgH DJ rearrangement and continue to rearrange its V gene segments until productive V_H–DJ_H alleles are generated in large pre-BII cells. These cells cease to express Rag1 and Rag2 and display the product of the rearranged IgH gene at the cell

surface; there, it assembles with the surrogate IgLs VpreB and λ5, together with the signaling molecules Igα and Igβ to form the pre-B-cell receptor (pre- BCR) (Reth 1992, Ghia *et al.* 1996, Blom and Spits 2006). Signals from the pre-BCR are responsible for the largest proliferation expansion of the B lineage cells in the BM and its expression is a crucial step in early B-cell development, at which the functionality of the heavy chain is monitored. In fact the phenomenon of allelic exclusion in the IgH locus ensures that in any B cell clone one heavy chain allele is productively rearranged and expressed whereas the other is either retained in the germline configuration or nonproductively rearranged. In case of unproductive rearrangement the gene on the second chromosome will undergo recombination (Vettermann and Schlissel 2010). Lastly signaling pathways induced by the pre-BCR leads to the generation of a conventional light chain that replaces the surrogate immunoglobulin light chains of the pre-BCR to form a mature BCR (Löffert *et al.*, Herzog *et al.* 2009). In addition, similar to the heavy chain recombination the kappa and lambda light chain rearrangement is tightly controlled, ensuring that only one chain (either kappa or lambda) is associated with the heavy chain to make a functional B cell receptor. Indeed during the light chain isotype exclusion process, the kappa locus rearranges first and only if this event generates a non-functional or a self-reactive protein, the lambda locus will undergo recombination (Perfetti *et al.* 2004). Newly formed immature B cells exit the BM to migrate into the spleen to complete their maturation process. Cells that are developmentally intermediate between immature BM B cells and fully mature naive B cells in the peripheral blood and secondary lymphoid organs are termed “transitional B cells”. This term was first used for mouse B cells (Carsetti *et al.* 1995) and three stages of murine transitional cells, namely T1, T2 and T3, have been identified (Allman *et al.* 2001). More recently, transitional B cells have been identified in humans and defined as new emigrant B cells, which have been selected for self-tolerance in the BM and have been exported to the periphery (Sims *et al.* 2005). During BM development, the Ig gene segment encoding V, D and J regions are rearranged in a unique stochastic process. While the random nature of this process ensures an unlimited variability of BCR, it includes the obvious risk to generate B cells able to recognize self-antigens. Thus, it has been suggested that negative selection should take place starting from the early phases of B cell development in the BM. Elegant mouse models have led to the characterization of three main mechanisms of negative selection taking place in early B cell development: clonal deletion, anergy and receptor editing, the latter one consisting in starting a new rearrangement of the light chain (Tiegs *et al.* 1993, Pelanda *et al.* 1997). Recently, the development of strategies to clone and express a BCR isolated from a single human B cell has allowed the analysis and quantification of the negative selection process. Wardemann et al demonstrated that while 76% of pre-B cells or early

immature B cells from BM are potentially self-reactive, this number drops to 40% in immature BM B cells and a 20% in mature, peripheral B cells, suggesting that most of the discrepancy between B-cell production in the BM and B-cell immigrants in the periphery is due to negative selection of autoreactive cells. Hence two distinct B cell development check points are delineated (Figure 7): a central tolerance check point in the BM which ensures a selection process between the early immature B cells and immature B cells stage and a peripheral checkpoint which removes autoantibodies from the transitional B cells before they enter the naive pool (Wardemann *et al.* 2003). It has been estimated that 20-50% of all peripheral naive B cells have undergone receptor editing (Meffre and Wardemann 2008). Furthermore, two additional check points have been suggested during the B cell development. Specifically one which ensures the exclusion of remaining autoantibodies from IgM memory pool (Tsuiji *et al.* 2006) and one which controls the differentiation towards IgG memory B cells (Tiller *et al.* 2007). Whether a positive selection of B cells exist is still not completely clear. Remarkably, it has been shown that selfrecognition is indeed crucial for the generation of B cells and the pre-BCR could function as an auto-reactive component (Köhler *et al.* 2008).

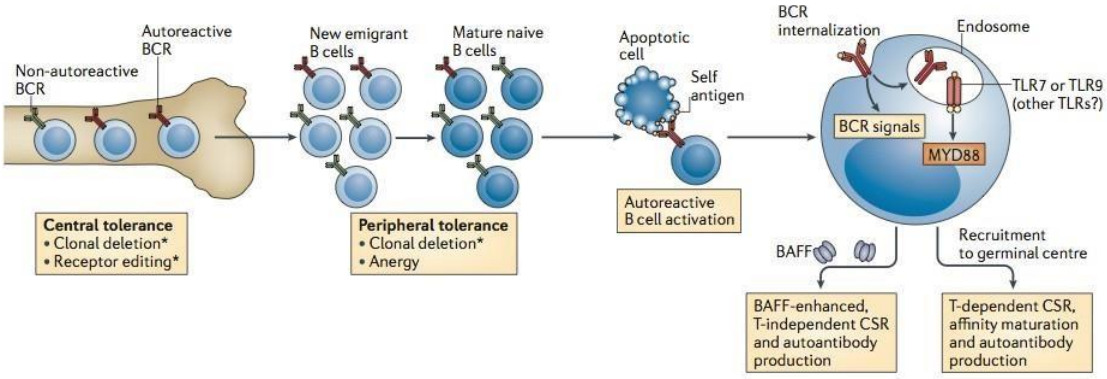

Figure 7. The role of B cell-intrinsic MYD88 signaling in B cell tolerance and autoimmunity.
B cell development frequently results in the generation of autoreactive B cells. Such cells are removed at distinct checkpoints in the bone marrow (central tolerance) and periphery (peripheral tolerance) via a combination of mechanisms, including B cell clonal deletion, receptor editing and functional anergy. Although the mechanisms remain to be determined, signaling through myeloid differentiation primary-response protein 88 (MYD88) may have an impact on tolerance mechanisms, as greater autoreactivity is noted in both new emigrant and mature naive B cell populations in patients with inborn errors in MYD88, *IL-1R-associated kinase 4 (*IRAK4*) or* UNC93B. *Despite intact tolerance mechanisms, autoreactive B cells also enter the mature compartment in healthy individuals. Mouse models have demonstrated the crucial importance of dual B cell receptor (BCR) and Toll-like receptor (TLR) signaling in the activation of autoreactive B cells. Antigen receptors on DNA- or RNA-reactive B cells can be engaged either directly by self-antigens on the surface of apoptotic cells and apoptotic debris or indirectly by self-antigens on APCs in the context of MHC class II molecules (not shown). Following engagement, BCR internalization shuttles DNA- or RNA-associated antigens to TLR7- and TLR9- containing intracellular compartments, resulting in MYD88-dependent B cell activation. The potential requirement for additional TLRs in*

activating autoreactive B cells with different specificities has not yet been addressed. Activated autoreactive B cells either undergo T-independent class-switch recombination (CSR) and produce autoantibodies (processes that are enhanced by B cell-activating factor (BAFF)), or recruit autoreactive T cells to germinal centres, where the autoreactive B cells undergo T-dependent CSR and affinity maturation and produce pathogenic autoantibodies (Rawlings *et al.* 2012).

Early events of B cell activation

The B cell antigen receptor, BCR complex is an hetero-oligomeric structure composed of immunoglobulin heavy (IgH) and light (IgL) chains, involved in the antigen recognition process, associated with two signaling components Igα and Igβ (CD79α and CD79β) which are characterized by a single ITAM. The unique binding specificity is determined by the combination of variable domains (VH and VL) in the heavy chain and the light chain (LC) while the Igα/Igβ heterodimer functions as the signaling subunit of the complex. Engagement of the BCR initiates signaling mechanisms that can lead to cell proliferation and differentiation (Reth 1989). Igα and Igβ share similar structural features, since they both carry an extracellular Ig domain, an evolutionarily conserved transmembrane domain and a cytoplasmic tail with an ITAM motif. Phosphorylation of ITAM-associated tyrosine residues creates docking sites for the recruitment and activation of Src family kinases, such as Lyn and Syk, which phosphorylate multiple downstream targets and adaptors like the SLP-65 and Nck proteins (Burkhardt *et al.* 1991, Hutchcroft *et al.* 1991). Such adaptors can bring together several effector proteins and promote multiple signaling pathways, for instance calcium signaling pathway, initiating the gene transcription process required for B cell functions (Ohhora and rao 2009). Following the activation phase, B lymphocytes can undertake a large number of distinct fates important for immune regulation (Duffy *et al.* 2012). The classical activation of naïve B cells to T-dependent antigens requires four types of signals. The first signal is given by the crosslinking of the BCR by multivalent antigens and is facilitated by complement receptors (CRs). The second signal is orchestrated by the help of $CD4^+$ T cells. The third signal is given by engaged toll like receptors (TLRs), which are expressed on the plasma membrane (TLR4) or in intracellular compartments (TLR3, TLR7 and TLR9) (Takeda *et al.* 2003, Hua and Hou 2013, Pihlgren *et al.* 2013). The fourth signal is given by soluble cytokines and membrane-bound ligands expressed by T helper cells, DCs or other myeloid cells (Traggiai *et al.* 2003). After antigen recognition, the BCR triggers a signaling cascade that leads to up-regulation of costimulatory molecules and efficient antigen internalization, followed by processing and presentation of antigenic peptides on MHC class II molecules for recognition by specific T cells (Chatterjee *et al.* 2012). This mechanism of

BCR-mediated antigen internalization allows specific B cells to present antigen to T cells with very high efficiency (Lanzavecchia 1985).

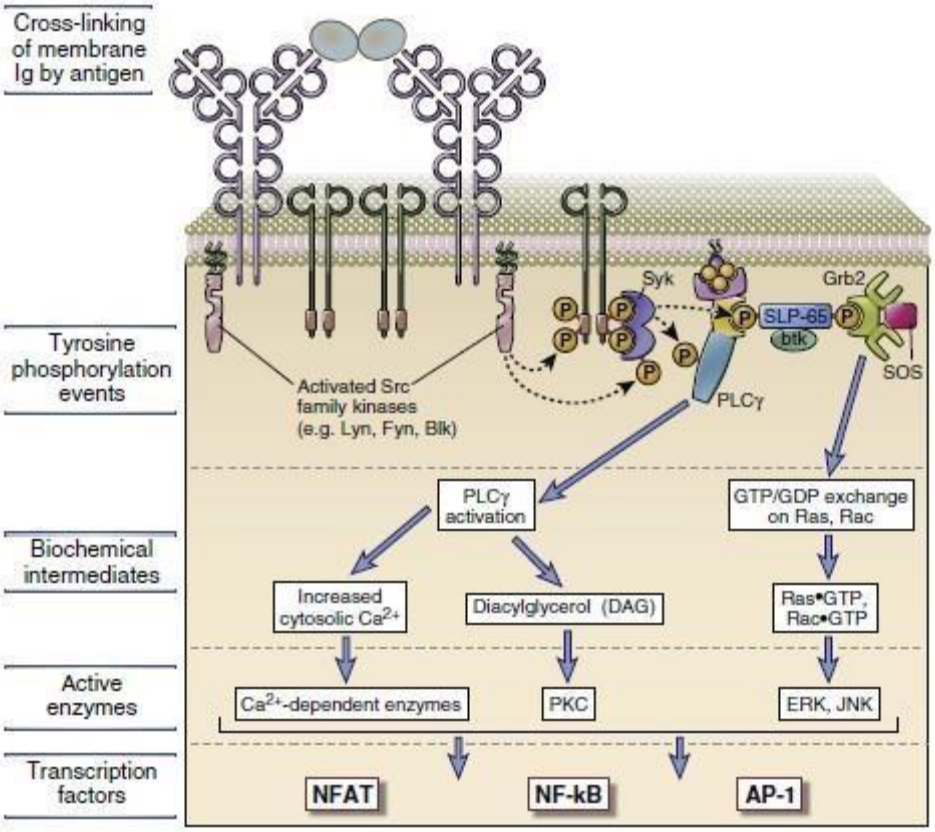

FIGURE 8. Signal transduction by the BCR complex. *Antigen-induced cross-linking of membrane Ig on B cells leads to clustering and activation of Src family tyrosine kinases and tyrosine phosphorylation of the ITAMs in the cytoplasmic tails of the Igα and Igβ molecules. This leads to docking of Syk and subsequent tyrosine phosphorylation events as depicted. Several signaling cascades follow these events, as shown, leading to the activation of several transcription factors. These signal transduction pathways are similar to those described in T cells* (Abbas et al. 2012).

Antigen modular responses of B lymphocytes

The features of B cells activation and the subsequent generated antibody response depend on the availability of different possible cellular help and on the type of activating antigens. Studies of athymic (nude) mice or mice that had undergone removal of the thymus provided early insights into the cellular factors needed to elicit antibody responses to immunizing antigens. Thymus dependent (TD) responses are absent in those mice because of the essential requirement for thymus-derived T cells for the full activation and subsequent proliferation of the B cells (Gershon

and Kondo 1970). TD antigens are generally monomeric soluble proteins that display single or few epitopes to antigen-specific B cells and require cognate T cell help for induction of highly specific antibody responses through GC reaction. After receiving a signal through the BCR, mature B cells migrate to the interface of the T cell zone (T zone) and the B cell follicle and present antigen-derived peptides on MHCII molecules for recognition by newly primed Tfh cells (Cyster 2010). As already discussed the key function of Tfh cells is to provide help to B cells and to support their activation, expansion and differentiation and the formation of the GC. Several features of Tfh cells enable them to carry out these functions (Crotty 2011). Possibly the best-characterized B cell helper signal provided by Tfh cells is CD40L, which is highly expressed on these cells. CD40L is a potent activator of the B cells, it is able to induce proliferation and, in combination with cytokines, isotype switching and cell differentiation (Noelle *et al.* 1992, Elgueta *et al.* 2009). Mutation of *CD40L* gene results in a disease called X-linked hyper-IgM syndrome which is characterized by defect in the TD antibody response, isotype switching, affinity maturation and memory B cell generation (Notarangelo and Hayward 2000).

The experimental results with the mutant mice showed that the in vivo TD-immunoglobulin class switching but not the thymus independent (TI) antibody responses is affected; indeed it is well know that TI antigens can induce IgG antibody responses in athymic nude mice (Mongini *et al.* 1981). Antigens that are expressed on the surface of pathogens in an organized, highly repetitive form can activate specific B cells in the absence of T-help by crosslinking of antigen receptors in a multivalent fashion, by a mechanism that depends on the expression of a functional Bruton's tyrosine kinase (Btk) (Bajpai *et al.* 2000). TI antigens are further classified in two main groups: type I (TI-1) and type 2 (TI-2). TI-1 antigens are mitogens that stimulate all B cells in a polyclonal manner and irrespective of antigen specificity. Physiological TI-1 antigens include Toll-like receptor (TLR) ligands, such as LPS which is expressed by gram negative bacteria (Coutinho *et al.* 1974), or certain viral coat proteins (Berberian *et al.* 1993, Blutt *et al.* 2004). TI-2 antigens are instead composed of repetitive epitopes that simultaneously engage multiple antigen receptors on the surface of antigen specific B cells. TI-2 antigens include polysaccharides found on encapsulated bacteria and viral capsid proteins and elicit robust IgM and IgG3 antibody production (Mongini *et al.* 1984, Bachmann *et al.* 1995, Bachmann *et al.* 1997). Furthermore, a third recent type of TI B cell activation (TI-3) pathway has been described that is based on blood-borne or gut bacteria and requires help of myeloid cells, such as neutrophils (with B help activity, NBH) (Puga *et al.* 2012), basophils, mast cells (Merluzzi *et al.* 2010), eosinophils (Wang and Weller 2008, Chu *et al.* 2011) or DCs (Gatto *et al.* 2013, Vinuesa and Chang 2013) (Figure 9).

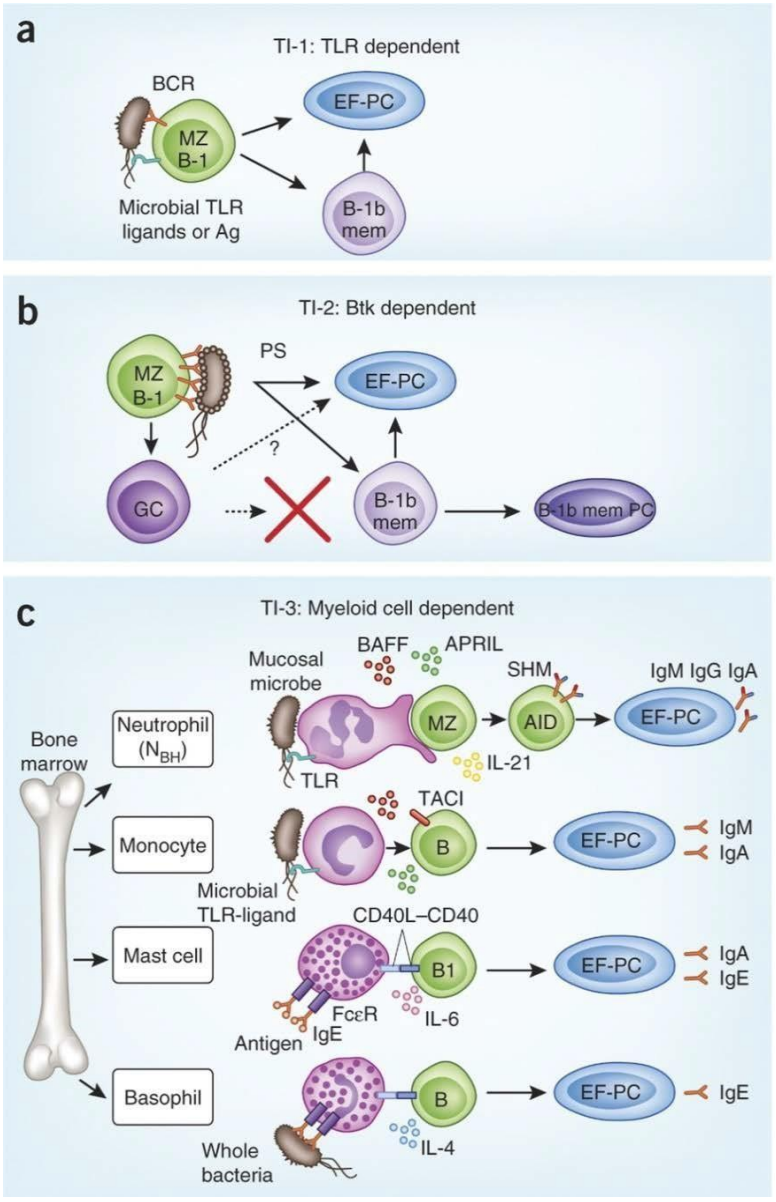

Figure 9. TI antibody responses. *(a) In TI-1 responses, B cells receive second signals through their TLRs. (b) In TI-2 antibody responses, extensive crosslinking of the BCR provides essential signals through Btk. (c) TI-3 antibody responses are elicited when B cells receive help from bone marrow– derived myeloid cells (neutrophils (NBH cells), monocytes, mast cells and basophils). Ag, antigen; PS, polysaccharide; B1-b mem, B-1b memory B cell; B1-b mem PC, B-1b memory plasma cell; MZ, marginal zone; AID, activation-induced cytidine deaminase; SHM, somatic hypermutation; FcεR, receptor for IgE* (Vinuesa and Chang 2013).

Peripheral B cell subsets

The mature peripheral B cells pool includes three distinct subsets according to their ability to migrate, their location and their propensity to generate a T-dependent or a T-independent

response: follicular B cells (FO B cells), marginal zone B cells (MZ B cells) and B-1 cells (Figure 10). Most mature naive B lymphocytes are FO B cells that constantly recirculate in the blood and migrate to the B cell areas of secondary lymph nodes where they might mediate TD immune responses to protein antigens. FO B cells enter secondary lymphoid organs (spleen, lymph nodes, mucosal lymphoid tissue) through blood vessels located in the T cell areas and then migrate into the follicle, precisely into the B cell zones and primarily make antibody response to protein antigens that require T cell help (Batista and Harwood 2009). The movement into the lymphoid follicle is mainly driven by the chemokine CXCL13 secreted by the stromal component of the follicle, above all follicular dendritic cells. Recirculating B cells express CXCR5, the chemokine receptor for CXCL13, and the specific interaction between the two partners attract the B cell into the follicle (Cyster 2010). The antigen can be delivered to naive B cells in lymphoid organs by multiple routes, for example the small antigens can be transported by afferent lymphatic vessels that drain into the subcapsular sinus of the nodes; large antigens instead can be captured by the subcapsular sinus macrophages or by the dendritic cells resident in the medullary region. In any cases the antigen is presented to B cells in a naive conformation and is not processed by antigen-presenting cells (Batista and Harwood 2009). This is one of the aspects that differ between the forms of antigens recognized by B and T lymphocytes.

FO B cells mainly mediate immune responses to TD protein antigens whereas MZ and B-1 subsets are primary responders to TI antigens. Early studies of MZ B cells in rodents showed that they are non-recirculating and restricted to the spleen with limited motility (MacLennan *et al.* 1982, Lu and Cyster 2002). Yet recent studies provided indirect evidence that this B cells population continuously exchanges between the marginal zone and follicle (Ferguson *et al.* 2004, Cinamon *et al.* 2008, Arnon *et al.* 2013). In rodents a small proportion of memory B cells may also populate the marginal zone, whereas in humans most MZ B cells are thought to represent a memory population. MZ B cells have long been considered critical determinants of host defense directed against encapsulated blood-borne bacterial TI antigens. There is, however, a growing appreciation of a broader role for MZ B cells in both T-independent and T-dependent immune responses (Weill *et al.* 2009).

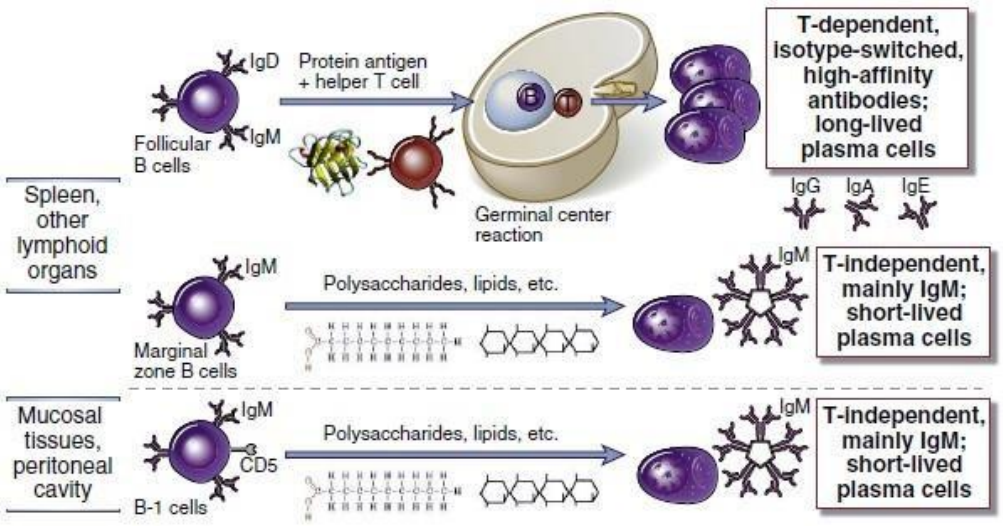

Figure 10. Distinct B cell subsets mediate different types of antibody response. *Follicular B cells respond to protein antigens and thus initiate T-dependent antibody responses. T-independent responses to multivalent antigens are mediated mainly by marginal zone B cells in the spleen and B-1 cells in mucosal sites. These functional distinctions between subsets are not absolute* (Abbas et al. 2012).

Dynamic of mature B lymphocytes fates: extra-follicular and germinal center responses

As already mentioned B cells responding to a TD antigen are considered to develop along two pathways; one leads to the rapid differentiation to extrafollicular plasma cells and the second to GC formation (MacLennan *et al.* 2003). After interaction with $CD4^+$ T helper cells in the T and B zones, activated B cells can migrate to the bridging channels the and red pulp of the spleen to form extrafollicular foci. Here they differentiate into plasma cells, many of which are short lived (Jacob and Kelsoe 1992) and secrete antibodies that may be either switched or un- switched (IgM) (Phan *et al.* 2005). During the extrafollicular response B cells do not undergo SHM and therefore the resultant antibodies are comprised almost exclusively of specificities encoded within the primary repertoire. Thus, the early extrafollicular response provides an immediate, germline encoded antibody response that can be crucial to fight the invading pathogen. The decision between extrafollicular differentiation and GC migration represents the main branch point during a TD B cell response and the mechanisms which direct B cells down one pathway versus the other are currently unknown (MacLennan *et al.* 2003). One theory counts on a stochastic process where the activated B cells can follow either pathway such that the original specificities are represented equally in both the extrafollicular and early GC populations (Dal Porto *et al.* 1998, Blink *et al.* 2005). Alternatively, according to the nonstochastic theory the nature of the interaction between antigen and BCR may influence the subsequent responses

causing different specificities in the extrafollicular and in the early GC populations (Paus *et al.* 2006). Therefore, what is really driving this process is still controversial (Newman *et al.* 1992, Roost *et al.* 1995).

Upon plasma cell differentiation there is an increase in the steady-state amount of Ig heavy and light chains mRNA and also an increased cytoplasmic to nuclear ratio. Because they are terminally differentiated, end-stage cells, plasma cells do not divide, however at a transient stage of plasmablast they can both secrete antibody and proliferate (McHeyzer-Williams and McHeyzer-Williams 2005). Differentiated plasma cells are endowed with prominent amounts of secretory vacuoles and rough endoplasmic reticulum and increased expression of syndecan1 (CD138) a membrane proteoglycan which is often used as a plasma cells surface marker (Sanderson *et al.* 1989). Others B cell specific surface proteins are instead downregulated, including MHCII, B220, CD19, CD21, and CD22 (Calame 2001). Furthermore differentiated plasma cells show increased chemotactic sensitivity to the CXCR4 ligand CXCL12 and in the meanwhile decreased responsiveness to the B and T zone chemokines CXCL13, CCL19, and CCL21 thanks to the downregulation of CXCR5 and CCR7 (Hargreaves *et al.* 2001). Several transcriptional factors required for early steps in B cell development are decreased or absent in plasma cells, for instance PAX5 which is involved in a B cell commitment and B cell lymphopoiesis in BM and spleen (Nutt *et al.* 1999, Nutt and Tarlinton 2011). Furthermore, the differentiation of mature B cells into antibody-secreting plasma cells is controlled by the presence of B lymphocyte-induced maturation protein 1 (BLIMP1) and the absence B cell lymphoma 6 (BCL6). BLIMP1 promotes B cell terminal differentiation by turning off the expression of genes that are associated with cell-cycle progression, DNA synthesis and repair, as well as those that are involved in mature B cell function (Turner *et al.* 1994, Piskurich *et al.* 2000, Shaffer *et al.* 2002). These include many transcriptional factors, such as

Bcl6 which is required for the GC-B cell formation (Chang *et al.* 1996, Dent *et al.* 1997, Fukuda *et al.* 1997). Of the genes that are turned on, particularly interesting is the transcriptional activator XBP1, which is a crucial regulator of plasma cells differentiation (Todd *et al.* 2009, Nutt *et al.* 2015). The second wave of TD responses is characterized by B cells migrating into specialized area called primary follicle where they give rise to the GC.

The GC was first described in 1884 by Walther Flemming, who observed a site of large lymphocytes undergoing mitosis in the follicles of many secondary lymphoid organs and proposed this site to be the major source of lymphocytes in the body (reviewed in (Nieuwenhuis and Opstelten 1984). Nowadays the association between the GC and the TD antibody response is well established, in which B cells undergo extensive round of proliferation, somatic

hypermutation, and antigen-affinity driven selection resulting in the generation of high-affinity antibody- secreting plasma cells and memory B cells (Liu *et al.* 1989, Honjo *et al.* 2002).

In secondary lymphoid organs, lymphocytes are segregated on the basis of chemokine sensitivity into discrete B cell zones (follicles) and T cell zones. TD antibody responses are initiated when rare B and T cells specific for an incoming antigen cluster at the boundary between B cell follicles and T cell zones and engage in cognate interactions (Garside *et al.* 1998, Okada *et al.* 2005). Localization to the B cell zones and the T cells zones depends on the chemokine receptors CXCR5 and CCR7, respectively. After being activated by antigen, B cells increase their expression of CCR7 and migrate to the interface between the zones, where they encounter cognate T cells (Cyster 2005). This results in a burst of proliferation in the outer follicle. The activated B cells can then adopt one of three potential fates: they can migrate in the extrafollicular areas where they undergo proliferation and terminal differentiation into short-lived plasma cells that produce a transient wave of relatively low affinity, antigen specific antibodies (Gatto *et al.* 2009, Pereira *et al.* 2009); they can return to the B cell follicle and proliferate further to establish a GC (Allen *et al.* 2007). Or finally, some of these expanding clones can assume a memory phenotype, forming the early memory B cells that enter the circulation (Blink *et al.* 2005).The precise kinetics of GC development vary depending on the system examined and are influenced by several factors like the species studied, the availability of T cell help, the immunizing antigen and the tissue in which the response occurs (Liu *et al.* 1991a, Liu *et al.* 1991b, Pape *et al.* 2003, Wang and Carter 2005). Despite these variables, some common principles of GC formation have emerged and decades of anatomical and functional studies have led to an overall model of how the GC reaction operates. Few days after the initiation of an immune response, activated B cells move to the center of B cell follicle in secondary lymphoid organs and start to proliferate within the follicular dendritic cells (FDCs) network (Liu *et al.* 1991b). FDCs are specialized stromal cells capable of capturing large amounts of antigen in the form of immune complexes and thought to be an important source of chemokines such as CXCL13 which is crucial for the localization of CXCR5 positive B and T cells within the GC (Allen and Cyster 2008, Vinuesa *et al.* 2010). From the very beginning, the GC was viewed primarily as an anatomical entity and conventional histology techniques allowed the identification of two main compartments termed “light” and “dark” zone (LZ and DZ, respectively) (Rohlich, 1930). The DZ consists almost entirely of B cells with a high nucleus-to-cytoplasm ratio, thus appearing “dark” by light microscopy. By contrast, B cells in the LZ are interspersed among a network of FDCs which give this zone its “lighter-appearance (Nieuwenhuis and Opstelten 1984, Cyster *et al.* 2000).

GC B cells in DZ and LZ were historically defined as centroblasts and centrocytes respectively, with centroblasts named on the basis of their mitotic activity and suggested to exit the cell cycle, re-express surface Ig and become smaller centrocytes that traveled to the LZ (MacLennan 1994). However recent works have demonstrated that cells progress through the cell cycle in both zones (Rahman *et al.* 2003, Wang and Carter 2005, Allen *et al.* 2007). The mechanism promoting cell movement from DZ to LZ seems likely to be regulated by the chemokine receptor CXCR4, which is needed for GC B cells positioning in the DZ and its ligand SDF-1 (CXCL12) which is more abundant in this zone than it is in the LZ (Allen *et al.* 2004). Therefore, based on expression levels of ‘‘signature’’ surface proteins centroblasts are defined by higher expression of CXCR4 but lower level of CD83 and CD86, whereas centrocytes are identified as being $CXCR4^{lo}$, $CD83^{hi}$, and $CD86^{hi}$ (Allen *et al.* 2004, Victora *et al.* 2010, Victora *et al.* 2012). The functional importance of the DZ became apparent when it was shown that these cells undergo somatic hypermutation (SHM) of their immunoglobulin variable region (IgV) genes which is the basis for the affinity maturation of antibodydependent immune responses (Jacob *et al.* 1991). The SHM process modifies the IgV of the rearranged antibody genes in B cells during an immune response. The mutations (mostly nucleotide substitutions) are introduced in the rearranged variable (V) region that encodes for the antigen binding sites of the antibody, allowing the generation of high-affinity BCR (Li *et al.* 2004). SHM is associated with DNA strand breaks and requires the activity of activationinduced cytidine deaminase (AID), an enzyme that catalyses the deamination of cytidines directly on DNA. The rate of mutation in the IgV regions during SHM in the GC is estimated to be as high as 10^3 per base pair per generation (Berek and Milstein 1987). A centroblast that expresses a newly generated antibody mutation needs to be tested for improved antigen binding and this process is thought to take place in the light zone and to be mediated by specific cell types located in this area, like antigen-presenting FDCs and TFH cells (MacLennan 1994). In fact the centrocytes within the LZ are selected according to their ability to bind antigen complexes on FDCs and to elicit help from cognate TFH cells (Tew *et al.* 1993, Fazilleau *et al.* 2009). Positively selected B cells have the 'choice' of recycling to the DZ for further mutation or exiting the GC reaction as memory cells or PCs (Allen *et al.* 2007, Victora *et al.* 2010). Another important process occurring in the LZ is the class-switch recombination (CSR) through which the heavy chain class of the antibody produced by an activated B cell changes from IgM and IgD to IgG, IgA or IgE. The process requires the enzymatic activity of AID and occurs not only within GCs, but also extensively outside of GCs in both T‑cell-dependent and T‑cell-independent responses (MacLennan 1994).

Isotype switching alters the effector functions of an antibody that can be specifically required to clear an antigen by leaving the antibody specificity defined by the rearranged V(D)J region of the heavy chain unchanged. CSR is AID driven and occurs by DNA recombination involving non-homologous end-joining processes between specific repetitive regions of several hundred base pairs (known as switch regions) that precede the immunoglobulin constant region genes (Muramatsu *et al.* 2000). The discovery of AID and the demonstration that it is required for SHM and CSR triggered many of the recent advances in this field (Muramatsu *et al.* 1999). Even though SHM and CSR occur in B cells at the same stage of differentiation, they appear to be very different biochemical processes. There are IgM antibodies expressing V regions that have undergone SHM, as well as IgG and IgA antibodies with no somatic mutations, suggesting that SHM and CSR are independent processes mediated by completely different sets of enzymes (Bemark *et al.* 2000, Manis *et al.* 2002, Reina-San- Martin *et al.* 2003). Recent study suggests that different AID domains recruit factors that differentially mediate the outcomes of SHM and CSR (Ta *et al.* 2003).

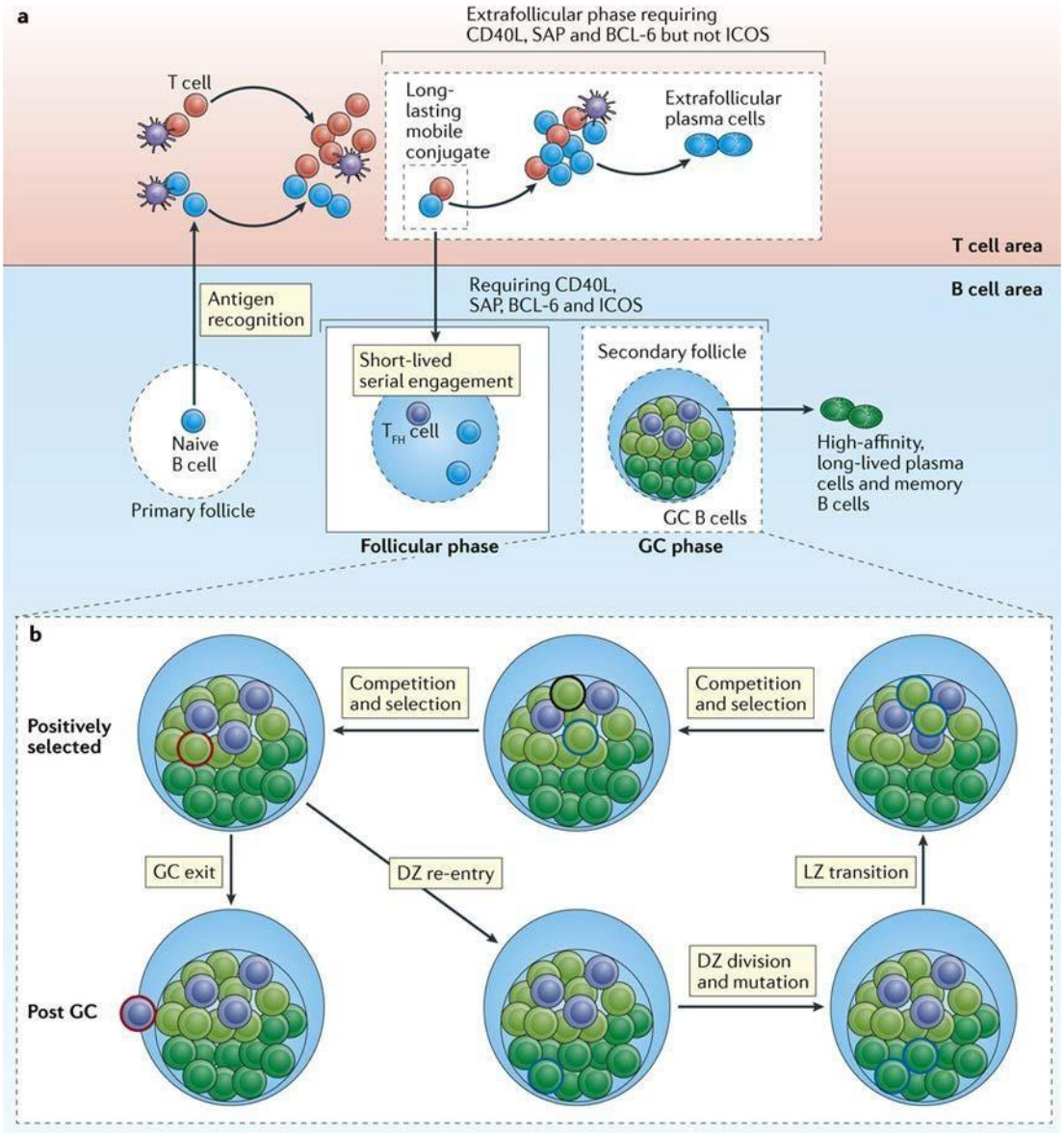

Figure 11. A T cell-dependent B cell response proceeds in three spatiotemporal phases in lymphoid tissues. *a | Upon initial activation by antigen, antigen-specific T cells and B cells interact in the border region between the T cell area and the B cell area, forming long-lived mobile conjugates. With T cell help, some of the clonally expanding B cells differentiate into plasma cells in extrafollicular foci. Some of the activated T cells and B cells move back into the follicle, interacting with each other in an increasingly short-lived manner and supporting continued expansion of the B cell populations. Germinal centres (GCs) develop as B cell clusters coalesce on the follicular dendritic cell network at the follicular centre (not depicted). GCs are divided into the light zone (LZ; light green) and dark zone (DZ; dark green); T follicular helper (TFH) cells are located primarily in the light zone. b | The GC reaction proceeds in a cyclic manner. A B cell in the light zone that receives sufficient T cell help becomes positively selected (red circled) for re-entering the dark zone for multiple rounds of cell division and somatic hypermutation (cyan circled); the resulting progenies transition back into the light zone, acquiring antigen and competing for access to TFH cell-derived help primarily through levels of antigen presentation. Some B cells die (black circled) while some are positively selected and give rise to cells that exit GCs eventually to become*

long-lived plasma cells and memory B cells. BCL-6, B cell lymphoma 6; CD40L, CD40 ligand; ICOS, inducible T cell costimulator; SAP, SLAM-associated protein (Qi 2016).

Memory B cells and long-lived plasma cells

Hallmark of adaptive immune responses is the generation of long-lived protection after primary exposure to a pathogen. In humoral responses, this protection stems from a combination of sustained antibody titers, deriving from long-lived plasma cells (PCs) and long-lived memory B cells. Both types of cells are thought to primarily derive from the germinal center (GC). Memory lymphocytes confer immediate protection in peripheral tissues and mount recall responses to antigens in secondary lymphoid organs (Blanchard-Rohner *et al.* 2009). The contribution of B cells to immunological memory involves two distinct populations of cells that are generated during primary immune responses: long-lived plasma cells (LLPCs), which continue to secrete high levels of functional antibodies for protracted periods of time after antigen clearance, and memory B cells, which can rapidly proliferate and differentiate into PCs following recurrent exposure to the initial immunizing antigen. The maintenance of serological memory by LLPCs was first shown in mice by the groups of Radbruch and Ahmed in the late 1990s. Manz et al. used bromodeoxyuridine (BrdU) incorporation to discriminate long-lived from short-lived PCs in defined murine immune responses. They found that 70% of the specific PCs entered the BM within the first month after immunization, followed by the 30% within the second month. These cells were found to persist for at least another 2 months without proliferation; furthermore despite the antigen clearance they were constantly secreting their antibodies (Manz *et al.* 1997). Slifka et al. investigated the role of PCs in maintaining long-term antibody production using the mouse model of infection with lymphocytic choriomeningitis virus (LCMV). They observed that infected mice continued to produce specific antibodies for 250 days despite being irradiated to inhibit the differentiation of new PCs (Slifka *et al.* 1998). A strong argument for LLPCs in humans comes from the analysis of patients with autoimmune diseases treated with the therapeutic anti-CD20 antibody (rituximab). CD20 is a B-cell restricted antigen expressed on B lymphocytes precursors and mature B cells, thereafter lost during differentiation to plasma cells. In human subjects depleted of $CD20^+$ B cells, total serum IgG and IgA antibody titers decline only initially to remain stable thereafter and still being within the normal range (Cambridge *et al.* 2003, Edwards *et al.* 2004). The long-term persistence of PCs is dependent on continued expression of the prosurvival molecule of the Bcl-2 family, Mcl1 (Peperzak *et al.* 2013). This in turn depends on survival signals derived from the BM microenvironment which contain monocytes, eosinophils, megakaryocytes, $CXCL12^+$ reticular stromal cells, basophils, osteoclasts and osteoblasts. Collectively, these cells provide survival factors that recruit, retain

and sustain BM PCs, like BAFF and APRIL (members of the tumor-necrosis factor superfamily), the cytokine IL-6, and the co-receptor CD28 ligands CD80 and CD86 (Tangye 2011). LLPCs appear an homogenous population whose preferential niche is the BM, whereas memory B cell compartment include different subpopulations harboring different effector functions and can either be tissue resident or recirculate through secondary lymphoid organs. Nevertheless, like LLPCs, memory B cells can persist in the absence of BCR signaling and T cell help (Vieira and Rajewsky 1990, Maruyama *et al.* 2000, Bernasconi *et al.* 2002). As already mentioned a vast majority of memory B cells are generated in GCs and are distinguished from naive B cells by an increased lifespan, faster and stronger response to stimulation and expression of somatically mutated and affinity matured immunoglobulin (Ig) genes (Rajewsky 1996, Klein *et al.* 1998). Memory B cells can be identified by higher expression of costimulatory molecules (SLAM, tumor necrosis factor, TLR7/9, CD27, CD80, CD86 and IL-21 receptor), antiapoptotic molecules (BCL2 family members) and signal transducers (STAT3) (Bernasconi *et al.* 2003, Good and Tangye 2007, Avery *et al.* 2010). Upon reactivation, memory B cells proliferate and either reenter GC reactions or differentiate into PCs which quickly produce large amount of high-affinity antibodies. This is essential to maintain the antibody serum pool as LLPCs lack proliferative potential and cannot supply an additional boost of high-affinity antibodies (Yoshida *et al.* 2010). If reactivated memory B cells reenter GC reactions, they may improve immunity to modified pathogens or infection conditions by downstream CSR and new rounds of affinity maturation (Dogan *et al.* 2009).

The study of long-term memory has generally focused on class switched IgG B cells, which represent approximately 15–20% of peripheral B cells in adults (Fecteau *et al.* 2006). Hence IgM^+IgD^+ B cells were generally assumed to represent antigen-inexperienced expressing variable region genes without somatic mutations. However somatically mutated IgM^+IgD^- B cells have been identified and further classified as IgM-expressing counterpart of classical class-switched memory B cells (Klein *et al.* 1993, 1997). It's now well established that in human two distinct populations of IgM memory can be identified: IgM only, with a GC derivation (Weill *et al.* 2009) and IgM^+IgD^+ memory B cells with a doubtful origin. Reynauld and Weill proposed that at least a fraction of $IgM^+IgD^+CD27^+$ B cells undergo somatic hypermutation in an antigen-independent pre-diversification process, rather than during antigen-driven B-cell responses (Weller *et al.* 2004, Weller *et al.* 2008). An historically method to discriminate memory B cells has been to use CD27 as a marker considering that its expression correlates with SHM in IgM^+IgD^+ cells (Klein *et al.* 1998). CD27 is a member of TNF-receptor family and the interaction with the ligand CD70, expressed on activated T cells, contributes to B cell expansion, differentiation, and

antibody production (Hintzen *et al.* 1994, Kobata *et al.* 1995, Lens *et al.* 1996, Arens *et al.* 2001). Nowadays is widely accepted to use CD27 and IgD as markers to distinguish four main populations: $CD27^{-}IgD^{+}$ antigeninexperienced cells, two subsets of $CD27^{+}$ memory cells (IgD^{+} and IgD^{-}) and $CD27^{-}IgD^{-}$ memory cells. The latter population shares similar morphology with $CD27^{+}$ and includes class switched B cells with mutated Ig genes. Furthermore this population lacks ABCB1 transporter, which discriminates human resting naive B cells from cycling transitional and memory B cells (Wirths and Lanzavecchia 2005, Fecteau *et al.* 2006, Wu *et al.* 2011). $CD27^{-}$ memory cells are present at birth (van Gent *et al.* 2009), and in healthy adult subjects they account for approximately 5% of the peripheral B cell population (Sanz *et al.* 2008). The increase of this subpopulation in the elderly has led to the idea that these cells are aged or exhausted memory B cells (Colonna-Romano *et al.* 2009). It is important to gain a better understanding of the $CD27^{-}$ cell population because it occurs at a greater frequency not only in elderly but also in pathologic situation like systemic lupus erythematosus (SLE), rotavirus infection, and human immunodeficiency virus (HIV) infection (Wei *et al.* 2007, Moir *et al.* 2008). Overall it is emerging that CD27 is not an appropriate marker and this highlight the need to have a precise method to define memory B cells. Despite the importance of memory B cells for vaccine and infection-induced protection (Bevan 2011) there is limited understanding of the nature of these cells, with most information coming from human studies. Presumably because of the constant exposure to antigens, humans have an abundance of memory-like cells. Using a transgenic mouse model in which relative numbers of antigen-specific memory B cells are elevated, several cell surface markers were investigated enabling the definition of memory B cells, which were confirmed in non-transgenic mice. In this mouse system, the costimulatory molecule CD80 has been identified as a memory B cell marker (Anderson *et al.* 2007). In later studies CD80 was combined with CD73 and PD-L2, which resulted in the identification of at least five different subsets of memory B cells in response to immunization (Tomayko *et al.* 2010). The authors suggested that there exists a spectrum of memory B cells consisting of naive-like memory B cells with few mutations and infrequently isotype-switched BCRs, and other more memory-like cells, expressing multiple memory-specific markers, with more frequently isotype-switched BCRs and containing high mutational content. These data demonstrate that there is significantly more heterogeneity within the memory B cell compartment than has been previously appreciated (Tomayko *et al.* 2010).

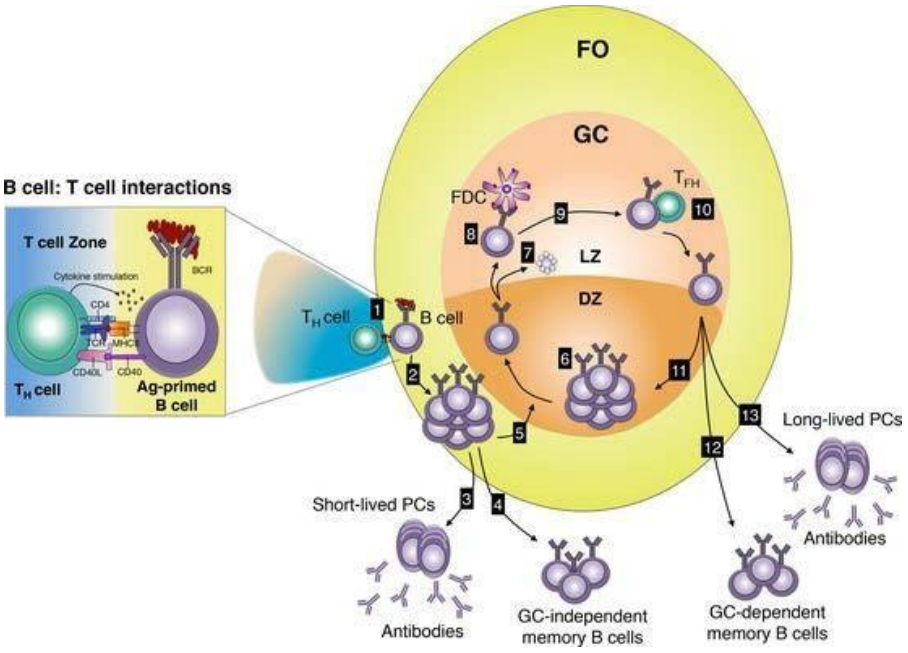

Figure 12. Pathways leading to memory B cell formation. *Two pathways are illustrated of which both are T-dependent, whereas one is germinal centre (GC)-dependent and the other is GC- independent. Depicted is a follicle (FO) with a GC that consists of a dark zone (DZ) and a light zone (LZ). (1) At the B and T cell border, an antigen-activated B cell interacts with its cognate $CD4^+$ helper T cell, here shown as interactions between MHC/antigen and CD40/CD40L, resulting in cytokine production; (2) This would lead to B cell proliferation and adaptation of one of three fates; (3) extrafollicular short-lived antibody-secreting plasma cells (PCs); (4) memory B cell formation independently of a GC reaction; or (5) GC formation in which the B cells can undergo somatic hypermutation (SHM) and/or class switch recombination(CSR); (6) In the DZ of the GC, B cells proliferate. In the LZ, GC B cells either; (7) undergo apoptosis; or (8) become positively selected by follicular dendritic cells (FDC) based on their affinity for antigen; and can then (10) receive further signals from TFH cells to either; (11) re-enter the DZ to initiate another GC cycle, or exit from the GC, to; (12) become a high-affinity memory B cell; or (13) a PC secreting high-affinity antibodies* (Bergmann *et al.* 2013).

1.4 Purinergic signaling

Purinergic pathway in the immune system regulation

Cellular ATP serves as an energy carrier implicated in virtually all metabolic processes. Its potent extracellular actions acting as a messenger were first recognized in 1929 by Drury and Szent-Gyorgy who reported the activities of adenine compounds on the mammalian heart (Drury and Szent-Györgyi 1929). It's now well established that these mediators act as signaling molecules, operating in virtually all tissues through the activation of G-protein–coupled or ligand-gated ion-channel receptors (Burnstock 1997). In addition, several families of ectonucleotidases that hydrolyse ATP to ADP, and AMP to adenosine have been found expressed on the surface of different mammalian cell types (Corriden and Insel 2010). The term purinergic receptor was first introduced to describe classes of membrane receptors that, when activated by either e-ATP (P2

receptors, P2R) or its breakdown product adenosine (P1 receptors, P1R), mediated relaxation of gut smooth muscle (Burnstock 1972, Burnstock *et al.* 1978). Controlled ATP release from intact cells was first discovered in neurons, which release it into neuronal synapses. Since then, many aspects of purinergic signaling in neurons have been elucidated (Abbracchio *et al.* 2009). Additional work revealed that purinergic mechanisms regulate key aspects of many other physiological processes, including activation of the different cell types of the immune system (Bours *et al.* 2006).

The P2R family is subdivided into the P2Y (eight members, P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11- P2Y14) and P2X (seven members, P2X1-7) subfamilies. P2Y receptors (P2YRs) are 7-membrane-spanning proteins, with aminoterminal domain in the extracellular environment and the carboxyterminal on the cytoplasmic side of the plasma membrane. P2YRs are coupled via G-proteins to $Ca2^{+}$ mobilization and cyclic AMP generation/inhibition, as well as to stimulation of the ERK/MAPK pathway (Abbracchio *et al.* 2006). Signal transduction occurs via the classical pathways triggered by the most 7-membrane-spanning receptors: activation of phospholipase C and/or stimulation/inhibition of adenylate cyclase. All the P2Y receptors are activated by ATP, but there is some selectivity, and for instance UTP preferentially triggers P2Y4 and P2Y6 while ATP and UTP are equipotent on P2Y2 (Lustig *et al.* 1993, Communi *et al.* 1996a, Communi *et al.* 1996b). P2X receptors (P2XRs) are homo-heterotrimeric ATPgated ion channels, characterized by two transmembrane hydrophobic domains and both the N-and C-termini on the cytoplasmic side (Stojilkovic and Koshimizu 2001). Signal transduction occurs via fast Na^{+} and Ca^{++} influx and K^{+} efflux, leading to depolarization of the plasma membrane and an increase in the concentration of cytosolic Ca^{++} (North 1996, Surprenant and North 2009). It is likely that the altered intracellular ion homeostasis caused by

P2X receptor opening activates additional intracellular messengers and enzyme pathways (Virgilio *et al.* 2001). Seven genes in vertebrates encode P2X receptor subunits, which are 40–50% identical in amino acid sequence. Each subunit has two transmembrane domains, separated by an extracellular domain. Channels form as multimers of several subunits and homomeric P2X1, P2X2, P2X3, P2X4, P2X5, and P2X7 channels and six functional P2XR heteromeric receptors have been characterized: P2X1/2R, P2X1/4R, P2X1/5R, P2X2/3R (North 2002, Spelta *et al.* 2002, Jiang *et al.* 2003, Aschrafi *et al.* 2004, Nicke *et al.* 2005). All homomeric and heteromeric receptors are activated by ATP but in a receptor-specific manner with EC50 values ranging from nanomolar to submillimolar concentrations. The gating of P2XRs usually consists of three phases: activation phase, triggered by the stimulation with the agonist and characterized by a rapid rising of inward current, desensitization phase with a slowly developing decay phase

in the presence of the agonist and a deactivation phase as a result of ATP removal with a rapid decay of current. The main difference among receptors is in their sensitivity for agonists and their activation and desensitization rates (North 2002).

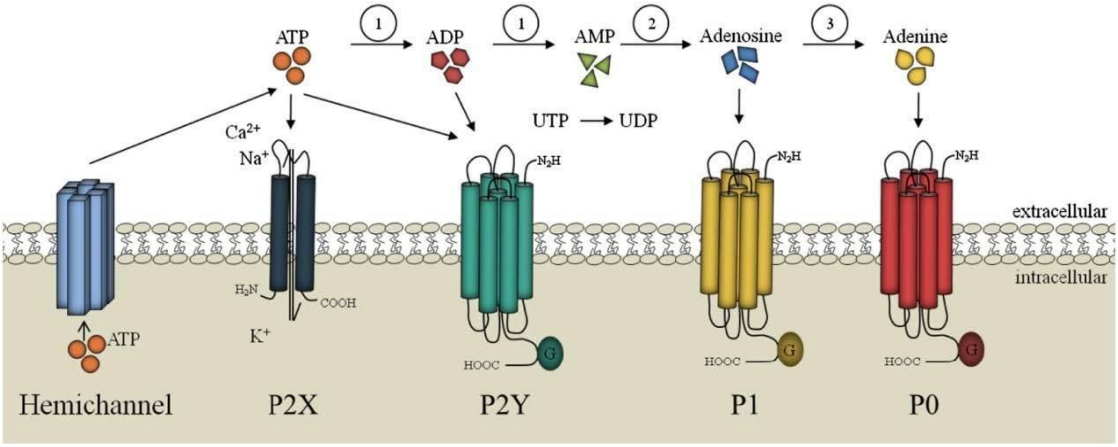

Figure 13. Purinergic receptors and their natural ligands. *Purinergic receptors are divided into P2 receptors which are activated by a variety of nucleotides and can be further subdivided into ionotropic P2X receptors activated by ATP and the metabotropic G-protein-coupled receptors (P2Y) which are stimulated by nucleotides, di- or triphosphates, purines or pyrimidines. In contrast, metabotropic P1 receptors are preferentially activated by adenosine. Recently, evidences for the functional expression of adenine receptors, designated as P0 receptors, have been found. 1: ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) e.g. CD39, 2: ecto-5'-nucleotidase (CD73), 3: purine nucleoside phosphorylase (PNP)* (Kaebisch *et al.* 2015).

Autocrine and paracrine actions of extracellular ATP

Immune cells have developed highly sensitive receptor systems that allow them to execute their many roles in immune surveillance and host defense. In this context extracellular ATP represents an ideal harbinger of cell stress. Indeed in their physiologic state mammalian cells contain high concentrations of ATP (5 to 8 mM) inside the cells, whereas in the extracellular environment the concentration is ~10 nM. The high nucleotide concentration inside the cells and its virtual absence extracellularly in healthy tissues renders ATP also an ideal harbinger of cell death; in fact, pathologic conditions cause the release of ATP from necrotic and apoptotic cells, which can serve as a "find-me" signal that, together with other mediators, attracts monocytes to phagocytose and remove dead or dying cells (Ravichandran 2010, Eltzschig and Eckle 2011). Interestingly it has been shown that inflammasomes activation involves ATP release through pannexin-1 and purinergic signaling through P2X7 receptors. Inflammasomes activation triggers

innate immune by inducing the maturation of pro-inflammatory cytokines, such as IL-1β (Trautmann 2009).

Concomitant to a necrosis dependent ATP release, autocrine/paracrine mechanisms regulating the function of healthy immune system by regulated ATP release, has been shown and three different mechanisms have been proposed so far:

1. Transmembrane diffusion via ATP permeable channels
2. Active transports
3. Exocytosis from vesicles

All these mechanisms of ATP release in the extracellular space contribute to regulate different function of the immune system. For instance, the directed migration of neutrophils, referred to as chemotaxis, requires the temporal and spatial regulation of intracellular signaling pathways allowing the neutrophil to detect a gradient of attractant, polarize, and migrate rapidly toward the highest concentration of the chemoattractant (Li Jeon *et al.* 2002). Interestingly autocrine purinergic feedback mechanisms control several aspects of this process and the stimulation of chemotaxis receptors induce a rapid release of ATP resulting in the accumulation of extracellular ATP near the cell surface closest to the source of chemoattractants. The released ATP can activate multiple adjacent P2Y2 receptors to provide autocrine signal amplification, which greatly increases the intracellular signals generated by chemotactic stimuli. Modifications in the extracellular ATP concentration, caused for example by blocking ATP release or P2Y2 receptors impairs chemotaxis by interfering with the autocrine signaling systems of neutrophils (Chen *et al.* 2006). Similar autocrine signaling systems were recently identified in macrophages (Kronlage *et al.* 2010). Furthermore a number of studies have shown that T cells can release ATP in response to various extracellular stimuli, suggesting that purinergic signaling may indeed play an active role in T cell activation (Filippini *et al.* 1990, Canaday *et al.* 2002). As already mentioned autocrine regulation mechanisms similar to those identified in neutrophils, also regulate T cell activation (Schenk *et al.* 2008, Woehrle *et al.* 2010). However, although neutrophils seem to amplify stimulatory signals using P2Y2 receptors, purinergic signal amplification in T cells occurs through P2X1, P2X4 and P2X7 receptors. Besides the ATP-permeable release channels the ATP-filled vesicles depict another important mechanism which allows the nucleotide release in the extracellular space (Schwiebert and Zsembery 2003). Indeed in human B cells ATP is stored in secretory vesicles released upon BCR triggering in a $Ca2^{+}$-sensitive fashion. Specifically, Schena et al in 2013 showed that in B cells ATP accumulates in late endosomal/vesicular compartments and pointed out the role of TI-VAMP vesicular protein

in the regulated ATP release. This role has been confirmed by the analysis of TI-VAMP-deficient B lymphocytes, which displayed reduced level of extracellular ATP upon stimulation (Schena *et al.* 2013). Extracellular ATP and P2 receptors play a direct role in the modulation of DCs response; expression of P2X7 on mouse DCs, for instance, correlates with antigen-presenting activity (Mutini *et al.* 1999, Ferrari *et al.* 2000). Chronic stimulation with low doses of extracellular ATP has been reported to affect the maturation and antigen presenting functions of DCs. Moreover mature DCs exposed to ATP showed an impaired ability to initiate Th1 response (la Sala *et al.* 2001).

P2X7 receptor

Among the P2RX family, the P2X7 receptor encoded by the *P2rx7* gene is the most abundant and prominent in the immune system (Coddou *et al.* 2011). Of the P2X family, the P2X7 monomeric subunit is the largest, with a length of 595 amino acids for the human, rat, mouse, dog, and Rhesus macaque receptors (Surprenant *et al.* 1996, Rassendren *et al.* 1997, Chessell *et al.* 1998, Roman *et al.* 2009, Bradley *et al.* 2011). Each subunit is characterized by relatively short and long intracellular amino and carboxyl termini respectively, as well as two hydrophobic membrane-spanning segments (transmembrane domains) separated by a long glycosylated extracellular ATP-binding domain. P2X7 receptor is distinguished structurally from other members of P2XRs by its long intracellular C terminus tail containing multiple protein, lipid interaction motifs and a cysteine-rich 18 amino acid segment. This receptor is widely expressed in cells of the immune system, such as macrophages/monocytes, dendritic cells, lymphocytes, and mast cells, as well as in glia cells, including microglia, astrocytes, oligodendrocytes and Schwann cells (Collo *et al.* 1997, Rassendren *et al.* 1997, Chessell *et al.* 1998, Di Virgilio *et al.* 2001, Franke *et al.* 2001, Di Virgilio *et al.* 2009, Skaper *et al.* 2010). Epithelial cells, fibroblasts, osteoblasts, pituitary cells, and some neuronal populations also express P2X7Rs (Groschel-Stewart *et al.* 1999, Koshimizu *et al.* 2000, Sim *et al.* 2004). Stimulation of the receptor with low ATP doses reversibly opens a membrane channel permeable to small cations (Na^+, Ca^{2+}, K^+). Upon sustained stimulation with higher ATP doses or repeated stimulation with sequential ATP pulses, the P2X7 receptor channel undergoes a large increase in conductance and a shift in selectivity, from cation-selective to nonselective. Under these conditions, the plasma membrane becomes permeable to solutes of molecular weight up to 900 Da. Up until now unequivocal demonstrations for the opening of such a peculiar pore are available only for the P2X7R subtype, where extensive studies have documented uptake of various low-molecular weight hydrophilic

fluorescent markers (Steinberg *et al.* 1987, Di Virgilio *et al.* 1989, Di Virgilio 1995). Pore formation has been functionally linked to the long intracellular C-terminus region of P2X7 (Becker *et al.* 2008). However, whether P2X7 it-self directly mediates pore formation by channel dilation or whether other P2X7-associated proteins such as pannexin1 form the pores is still a matter of debate. Activation of P2X7 by ATP results in a number of downstream signaling events which depend on cell type, extracellular conditions and the concentration of extracellular ATP, (Burnstock 2007). The activation requires cell surface ATP levels >100 μM sustained over some minutes. For this reason P2X7 receptors are possibly only activated after injury, infection, or in tumor microenvironments when the concentration of ATP increases locally or when ectonucleotidases are downregulated (Lenertz *et al.* 2011). P2X7 activation acts as a costimulus for the formation of the NALP3 inflammasome and t h e secretion of the proinflammatory cytokines IL-1β and IL-18 and nitric-oxide release (Sperlagh *et al.* 1998, Mariathasan *et al.* 2006, Piccinni *et al.* 2008). Ligation of the P2X7 receptor leads to the activation of NF-kB which controls cytokine expression and apoptosis (Ferrari *et al.* 1997). Furthermore it is well documented that prolonged stimulation of cells using high concentrations of ATP causes cell death via P2X7-induced apoptosis characterized by phosphatidylserine externalization and membrane blebbing (MacKenzie *et al.* 2001). Contrary to the aforementioned role in apoptotic cell death, P2X7 has also been proposed to have a role in cell proliferation. For example T cell activation induces the release of ATP through pannexin-1 channels that translocate with P2X7 receptor to the immune synapse, where they promote calcium influx and cell activation through autocrine purinergic signaling (Figure 14) (Schenk *et al.* 2008, Yip *et al.* 2009). Schenk et al in 2011 showed that P2X7 activity regulates the immunosuppressive function of Treg cells. Particularly they showed that the activation of P2X7 by ATP inhibits the suppressive potential of Tregs and that the pharmacological antagonism of P2X receptors promoted the cell-autonomous conversion of naïve $CD4^+$ T cells into Tregs after TCR stimulation (Schenk *et al.* 2011). Further, Proietti et al identified a novel role of the P2X7 receptor as an important regulator of the adaptive IgA response in the small intestine. Deletion of *P2rx7* resulted in the expansion of TFH with consequent enhanced germinal center reaction which in turn resulted in higher affinity IgA production and depletion of mucosal bacteria in *P2rx7*$^{-/-}$ mice (Proietti *et al.* 2014).

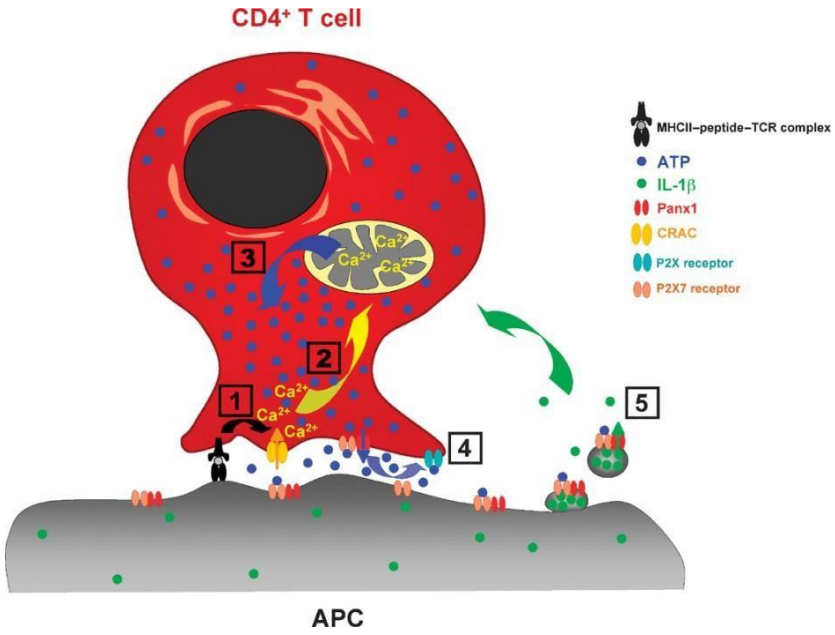

Figure 14. Purinergic control of T cell activation. *Schematic representation of a T cell (red) interacting with a professional APC (gray). The early phase of T cell activation, which is triggered by the interaction of the TCR with the MHC–peptide complex, is characterized by CCE (step 1), which is accompanied by mitochondrial uptake of Ca2+ (step 2) and Ca2+-dependent stimulation of ATP synthesis (step 3). ATP is released from activated T cells through pannexin hemichannels, which open when the cytosolic [Ca2+] is elevated. This mechanism guarantees a high concentration of pericellular ATP, which by binding to P2XRs, serves as an indispensable autocrine costimulus for productive T cell activation (step 4). Finally, ATP released from activated T cells might also modulate APC function, such as secretion of IL-1β (step 5) (Schenk et al. 2008).*

Extracellular ATP metabolism: role of CD39 and CD73

Following its release into the extracellular space, ATP is rapidly hydrolysed by ectonucleotidases in a two-step enzymatic process which is important for calibrating the duration, magnitude and composition of the "purinergic halo" surrounding the immune cells. The first step provides for ATP and ADP conversion to AMP through the ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and it is followed by a second step mediated by ecto-5'-nucleotidase (CD73) which converts extracellular AMP to adenosine (Yegutkin 2008). Besides CD39 and CD73, which are the major ectonucleotidases that regulate immunity and inflammation, there are other less understood enzymes involved in the catabolism of extracellular nucleotides, which include alkaline phosphatases, pyrophosphatases, and phosphodiesterases as well as the counteracting ATP-regenerating ectoenzymes adenylate kinase and nucleoside-diphosphate kinase (Yegutkin 2008). The expression and activity of both CD39 and CD73 undergo dynamic changes in accordance with environment context, contributing to the outcome of several pathophysiological conditions, such as infections, autoimmune diseases and cancer (Deaglio and Robson 2011, Zhang 2012, Bastid *et al.* 2013). Ectonucleoside triphosphate diphosphohydrolases (ENTPDases) hydrolyze nucleotide diphosphates and triphosphates to generate nucleotide monophosphates, and they are the largest family of ectonucleotidases, consisting of ENTPDase1 to 8, including E-NTPDase1 (CD39), E-NTPDase2 (CD39L1), E-NTPDase3 (CD39L3), E-NTPDase5 (CD39L4), and ENTPDase6 (CD39L2) (Zimmermann 2000). CD39 is a 510-amino acid membrane protein which hydrolyzes ATP, and less efficiently ADP, in a $Ca2^{+}$- and Mg^{2+}-dependent manner (Heine *et al.* 2001). Structurally it is characterized by two transmembrane domains, a small cytoplasmic domain and a large extracellular domain consisting of five regions, known as apyrase conserved regions (ACR) 1-5, important for the enzymatic activity. The localization on the cell surface is essential for the catalytic activity and post translational glycosylation is crucial for correct protein folding, membrane targeting and enzyme activity (Smith and Kirley 1998). CD39 expression is regulated by several pro-inflammatory cytokines, oxidative stress and hypoxia and is primarily associated with endothelial cells and immune cell populations, specifically B cells, natural killer (NK) cells, dendritic cells, Langerhans cells, monocytes, macrophages, neutrophils and Treg cells (Dwyer *et al.* 2007). In addition, the expression of CD39 is increased in several solid tumors (colorectal cancer, head and neck cancer, pancreatic cancer) as well as in chronic lymphocytic leukemia, suggesting this enzyme is also involved in the development and progression of malignancies (Bastid *et al.* 2013). The second step in the metabolism of purine nucleotides is catalyzed by ecto-5'-nucleotidase CD73, which

is considered as the rate limiting enzyme responsible for the irreversible dephosphorylation of AMP into adenosine (Hunsucker *et al.* 2005). Notably the hydrolysis of ATP to AMP by CD39 is reversible thanks to the action of membrane-bound ATP regenerating enzymes (Yegutkin 2008); CD73-mediated conversion of AMP to adenosine is instead virtually irreversible but is actually possible after the uptake of extracellular adenosine and the conversion into AMP by intracellular adenosine kinase (AK) (Beavis *et al.* 2012). In this context, CD73 can be considered as a crucial regulator of the extracellular ATP/adenosine balance.

CD39 and CD73 in the immune system

The generation of adenosine by CD39 and CD73 can be viewed as "immunological switches" that shift ATP-driven pro-inflammatory activity toward an anti-inflammatory state mediated by adenosine. This system has to be tightly regulated in order to preserve and restore homeostasis. Several regulatory mechanisms are used to maintain immune equilibrium and in this regard Treg cells are considered the primary mediators of peripheral tolerance (Sakaguchi *et al.* 2008). It has been shown that CD39 and CD73 expression in $Foxp3^+$ Tregs synchronized with the activation status of these cells. Indeed murine Treg display increased ATPmetabolizing activity and this feature appears to be crucial for the immunosuppressive activity of these cells (Mandapathil *et al.* 2009, Ernst *et al.* 2010). Deaglio et al showed that the inhibitory control by Treg-derived adenosine is mediated by the engagement of adenosine A2A receptors on effector T cells (Deaglio *et al.* 2007). In humans, it has been reported that the expression of CD39 on peripheral $CD4^+$ T cells is associated with regulatory $Foxp3^+$ signatures and the acquisition of a memory phenotype. In contrast to murine Treg phenotype, CD73 is not substantially co-expressed but it accumulates in the cytoplasmic compartment (Mandapathil *et al.* 2010). It has been suggested that human Treg cells secrete CD73 which may in turn convert AMP to adenosine. Alternatively other AMP-metabolizing enzymes may contribute to the adenosine generation (Antonioli *et al.* 2013). In addition to Treg other T cell subsets express the CD39 and CD73 ectonucleotidases. For instance in Th17 cells the expression of both enzymes is tightly regulated by cytokines like IL6 and TGFβ which control the immunosuppressive function of these cells (Chalmin *et al.* 2012).

As already mentioned, ATP can be released by neutrophils and act in an autocrine fashion modulating the migration capacity of these cells (Chen *et al.* 2006). Additionally it has been reported that neutrophils widely express CD39 and to some extent CD73 and that adenosine also plays a critical role in the polarization and migration of these cells (Pulte *et al.* 2007, Flogel *et*

al. 2012). Hence inadequate enzymatic activity has been associated with uncontrolled cell activation and amplified chemotactic functions (Corriden *et al.* 2008). The purine concentration in the extracellular space is also important for the regulation of macrophages activity and differentiation. In particular pro-inflammatory M1 macrophages decrease the expression and the activity of both CD39 and CD73 resulting in reduced level of ATP degradation. By contrast, anti-inflammatory M2 macrophages showed increased expression and activity of both catalytic enzymes, resulting in the generation of adenosine-rich environment (Csoka *et al.* 2012). Ectonucleotidases are involved in the modulation of dendritic cells functions. For instance CD39 has been proposed to prevent the desensitization of P2 receptors, which are required for the optimal T cells stimulation by dendritic cells (Mizumoto *et al.* 2002).

CD73 is a human orthologue of bacterial monomeric 5'-nucleotidases, indicating a common evolutionary origin of these enzymes (Zimmermann 1992). The catalytic properties are instead different, since the human enzyme catalysis the hydrolysis of AMP into adenosine and in contrast the bacterial ecto-nucleotidases are able to hydrolyze AMP, ADP and ATP and other 5-ribo- and 5-deoxyribonucleotides (Neu 1967). Structurally CD73 is a dimer of two identical 70 kD subunits, anchored to the plasma membrane via a C-terminal serine residue, linked to glycosyl-phosphatidylinositol (GPI). Each subunit of the dimer consists of two structural domains: the N-terminal, which contains the metal ion binding site and the C-terminal domain which contains the substrate binding site and dimerization interface. The two domains are linked by a small hinge region, which enables the enzyme to undergo large domain movements and thereby switch between the open and closed conformations (Knapp *et al.* 2012). Switching movements from one conformation to another are responsible for substrate selectivity and catalytic reaction (Knapp *et al.* 2012). A soluble form of CD73 can be shed from the membrane through proteolytic cleavage or hydrolysis of the GPI anchor by phosphatidylinositol-specific phospholipase (Strater 2006, Heuts *et al.* 2012). Those soluble forms have been described in several tissues, including serum (Chuang *et al.* 1984) as well as seminal and synovial fluids (Johnson *et al.* 1999, Fini *et al.* 2003).

CD73 can be detected in several mammalian tissues including colon, brain, kidney, liver, lung and heart, spleen, lymph nodes, thymus and BM. The expression and the function of the enzyme are both up-regulated under hypoxic conditions and by pro-inflammatory mediators like TGF-β, INFs, TNF-α and IL-1β (Thompson *et al.* 2004, Regateiro *et al.* 2011). Plasma membrane-bound CD73 has been shown to be involved in lymphocytes-endothelial cells interaction, cell-matrix interactions and a signal transducing molecule in the immune system. A subpopulation of peripheral blood lymphocytes expresses CD73, and specifically the majority of B cells and $CD8^+$

T cells and only about 10% of $CD4^+$ T cells (Thompson *et al.* 1987, Airas *et al.* 1995). Owing to its ubiquitous expression and to its pivotal role in adenosine homeostasis, CD73 has been implicated in many physiological functions and disorders (Colgan *et al.* 2006). In particular, its role as a costimulatory molecule in T cell activation has been well established (Resta *et al.* 1994, Gutensohn *et al.* 1995). Furthermore, recently CD73 has been described as functional enzymatic marker expressed on peripheral B cell subsets (Schena *et al.* 2013). Peripheral human B cells co-expressing CD73 and CD39 have a more prompt capacity to expand and differentiate into antibody secreting cells *in vitro*. This property has been linked to the hydrolyzing enzymatic function of CD39 and CD73, which generate in coordination adenosine starting from extracellular ATP, pointing out for the first time a role of adenosine in activating instead of inhibiting the immune system (Figure 15). Remarkably common variable immunodeficiency patients displaying low levels of IgG and IgA showed a reduced CD73 surface expression (Schena *et al.* 2013). In addition, it has been observed that the expression of CD73 on follicular dendritic cells is crucial for the adhesive interaction between these cells and the germinal center B cells (Airas and Jalkanen 1996, Airas 1998). Recently, more studies have been focused in understanding the role of adenosinergic pathway in autoimmune disease development. Rheumatoid arthritis (RA) for instance is a chronic autoimmune disease associated with joint destruction and characterized by synovial infiltration of inflammatory cells secreting proinflammatory cytokines. A protective role of CD73 in RA pathogenesis has been pointed out (Chrobak *et al.* 2015). Specifically by using collagen-induced arthritis (CIA) mouse models, which is a commonly used mouse model for RA study, Chrobak et al. found that CD73-deficient mice were significantly more susceptible to CIA than wild-type (WT) mice. CD73 deficiency resulted in an increased production of proinflammatory cytokines in joint and increased joint destruction (Chrobak *et al.* 2015). Furthermore increasing evidence has suggested that adenosinergic system may be an important factor in multiple sclerosis (MS) pathophysiology. MS is a chronic inflammatory autoimmune disease of the central nervous system (CNS), due to an immune reaction against myelin proteins. The etiology of MS is still unclear (Loma and Heyman 2011). Mills et al. demonstrated that CD73-deficient mice were more resistant to experiment autoimmune encephalomyelitis (EAE), which is the classical animal model of MS. In addition, it was observed that the infiltration of lymphocytes into CNS in CD73-deficient mice was fewer than that in WT mice (Mills *et al.* 2008). CD73 has also been found to be involved in autoimmune type 1 diabetes development. Tai et al. have showed that TLR9 deficiency in non-obese diabetic mice could significantly protect from disease development via upregulating CD73 expression (Tai *et al.* 2013). Additionally, lower expression of CD73 has been found on synovial

fluid mononuclear cells (SFMCs) extracted from juvenile idiopathic arthritis (JIA) patients, compared to peripheral cells extracted from both JIA patients and healthy controls (Botta Gordon-Smith *et al.* 2015). JIA is a chronic autoimmune rheumatic disease of childhood with unknown etiology, characterized by swelling of the joints and thickening of synovial lining (Woo and Wedderburn 1998). Moreover, low expression of CD73 on T and B cells in inflamed site is correlated with the clinical severity of JIA patients. This finding suggested that the decreased CD73 expression in SFMCs would lead to a decreased potential for anti-inflammatory activity and then, the deterioration of disease (Botta GordonSmith *et al.* 2015). Like CD39, also CD73 is found overexpressed in several type of cancer, including bladder cancer, leukemia, glioma, glioblastoma, melanoma, ovarian cancer, colon cancer and breast cancer (Allard *et al.* 2012, Beavis *et al.* 2012). CD73 transcripts are induced by hypoxia-inducible factor (HIF)-1 (Synnestvedt *et al.* 2002) and the overexpression observed in tumor is at least in part a consequence of the hypoxic nature of the tumor microenvironment. Lately, CD73 has been widely used as a marker for mouse B cell memory. In T-dependent model of immunization, CD80 combined with CD73 and PD-L2 resulted in the identification of at least five different subsets of memory B cells. These data indicate that the diversity of memory B cells is considerable and the authors suggested the presence of a spectrum of memory B cells, where the high and concomitant expression of CD80, PD-L2 and CD73 define a "more memory like" phenotype (Tomayko *et al.* 2010).

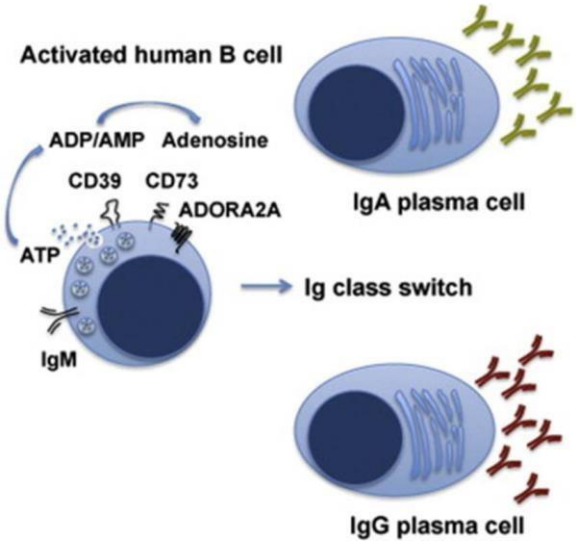

Figure 15. Dependence of Immunoglobulin CSR in B Cells on vesicular release of ATP and CD73 ectonucleotidase activity. *In human B cells, ATP is stored in secretory vesicles that are released in a Ca^{2+}-sensitive fashion upon BCR stimulation. CD73-dependent adenosine generation favors CSR, endowing the B cell with an intrinsic control of differentiation toward Ig class-switched plasma cells* (Schena *et al.* 2013).

Effect of adenosine on the immune system

Under physiological conditions, adenosine is constantly present at low level in the interstitial fluids of unstressed tissues. However the concentration of this nucleoside can rapidly increase in response to pathophysiological conditions, such as hypoxia, ischaemia, inflammation or trauma. In these conditions, after being released in the extracellular space, adenosine behaves like a danger signal and activates specific receptors on target cells aiming to restore tissue homeostasis and promote healing processes (Hasko *et al.* 2008). Four distinct G-protein- coupled P1 receptors have been cloned: A1, A2A, A2B and A3. A1, A2A and A2B receptors are conserved throughout evolution and are highly homologous (80-95%), whereas A3 receptors vary substantially among species (Antonioli *et al.* 2008). Unlike A1, A2A and A3 receptors that can be stimulated by levels of adenosine ranging between 30 and 300 nM, the low-affinity A2B receptor requires higher concentrations of adenosine to be activated, such as those encountered for example in hypoxic microenvironments (Fredholm *et al.* 2001). The A1 and A3 receptors are coupled to G proteins from the Go and Gi family and their activation is linked to the inhibition of adenylyl cyclase, decreasing intracellular cyclic AMP (cAMP) levels. The stimulation of A1 and A3 receptors can also elicit the release of calcium ions from intracellular stores (Hoskin *et al.* 2008). On the other hand signaling mechanism of A2A and A2B receptors relies on the stimulation of adenylyl cyclase by Gs proteins, increasing cAMP synthesis (Ralevic and Burnstock 1998, Klinger *et al.* 2002). As a consequence, activation of A2A and A2B receptors on immune cells induce strong immunosuppressive effects through the cAMP/PKA-mediated inhibition of NF-kB, TCR and JAK-STAT signaling pathways (Palmer and Trevethick 2008). Moreover, all adenosine receptors couple to mitogen-activated protein kinase (MAPK) pathways, which include extracellular signal-regulated kinase 1 (ERK1), ERK2, JUN N-terminal kinase and p38 MAPK (Hoskin *et al.* 2008). Adenosine receptors signaling depend on the level of extracellular adenosine, which is determined by a complex ecto-enzyme machinery and uptake system. Adenosine biological activity is further regulated by an ecto-adenosine deaminase (ADA) which degrades it into inosine, or via the reuptake of adenosine by equilibrative and/or concentrative transporters (Baldwin *et al.* 2004, Gray *et al.* 2004, Antonioli *et al.* 2012).

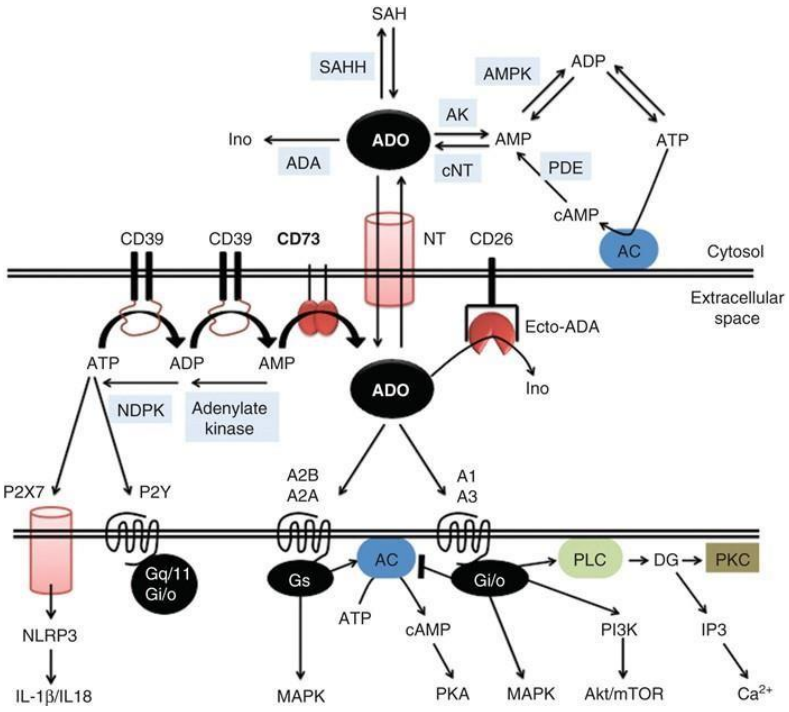

Figure 16. The adenosinergic system. *Extracellular adenosine production results from the concerted action of CD39 and CD73. CD39 catalyses the degradation of ATP into AMP, whereas CD73 hydrolyses AMP into adenosine. ATP breakdown into AMP is reversible through the action of membrane-bound regenerating enzymes, namely, adenylate kinase and NDPK. In contrast, AMP hydrolysis into adenosine by CD73 is irreversible. Nevertheless, CD73-generated adenosine can regenerate AMP after reuptake in the intracellular medium through NT and subsequent phosphorylation by AK. Of note, adenosine can also be produced intracellularly through the dephosphorylation of AMP by cytosolic 5'-nucleotidase (cNT). Biologically active adenosine can be catabolized into inosine by intracellular or membrane-bound ADA. Importantly, CD26 or dipeptidyl peptidase 4 can form a complex with ADA and allows its expression at the cell surface. Extracellular adenosine exerts its biological effects through the engagement of four distinct subtypes of adenosine receptors, namely, A1, A2A, A2B and A3 receptors. Those receptors are differently coupled to intracellular G proteins, which can then trigger various signaling pathways. In general, A1 and A3 receptors are coupled to Gi/o and inhibit the production of cAMP by AC. On the contrary, A2A and A2B receptors are commonly coupled to Gs, which activates the cAMP/PKA pathway. Furthermore, activation of the PLC/IP3/calcium pathway has also been documented for A1 and A3 receptors. In addition to the activation of the canonical G-protein-associated pathways, adenosine receptors have been shown to trigger the MAPK and PI3K/Akt/mTOR pathways. In the case extracellular ATP is not degraded by the ectoenzymatic machinery, ATP can activate purinergic P2X and P2Y receptors. Signaling through the P2X7 receptor has been shown to trigger the NLRP3-dependent production of inflammatory cytokines such as IL-1β and IL-18* (Allard et al. 2014).

Adenosine receptors are abundant on the surface of many innate immune cell types, including neutrophils which express all four P1 receptors. Low concentration of adenosine enhances adhesion property of this cell subset, whereas higher levels of adenosine, mainly acting through A2A receptors, inhibit neutrophils recruitment to the endothelium probably by decreasing neutrophil integrin expression (Sullivan *et al.* 2004). Furthermore, the involvement of A2B receptors in regulating neutrophil transmigration is as well documented. Thus activation of A2B

on neutrophils diminishes the ability of these cells to transmigrate through endothelial membranes (Wakai *et al.* 2001).

The pattern of adenosine receptor expression on DCs changes during their development. Indeed immature human myeloid DCs (mDC)s express dominantly A1 and A3 receptors, while mature mDCs predominantly express A2A receptors (Panther *et al.* 2001, Fossetta *et al.* 2003). Similarly, functional A1 receptors have been found expressed in human immature plasmacytoid DCs (pDCs). Upon pDCs maturation, the level of A1 receptor decreases and the expression of A2A receptor is instead robustly increased (Schnurr *et al.* 2004). Stimulation of A1 and A3 adenosine receptors on immature DC cells promotes their migration to the site of infection or tissue injury. On the other hand, activation of A2A receptors on mature human DCs changes their cytokine expression profile, shifting toward an anti-inflammatory type, with reduced IL-12, IL-6, IFN-α, and TNF-α release and enhanced IL-10 production (Panther *et al.* 2001, Panther *et al.* 2003, Schnurr *et al.* 2004). Furthermore adenosine receptors regulate many functions of adaptive immune cells. T cells for instance mainly express A2A receptor and studies in a knock out mouse model have shown a major inhibitory effect of A2A activation on IL-2 production and proliferation of $CD4^+$ cells after TCR engagement (Naganuma *et al.* 2006, Sevigny *et al.* 2007). A2A receptor activation also suppresses the synthesis of both Th1 and Th2 cytokines by naïve $CD4^+$ (Lappas *et al.* 2005, Naganuma *et al.* 2006), as well as polarized Th1 and Th2 lymphocytes (Csoka *et al.* 2008). As already mentioned Treg cells can produce adenosine and thereby regulate immune cell function. The first evidence that adenosine might be involved in Treg functions was derived from an in vivo experimental colitis study, where Tregs failed to control colitis by effector T cells that lacked the A2A receptor, suggesting that adenosine contributed to the anti-inflammatory effect conferred by Tregs (Naganuma *et al.* 2006). Tregs express A2A receptors and A2A receptor activation augments the level of FOXP3 transcription factor, which is a key Treginducing factor (Zarek *et al.* 2008). Additionally, FOXP3 upregulates CD39 expression thereby creating a positive feedback circle for amplifying adenosine signaling by Tregs (Borsellino *et al.* 2007). Altogether, these data strongly support the concept that adenosine is a crucial mediator of Treg responses.

As already mentioned, human B cells store ATP in secretory vesicles which are released in the extracellular space upon BCR stimulation. ATP is further metabolized in a CD73-dependent mechanism generating adenosine, which in turn promotes class switch recombination (Schena *et al.* 2013). Schena et al pointed out for the first time a role of adenosine in activating instead of inhibiting the immune system.

Worthy of note, alternative pathways for extracellular adenosine generation have been described. This includes the production of adenosine from nicotinamide dinucleotide (NAD) instead of ATP, by the concerted action of CD38, CD203a and CD73 (Horenstein *et al.* 2013), and the CD73-independent production of adenosine by other phosphates such alkaline phosphatase or prostate-specific phosphatase (Street *et al.* 2013).

2. Results

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ATP-Gated Ionotropic P2X7 Receptor Controls Follicular T Helper Cell Numbers in Peyer's Patches to Promote Host-Microbiota Mutualism

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SUMMARY

Microbial colonization of the gut induces the development of gut-associated lymphoid tissue (GALT). The molecular mechanisms that regulate GALT func- tion and result in gut-commensal homeostasis are poorly defined. T follicular helper (Tfh) cells in Peyer's patches (PPs) promote high-affinity IgA responses. Here we found that the ATP-gated ionotropic P2X7 receptor controls Tfh cell numbers in PPs. Lack of P2X7 in Tfh cells enhanced germinal center reactions and high-affinity IgA secretion and binding to com- mensals. The ensuing depletion of mucosal bacteria resulted in reduced systemic translocation of micro- bial components, lowering B1 cell stimulation and serum IgM concentrations. Mice lacking P2X7 had increased susceptibility to polymicrobial sepsis, which was rescued by Tfh cell depletion or adminis- tration of purified IgM. Thus, regulation of Tfh cells by P2X7 activity is important for mucosal colonization, which in turn results in IgM serum concentrations necessary to protect the host from bacteremia.

INTRODUCTION

Host physiology and diet influence the development of the gut ecosystem (Muegge et al., 2011). Reciprocally, through interactions with the host, the gut microbiota influences physiology and disease susceptibility (Lozupone et al., 2012), a beneficial homeostatic relationship that is ensured by several layers of control, including mucus, antimicrobial factors, and innate and adaptive immunity (Henao-Mejia et al., 2012; Littman and Pamer, 2011; Sun et al., 2007; Vaishnava et al., 2011).

The gut microbiota promote the development of the gut-associated lymphoid tissue (GALT) (Macpherson and Harris, 2004), which is responsible for intestinal IgA production in response to microbial stimulation (Cerutti and Rescigno, 2008; Craig and Cebra, 1971). In the Peyer's patches (PPs) of the small intestine, T follicular helper (Tfh) cells support affinity maturation of B cells in germinal centers (GCs) (Crotty, 2011) and differentiation of B cells to IgA-secreting plasma cells, a process that is critical in maintaining intestinal homeostasis and efficient mucosal defense (Wei et al., 2011). Moreover, some evidence suggests that affinity maturation of IgA can also affect systemic immunity. For example, it has been shown that recognition of luminal bacteria by secretory IgA prevents their access to the lamina propria and systemic proinflamma- tory stimulation by bacterial products (Johansen et al., 1999; Macpherson and Uhr, 2004; Sait et al., 2007), a result in agree- ment with the observation that IgA with reduced bacterial bind- ing capacity results in systemic hyperactivation of the immune system (Kawamoto et al., 2012).

Purinergic receptors in the plasma membranes of eukaryotic cells comprise ATP-gated ionotropic P2Xs (P2X1-7) and guanine nucleotide-binding protein (G protein)-coupled P2Ys (P2Y1, 2, 4, 6, 11–14, which bind also ADP, UDP, UTP, or UDP-glucose) (Burnstock, 2007). ATP released during tissue damage acts as a danger-associated molecular pattern (DAMP) for cells of the innate immune system through stimulation of P2 receptors. P2rx7, which encodes the ATP-gated P2X7 receptor, is a signature gene of effector T cell subsets (Choi et al., 2013; Gavin et al., 2007; Hale et al., 2013; Hill et al., 2008). We have previously shown that P2X7 activity regulates the immunosuppressive function of regulatory T (Treg) cells (Schenk et al., 2011), and persistent P2X7 stimulation by ATP results in pyroptosis-like cell death of Treg cells (Taylor et al., 2008). In fact, sustained P2X7 activation leads to the formation of a pore permeable to molecules up to 900 Da.

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We found that P2rx7 is selectively and highly expressed in Tfh cells. Deletion of P2rx7 in Tfh cells resulted in resistance to cell death, enhanced GC reaction in PPs, increased IgA binding to commensals, and reduction of mucosal bacteria. Our results unravel P2X7 as a regulator of the adaptive IgA response in the small intestine to allow commensalism and systemic stimulation of the immune system.

RESULTS

Tfh Cell Numbers Are Increased in the PPs of P2rx7$^{-/-}$ Mice

Histochemical analysis of the intestine from P2rx7$^{-/-}$ mice revealed enlarged PPs with increased cellularity as compared to wild-type (WT) mice (Figure 1A); ileal and colonic epithelium were otherwise histologically normal (Figure S1A available online). Flow cytometry of cells from P2rx7$^{-/-}$ PPs revealed an increase of Tfh cells identified by expression of the C-X-C chemokine receptor type 5 (CXCR5) (Fo¨ rster et al., 1996), CD28 family member inducible T cell costimulator (ICOS) (Choi et al., 2011), the transcription factor B cell lymphoma 6 (Bcl6) (Nurieva et al., 2009; Yu et al., 2009), programmed cell death-1 (PD1) (Haynes et al., 2007), and CD40L (Figure 1B). In addition to the increase in Tfh cell numbers, mean fluorescence intensities (MFIs) of ICOS and CD40L were increased in Tfh cells from P2rx7$^{-/-}$ mice (Figure 1C). Quantitative real-time PCR on various cell subsets purified from PPs of WT mice, namely CD19^{+} B cells, Fas^{+}PNA^{+} GC B cells, CD3^{+}CD4^{+} T cells, Treg cells, and Tfh cells, revealed highest expression of P2rx7 transcripts in Tfh cells (Figure 1D). P2X7 was the only P2X receptor subtype expressed in PPs Tfh cells (Figure 1E). In fact, these cells showed the strongest response to stimulation with the P2X7 agonist 3^{0}-O- (4-Benzoyl)benzoyl ATP (BzATP) (see below). In Tfh cells isolated from P2rx7$^{-/-}$ PPs, we observed higher expression of Bcl6 transcripts and concomitant reduction in transcripts encoding the Bcl6 antagonist Blimp1 (Figure 1F; Johnston et al., 2009). Expression of Bcl6 inhibits CD4 T cell polarization toward T help- er 1 (Th1) and Th17 phenotypes (Nurieva et al., 2009; Yu et al., 2009). Accordingly, the Th17-cell-specific transcripts Rorc and Il17a and Th1-cell-specific transcripts Tbx21 and Ifng were all reduced in Tfh cells from P2rx7$^{-/-}$ PPs (Figure 1F).

The activity of the P2X7 receptor is influenced by a strain poly- morphism (P451L) in the cytoplasmic domain. Proline-bearing BALB/c mice are more sensitive to P2X7 stimulation by ATP than leucine-bearing C57BL/6 mice (Adriouch et al., 2002). We observed a progressive increase in Tfh cells across BALB/c, C57BL/6, and finally P2rx7$^{-/-}$ mice, indicating that P2X7 respon- siveness inversely correlates with Tfh cell number (Figure S1B). These observations suggest that P2X7 activity limits the abun- dance of Tfh cells in PPs.

Expansion of Tfh Cells in PPs of P2rx7$^{-/-}$ Mice Is Cell Intrinsic

To rule out that other cell subsets contributed to the increase of Tfh cell numbers in PPs from P2rx7$^{-/-}$ mice, we adoptively transferred either WT or P2rx7$^{-/-}$ CD4 Foxp3^{EGFP} T cells into Cd3e$^{-/-}$ mice. This model of transfer into lymphopenic mice has previously demonstrated selective conversion of FoxP3-expressing cells into Tfh cells in PPs (Tsuji et al., 2009).

Foxp3^{EGFP} cells from P2rx7$^{-/-}$ mice did not differ from the WT counterpart in the expression of gut-homing receptors (CD103 and CCR9) (Annacker et al., 2005; Zabel et al., 1999) (data not shown). PPs from Cd3e$^{-/-}$ mice adoptively transferred with Foxp3-expressing P2rx7$^{-/-}$ cells showed a prominent increase of Tfh cells (Figure 2A). To more directly address the role of P2X7 in Tfh cells, we competitively transferred Cd3e$^{-/-}$ mice with Tfh cells purified from PPs of WT (CD45.1^{+}) and P2rx7$^{-/-}$ (CD45.2^{+}) mice at a 1:1 ratio. After 1 month, more than 90% of the Tfh cells recovered from PPs of reconstituted mice originated from P2rx7$^{-/-}$ mice, indicating that P2rx7 deletion conferred greatly enhanced reconstitution potential to Tfh cells (Figure 2B).

To exclude the possibility that the expansion of Tfh cells in P2rx7$^{-/-}$ mice was driven by inheritable differences in intestinal microbiota we analyzed Tfh cells in PPs from cohoused WT and P2rx7$^{-/-}$ mice, which were either delivered through caesarian and cofostered (CoF) with CD1 adoptive mothers (Figure 2C) or cross-fostered (CrF) with reciprocal WT and P2rx7$^{-/-}$ mothers

(Figure 2D) or cobred (CoB) with WT and P2rx7$^{-/-}$ mothers (Figure 2E). In all these breeding conditions, P2rx7$^{-/-}$ mice sacrificed at 8 weeks of age showed an increase in Tfh cell numbers. Finally, reconstitution of cohoused Cd3e$^{-/-}$ mice with WT or P2rx7$^{-/-}$ Foxp3-expressing T cells also showed the selective expansion of Tfh cell numbers in mice transferred with P2rx7$^{-/-}$ cells (Figure 2A). Taken together, these results demonstrate that expansion of Tfh cell numbers is T cell intrinsic and not secondary to inheritable dysbiosis in P2rx7$^{-/-}$ mice.

P2X7 Mediates Tfh Cell Death

We analyzed PPs from WT and P2rx7$^{-/-}$ mice by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). This assay revealed the reduction of positive cells in P2rx7$^{-/-}$ mice (Figure 3A). Because P2X7-mediated cell death is charac- terized by phosphatidylserine (PS) exposure, we analyzed PP cells for Annexin V staining by flow cytometry; more than 20% of ex vivo Tfh cells were labeled by Annexin V in WT but not P2rx7$^{-/-}$ mice, whereas Annexin V staining in other PP cell subsets was consistently lower and indistinguishable between the two strains (Figure 3B). These results indicate that P2X7 expression correlates with selective Tfh cell death in PPs.

In peripheral lymph nodes and spleen of nonimmunized animals, Tfh cells are virtually absent, and P2rx7$^{-/-}$ mice did not show differences with respect to WT animals (Figure S2A). In contrast, Tfh cells could be detected in mesenteric lymph nodes (mLNs) because of constant antigenic challenge. As observed in PPs, significant increases in Annexin V^{+} Tfh cells were detected in mLNs from WT mice, suggesting that P2X7 also regulates Tfh cell representation in LNs (Figure S2B; Wilhelm et al., 2010). The increase in cell death of PP Tfh cells in WT mice was confirmed with VAD-FMK staining detecting acti- vated caspases and by increased expression of proapoptotic bik (Boyd et al., 1995) and bax (Oltvai et al., 1993) transcripts (Fig- ure S2C). An analogous result together with selective increase in Annexin V^{+} Tfh cells was obtained with converted WT but not P2rx7$^{-/-}$ Tfh cells in adoptively transferred Cd3e$^{-/-}$ mice (Fig- ure S2D), indicating the cell-autonomous nature of P2X7-medi- ated Tfh cell death. This conclusion was further supported by the significant increase of Annexin V^{+} cells in Cd3e$^{-/-}$ mice recon- stituted with Tfh cells purified from PPs of WT mice (Figure S2E).

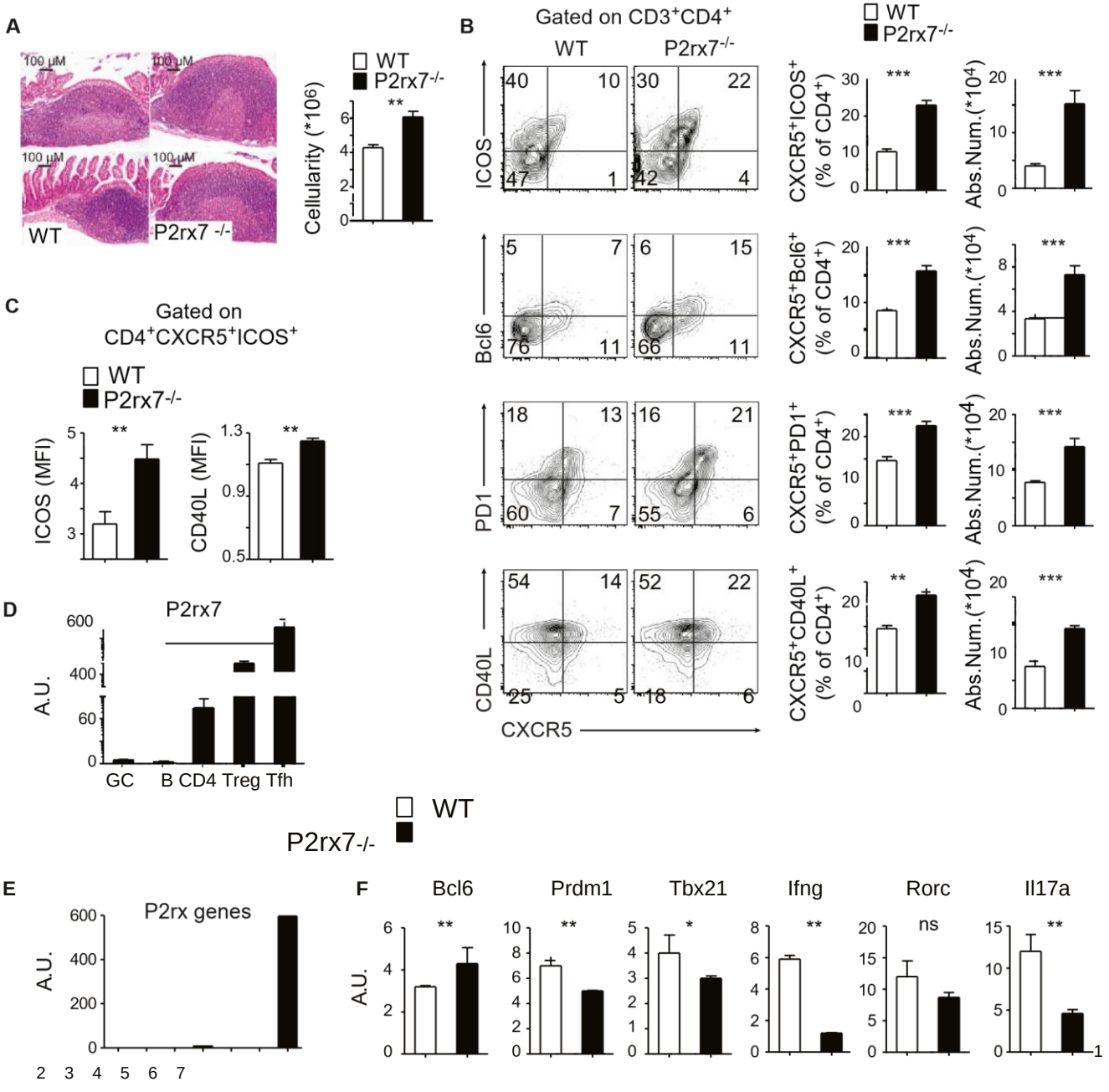

Figure 1. P2rx7$^{-/-}$ Mice Have Increased Tfh Cell Numbers in the PP

(A) Ileal PPs from 8-week-old mice (scale bars represent 100 mm); histograms show the number of total cells (mean ± SD, n = 5) recovered from PPs harvested from the entire small intestine.

(B) Dot plot and statistical analyses of WT and P2rx7$^{-/-}$ CD3$^+$CD4$^+$ PPs cells stained with ICOS, Bcl6, PD1, CD40L, and CXCR5 antibodies. Percentages are shown in each gate, histograms show mean percentages ± SD (n = 5).

(C) Mean fluorescence intensity (MFI) of ICOS and CD40L on Tfh cells from WT or P2rx7$^{-/-}$ quantified by flow cytometry.

(D) Quantitative real-time PCR of P2rx7 transcripts in purified CD19$^+$B220$^+$, Fas$^+$PNA$^+$ GC B cells, CD19$^+$ B cells, CD3$^+$CD4$^+$ T cells, CD3$^+$CD4$^+$CD25^{hi} Treg cells, and CD3$^+$CD4$^+$CXCR5$^+$ICOS$^+$ Tfh cells from WT mice.

(E) Quantitative real-time PCR of P2rx gene transcripts in purified CD3$^+$CD4$^+$CXCR5$^+$ICOS$^+$ Tfh cells from WT mice.

(F) Quantitative real-time PCR of Bcl6, Prdm1, Tbx21, Ifng, Rorc, and Il17a transcripts in purified CD3$^+$CD4$^+$CXCR5$^+$ICOS$^+$ Tfh cells from WT or P2rx7$^{-/-}$ mice. Data are representative results of three independent experiments with at least five mice per experiment. *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not significant. Error bars represent SD.

Analysis of permeability to DAPI upon stimulation with the P2X7 agonist BzATP showed that Tfh cells were the most sensitive subset to P2X7-mediated pore formation in PPs (Figure 3C). Time monitoring of PS exposure and DAPI uptake upon P2X7 stimulation using the natural ligand ATP confirmed the lack of PS exposure and DAPI uptake in P2rx7$^{-/-}$ Tfh cells (Figure 3D).

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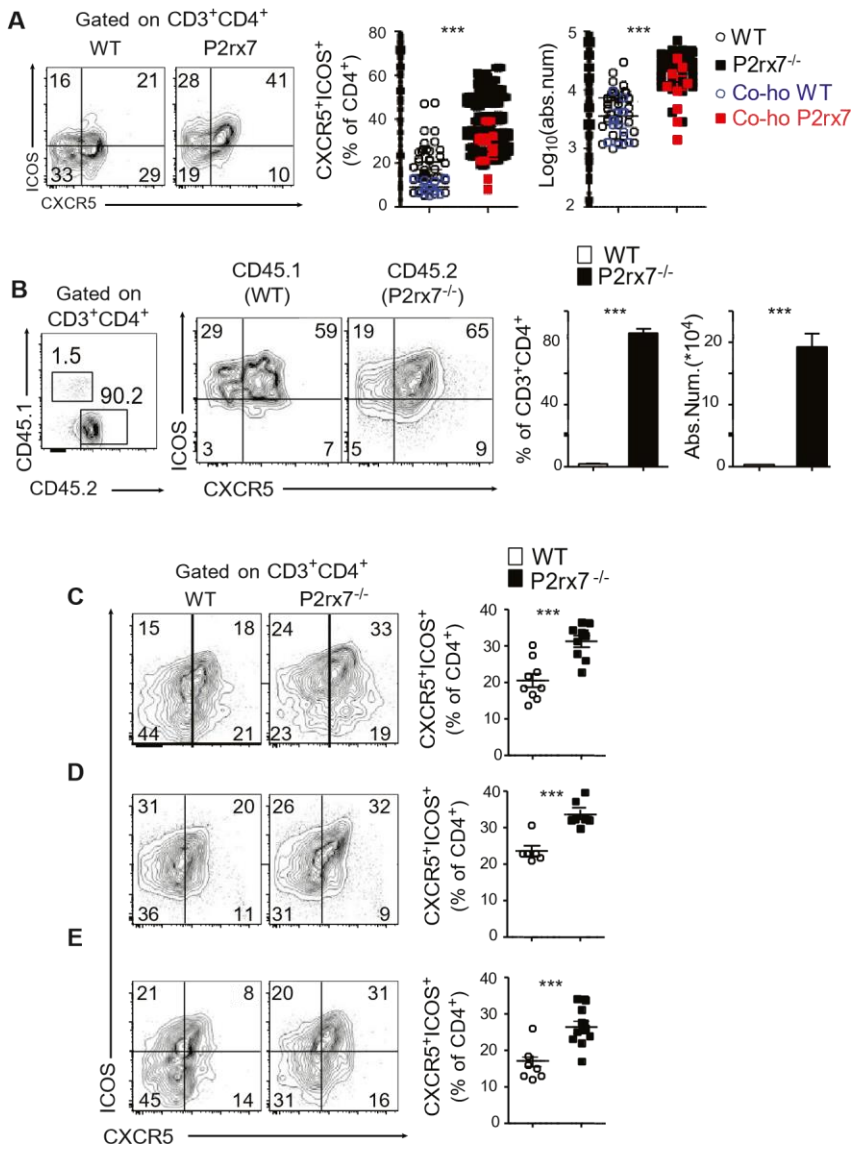


Figure 2. Expansion of Tfh Cell Numbers in the PPs of P2rx7$^{-/-}$ Mice Is Cell Autonomous

(A) Dot plot and statistical analyses of PPs cells from Cd3e$^{-/-}$ mice reconstituted with WT or P2rx7$^{-/-}$ Foxp3^{EGFP} cells. In two independent experiments, cohoused Cd3e$^{-/-}$ mice were analyzed 4 weeks after the reconstitution and are shown as superimposed blue and red results for mice reconstituted with WT or P2rx7$^{-/-}$ Foxp3^{EGFP} cells, respectively.

(B) Dot plot and statistical analyses of PPs cells from Cd3e$^{-/-}$ mice reconstituted with purified WT (CD45.1) and P2rx7$^{-/-}$ (CD45.2) Tfh cells at 1:1 ratio. Cells were gated for surface CD4 and CD3, and ICOS^{+}CXCR5^{+} cells were analyzed for CD45.1 and CD45.2 to score WT and P2rx7$^{-/-}$ cells (left dot plot). Middle panels show staining with CXCR5 and ICOS antibodies on gated WT and P2rx7$^{-/-}$ CD4 cells. Histograms on the right show statistics for percentage and absolute cell number of WT and P2rx7$^{-/-}$ Tfh cells recovered from PPs of reconstituted Cd3e$^{-/-}$ mice (n = 3).

(C–E) Flow cytometry of PPs CD3^{+}CD4^{+} T cells stained with CXCR5 and ICOS antibodies; cells were recovered from cohoused WT and P2rx7$^{-/-}$ mice. Before cohousing, mice were delivered through caesarean and cofostered with CD1 adoptive mothers (C), cross-fostered with recip- rocal WT and P2rx7$^{-/-}$ mothers and cohoused for 4 weeks (D), or cobred with WT and P2rx7$^{-/-}$ mothers and cohoused for 4 weeks (E).

Data are representative results of six (A), two (B), and three (C–E) independent experiments with three to five mice per experiment. ***p < 0.001. Error bars represent SD.

Analogous sensitivity to ATP stimulation was observed in Tfh cells from WT but not P2rx7$^{-/-}$ mLNs (Figure S2F), indicating that Tfh cells are exquisitely sensitive to P2X7-mediated cell death by extracellular ATP.

Importantly, extracellular ATP-mediated Tfh cell death via P2X7 does not affect Tfh cells actively responding to cognate antigen stimulation. Indeed, p2rx7 transcription in Tfh cells was robustly downregulated by CD3 and CD28 stimulation and Tfh cells became resistant to P2X7-mediated DAPI permeability

P2X7 in Tfh Cells Limits GC Reaction in PPs

The increase of Tfh cells in P2rx7$^{-/-}$ mice correlated with an enhanced GC reaction in the PPs (Figure 4A); the increase in (Figure 3E). Further, we generated antigen-specific Tfh cells by feeding TCR transgenic OT-II mice (recognizing OVA peptide 323-339) with OVA. After 7 days we analyzed the amount of P2rx7 transcripts in ex vivo sorted OT-II Tfh cells from PPs as well as in OT-II Tfh cells sorted after in vitro stimulation for 16 hr with dendritic cells pulsed with OVA peptide. This analysis revealed the robust P2rx7 downregulation in antigen-stimulated cells (Figure 3F), thereby supporting the notion that acute TCR stimulation spares Tfh cells from death via P2X7.

Fas^+PNA^+ GC B cells was associated with increased expression of Aicda mRNA encoding activation-induced cytidine deaminase (AID), which controls Ig somatic hypermutation (SHM) and class switch recombination (CSR) (Figure 4B; Muramatsu et al., 2000). In vitro stimulation of both T and B cells results in extracellular ATP release (Schena et al., 2013; Schenk et al., 2008). To see whether P2X7 activation affected Tfh cell activity in vitro, WT IgM^+ B cells were cocultured with either WT or $P2rx7^{-/-}$ Tfh cells isolated from PPs; when $P2rx7^{-/-}$ Tfh cells were present in the culture, there was increased Ig CSR to IgA with more abundant IgA secretion, suggesting that lack of P2X7 results in enhanced B cell helper function (Figure 4H). The number of IgA-secreting B cells measured by ELISPOT was significantly increased in the small but not large intestine lamina propria of $P2rx7^{-/-}$ mice, consistent with increased Tfh cell activity in vivo (Fig- ure 4C). Accordingly, fecal IgA concentrations in $P2rx7^{-/-}$ mice were elevated, whereas IgM were reduced (Figure 4D). The same phenotype was observed in $Cd3e^{-/-}$ mice reconstituted with $P2rx7^{-/-}$ $Foxp3^+$ T cells, indicating the dependence of

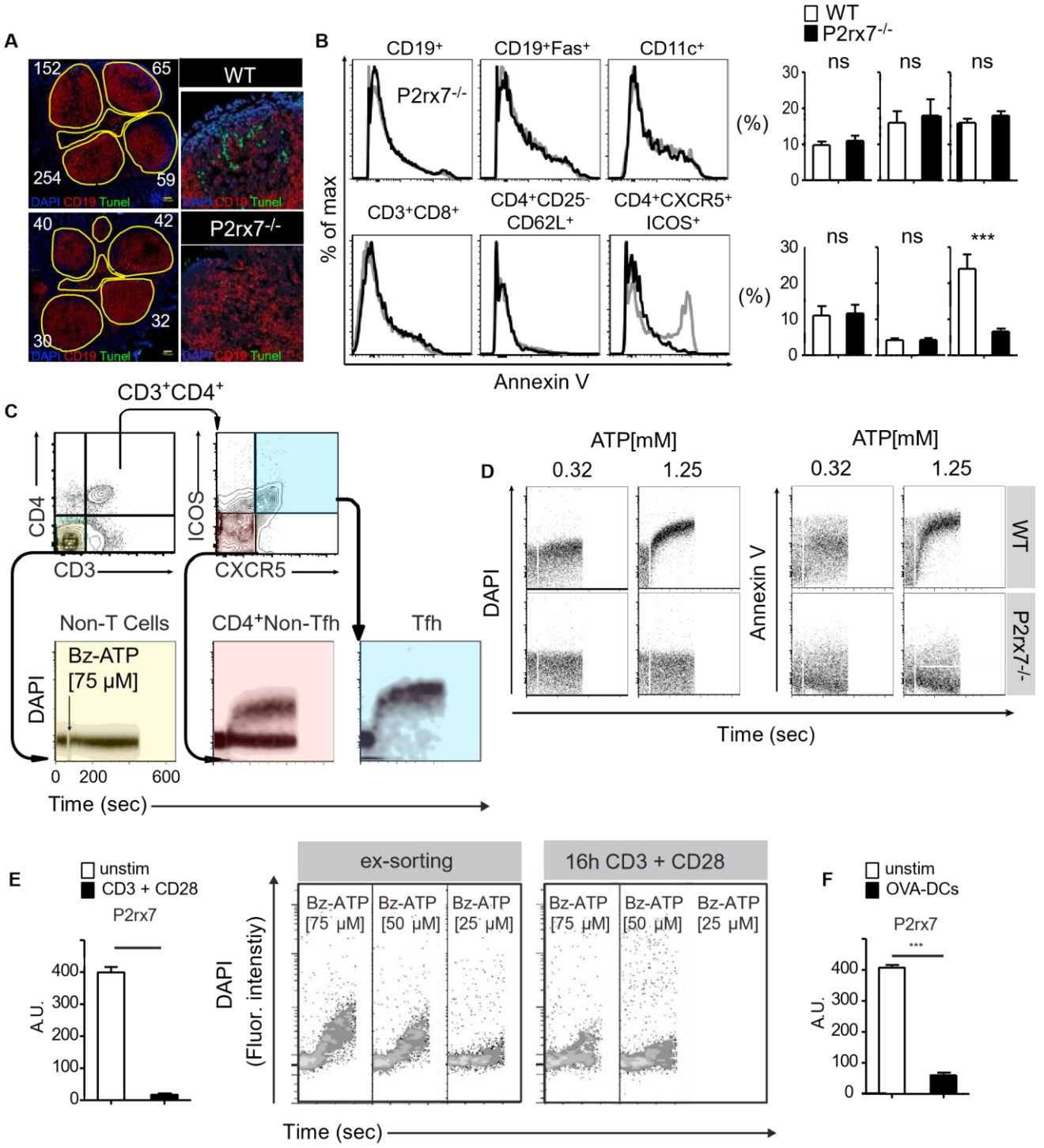


Figure 3. Extracellular ATP Promotes Tfh Cell Death

(A) Representative TUNEL assay on ileal PPs from WT and P2rx7$^{-/-}$ mice (scale bars represent 100 mm); white numbers represent TUNEL-positive cells.
(B) Annexin V staining and corresponding statistical analysis of freshly isolated WT and P2rx7$^{-/-}$ PP cell subsets gated as indicated (n = 5).
(C) Time monitoring of electronically gated PPs cell subsets for DAPI uptake after stimulation with Bz-ATP (75 mM).
(D) Time monitoring of electronically gated PPs WT or P2rx7$^{-/-}$ Tfh cells for DAPI uptake and PS exposure after stimulation with the indicated doses of ATP. (E) Real-time PCR of P2rx7 transcripts in Tfh cells either unstimulated or stimulated for 16 hr with anti-CD3 and CD28 mAbs; Bz-ATP responsiveness measured by time-monitoring of DAPI staining in the same cells.
(F) Real-time PCR of P2rx7 transcripts in OT-II Tfh cells either unstimulated or stimulated for 16 hr with OVA peptide-pulsed dendritic cells. Data are representative results of three to six independent experiments. ***$p < 0.001$; ns, not significant. Error bars represent SD.

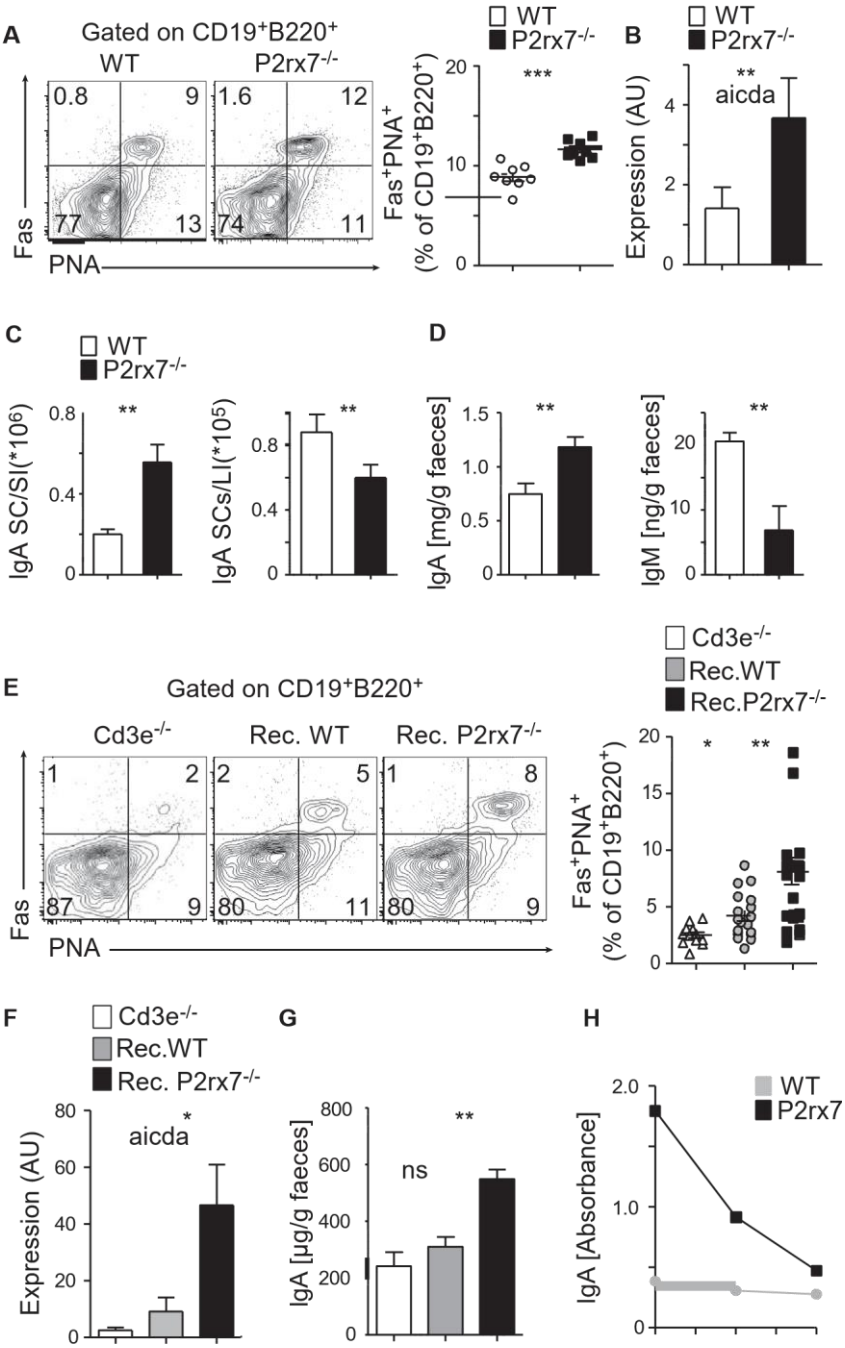


Figure 4. P2X7 Deficiency in Tfh Cells Leads to Enhanced GC Reactions and IgA Response

(A) Representative dot plots and statistical analysis of Fas^+PNA^+ GC B cells.

(B) Real-time PCR of Aicda in purified $CD19^+$ cells (n = 5).

(C) IgA-secreting plasma cells in lamina propria of small (SI) or large (LI) intestine quantified by ELISPOT (n = 3).

(D) Fecal IgA (n = 10) and IgM (n = 5) concentrations in WT and $P2rx7^{-/-}$ mice.

(E–G) Representative dot plots and statistical analysis of Fas^+PNA^+ GC B cells (E), real-time PCR of Aicda in purified $CD19^+$ cells (n = 5) (F), and fecal IgA concentrations (n = 6) (G) in $Cd3e^{-/-}$ mice either nonreconstituted or reconstituted with WT or $P2rx7^{-/-}$ $Foxp3^{EGFP}$ cells.

(H) Absorbance values in ELISA-detecting IgA in the supernatants of B cells cocultured with WT or $P2rx7^{-/-}$ Tfh cells purified from PPs.

Data are representative results of at least two independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not significant. Error bars represent SD.

Wild-type Tfr cells were more resistant to cell death than Tfh cells, as determined by cell labeling with Annexin V (Figure S3C). Interestingly, in Tfr cells we

observed increased surface expression of the plasma membrane ectonucleoti- dase CD39 (that degrades ATP to ADP/ AMP) with respect to Tfh cells, likely reducing P2X7 sensitivity to extracellular ATP (Figure S3D). These observations suggest that P2xr7 deletion does not influence Tfr cells. In mice reconstituted with Foxp3^{+} cells, we observed more than 90% conversion of P2rx7$^{-/-}$ Treg to Tfh cells in PPs. P2rx7$^{-/-}$ Treg cells converted to Tfh cells more efficiently than WT Treg cells (Figure S3E), sug- gesting that lack of P2X7 results in skewing and/or expansion of Tfh but not Tfr cells. Finally, transfer into Cd3e$^{-/-}$ mice of CD4^{+}CXCR5^{+}ICOS^{+} cells, com- prising both Tfh and Tfr cells, resulted in the observed phenotype on P2rx7 deletion in Tfh cells (Figures 4E–4G).

Follicular regulatory (CD4^{+}CXCR5^{+}ICOS^{+}Foxp3^{+}) T (Tfr) cells play a crucial role in GC responses by limiting Tfh and GC B cell numbers as well as plasma cells differentiation (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Because P2rx7 is expressed in Tfr cells (Figure S3A), P2rx7 dele- tion might result in increasing Tfr cells in PPs. However, similar Tfr cell numbers were found in WT and P2rx7$^{-/-}$ mice, while the percentage was reduced in p2rx7$^{-/-}$ mice (Figure S3B). the recovery of mostly CD4^{+}CXCR5^{+} ICOS^{+}Foxp3^{-} Tfh cells after 4 weeks (Figure S3F), indicating that lack of P2X7 does not result in Tfr cell expansion.

P2X7 Signaling Regulates Human Tfh Cells

TUNEL-positive cells were observed in the T cell area of human PPs (Figure 5A) as well as selective PS exposure by FACS in Tfh cells from PPs of three healthy subjects (Figure 5B). On-line monitoring of Annexin V staining and DAPI permeability of PP cells exposed to ATP revealed an increase in Tfh but not other PP cells (Figure 5C). Indeed, naive B cells do not express detectable amounts of P2rx7 (Figure 5E), and P2rx7 transcripts were not detected in plasma blasts (PBs), GC, and memory (ME) B cells by real-time PCR (Figure 5D). These observations suggest that luminal ATP can also modulate Tfh cell function in the human small intestine. To address the role of P2X7 in regu- lating B cell help by Tfh cells, an in vitro assay of Ig CSR was per- formed in the presence of a selective P2X7 pharmacological antagonist (A438079). Naive B cells isolated from peripheral blood as $CD19^+Lin^-CD27^-IgG^-IgA^-$ cells predominantly ex- press P2rx5 and to a lesser extent P2rx1 and P2rx4 (Figure 5E), and therefore they were not directly influenced by the drug. Coculturing of naive B cells with Tfh cells isolated from peripheral blood as $CD4^+CXCR5^+CD45RA^-$ cells (Morita et al., 2011) re- sulted in substantial Ig CSR to IgG. Notably, inhibition of P2X7 by A438079 resulted in an increase of IgG concentration in the culture medium

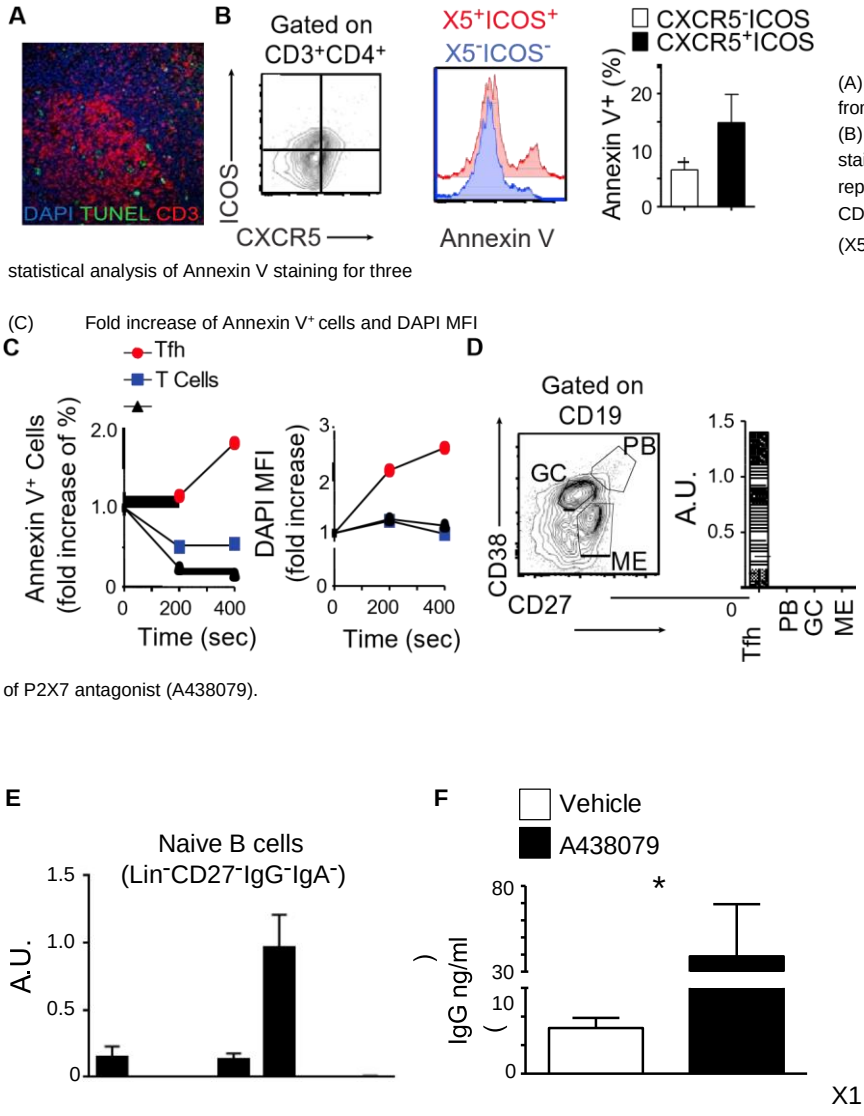


Figure 5. Purinergic Regulation of Tfh Cells Is Conserved in Humans

(A) Representative TUNEL assay on ileal PPs from a healthy subject.

(B) Cell suspensions from human PPs were stained with CD3, CD4, CXCR5, and ICOS antibodies. A representative staining with Annexin V of $CD3^+CD4^+ICOS^-CXCR5^-$ ($X5^-ICOS^-$) and Tfh ($X5^+ICOS^+$) cells is shown. Bars on the right show statistical analysis of Annexin V staining for three subjects.

(C) Fold increase of Annexin V^+ cells and DAPI MFI in the indicated cell subsets recovered from PPs of healthy subject upon stimulation with 3 mM ATP.

(D) Gating strategy for the isolation of Tfh cell subsets and real-time PCR of P2rx7 transcripts in germinal center B cells (GC), plasmablasts (PB), memory B (ME), and Tfh cells purified in flow cytometry from ileal PPs of healthy subjects. (E) Real-time PCR of P2rx7 transcripts in naive B cells sorted as $CD19^+IgM^+IgD^+CD27^-$ from blood of three healthy donors.

(F) Quantification of IgG by ELISA in the supernatants of blood naive B cells cultured with purified memory $CXCR5^+CD45RA^-CD4^+$ T cells in presence of P2X7 antagonist (A438079).

Results are means ± SD of five independent experiments with cells from five different healthy donors. *$p < 0.05$. Error bars represent SD.

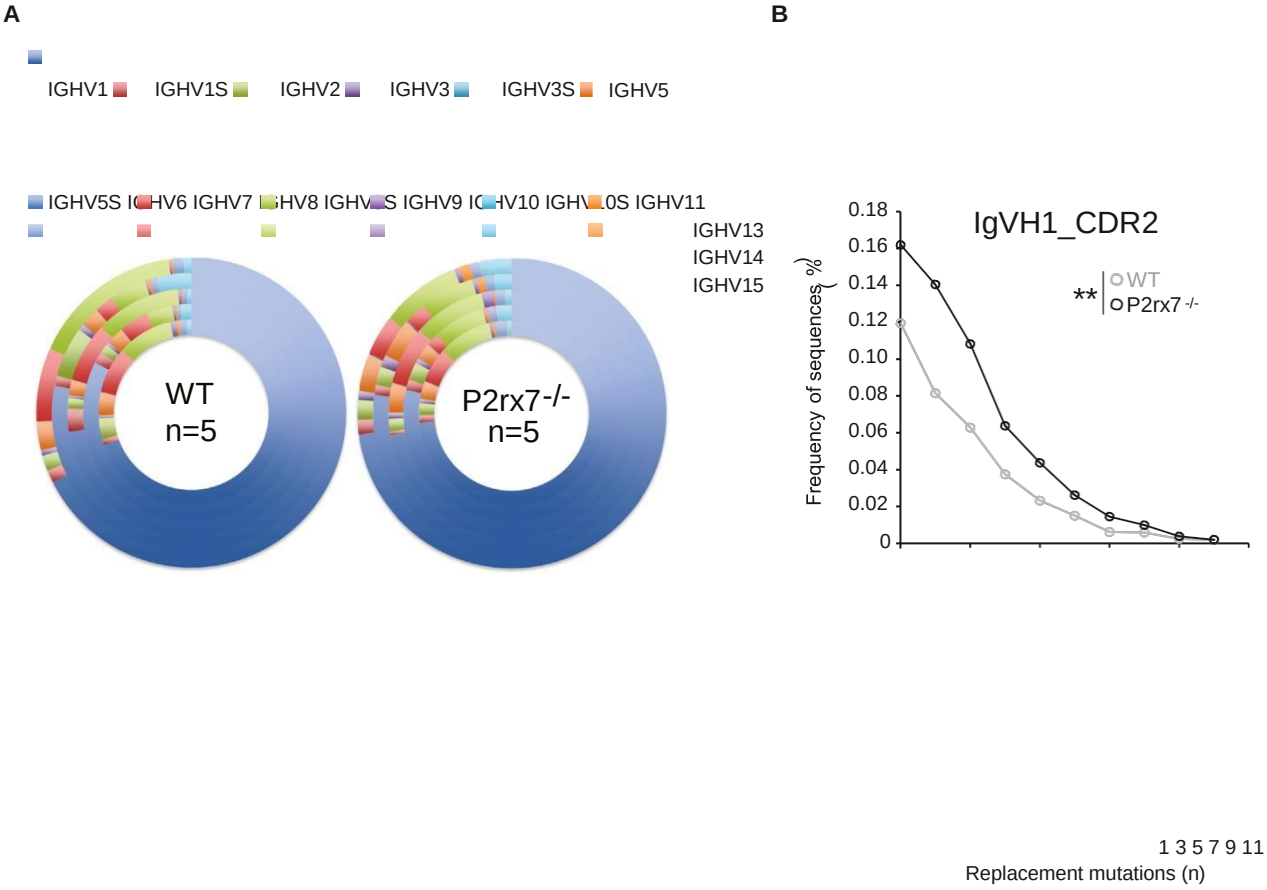
2004; Peterson et al., 2007). Low-affinity T-cell-independent IgA is sufficient for controlling benign commensal bacteria. Conversely, T-cell-dependent affinity-matured IgA responses are critical for efficient mucosal defense and contain-

X1 X2 X3 X4 X5 X6 X7 ment of more invasive commensal spe- cies, such as segmented filamentous

(Figure 5F), suggesting that P2X7 activity is also important for regulating human Tfh cells.

P2rx7$^{-/-}$ Mice Have Enhanced High-Affinity IgA Responses and Reduced Mucosal Colonization

Mucosal IgA inhibits intestinal bacteria from penetrating the epithelial cell layer (Burns et al., 1996; Macpherson and Uhr, bacteria (SFB), which tightly adhere to epithelial cells in the terminal ileum (Ivanov et al., 2009; Suzuki et al., 2004). High-throughput sequencing of Ig V_H regions in B cells isolated from PPs revealed minor differences between WT and P2rx7$^{-/-}$ mice in the distribution of the V_H repertoire of IgA (Figure 6A). However, an increase in the frequency of replacement (R) mutations (i.e., encoding a different amino acid) in V_{H1} family's CDR2 was detected in P2rx7$^{-/-}$ mice, suggesting enhanced affinity maturation of IgA responses (Figure 6B). Analysis in flow cytometry of IgA-coated bacteria from the small intestine revealed the increase of IgA-coated cells in P2rx7$^{-/-}$ mice. Conversely, bacteria coated by IgG_3, a T-cell-independent Ig subclass present in the small intestine and specific for bacterial polysaccharides (McLay et al., 2002), were the same for both strains (Figure 6C). Inspection of the ileum from WT and P2rx7$^{-/-}$ mice by scanning electron micro- scopy (SEM) revealed the striking reduction of mucosal coloniza- tion by SFB in P2rx7$^{-/-}$ mice (Figure 6D). To demonstrate enhanced high-affinity IgA responses in P2rx7$^{-/-}$ mice, we gav- aged mice with E. coli and analyzed the specific IgA response. In fact, the E. coli-specific IgA response is fully dependent on PPs (Le´ cuyer et al., 2014). Total IgA concentration in intestinal washes was quantified by ELISA. Undiluted aliquots of the



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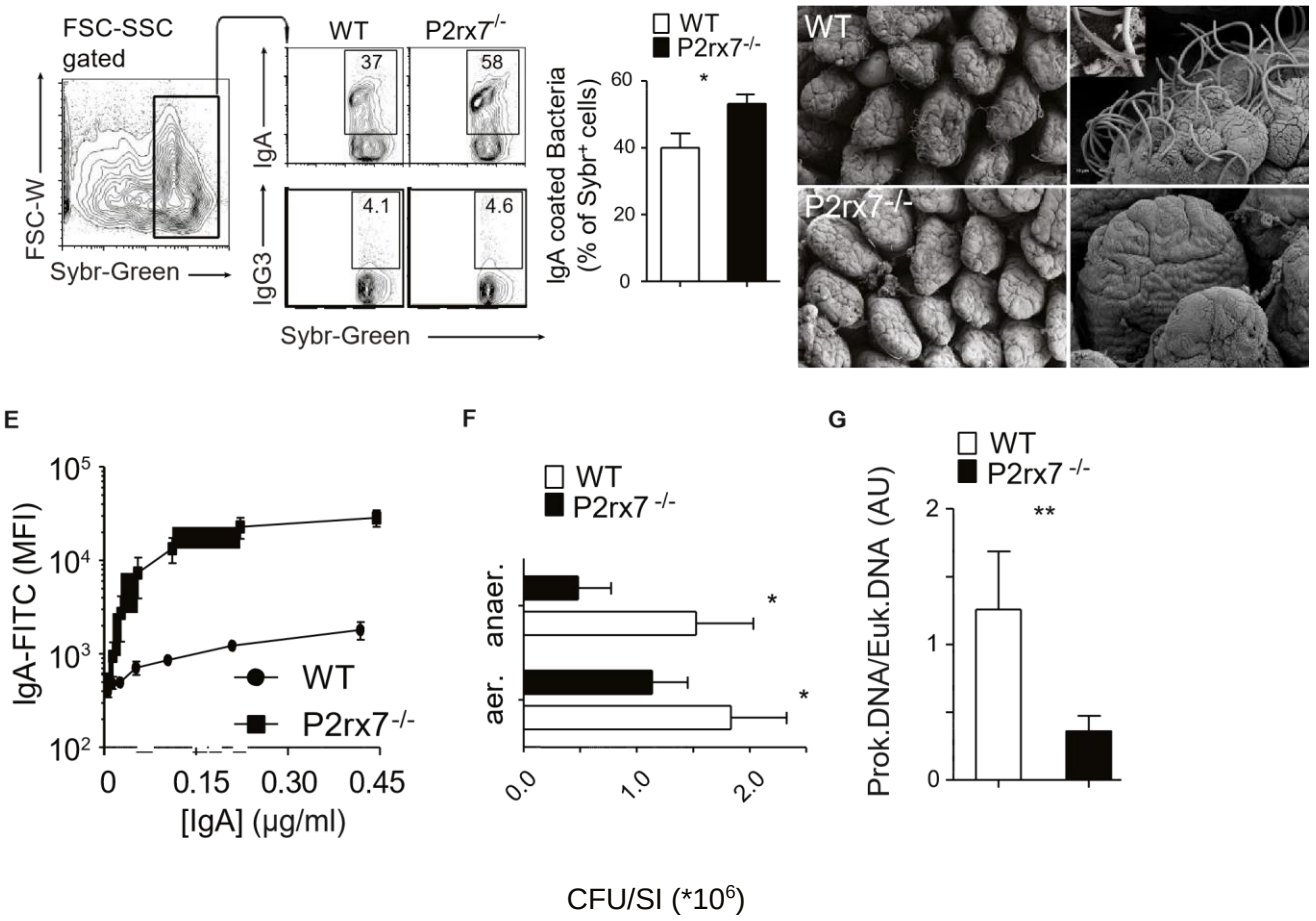

Figure 6. P2X7 Deficiency Leads to Enhanced IgA Affinity and Depletion of Mucosal Commensals

(A and B) V_H family distribution in five mice per genotype (A) and frequency of R mutations in IgV_{H1} family CDR2 (B) in IgA-expressing $CD19^+$ B cells isolated from PPs of WT and $P2rx7^{-/-}$ mice.

(C) Representative dot plots and percentages of IgA (n = 6)- and IgG_3 (n = 6)-coated bacteria from the small intestine of WT and $P2rx7^{-/-}$ mice as determined by flow cytometry.

(D) SEM of the terminal ileum from WT and $P2rx7^{-/-}$ mice. Inset in WT image shows SFBs in more detail.

(E) Quantification by flow cytometry of E. coli-specific IgA in intestinal lavages from WT and $P2rx7^{-/-}$ mice gavaged with E. coli.

(F) Statistical analysis of colony forming units (CFU) of aerobic and anaerobic bacteria recovered from the SI of WT and $P2rx7^{-/-}$ mice (n = 10).

(G) Quantitative real-time PCR of ileal adherent bacteria in WT and $P2rx7^{-/-}$ mice expressed as prokaryotic/eukaryotic DNA ratio (n = 5). Data are from one experiment (A and B) or at least two independent experiments. *$p < 0.05$, **$p < 0.01$. Error bars represent SD.

same samples were used to stain E. coli for FACS analysis. Mean fluorescence intensities of E. coli-specific IgA plotted against IgA concentrations revealed an increase of E. coli-specific IgA in $P2rx7^{-/-}$ mice, thereby indicating that PP-dependent IgA response was enhanced (Figure 6E). Taxonomic analysis of total gut microbiota in WT and $P2rx7^{-/-}$ mice did not reveal differ- ences in the representation of bacterial families (Figure S4A).

However, bacterial cultures from small intestine of $P2rx7^{-/-}$ mice showed a decrease of both aerobic and anaerobic species (Figure 6F). Finally, after removal of fecal pellet, we isolated whole ileal mucosa comprising epithelium and mucus (Figure S4B) to quantify adherent bacteria per tissue mass by real-time PCR. In $P2rx7^{-/-}$ mice we could detect a decreased prokaryotic to eukaryotic DNA ratio (Figure 6G), indicating that P2X7 in Tfh cells is important in dampening high-affinity IgA

responses in the small intestine and that P2rx7 deletion results in an enhanced clearance of mucosal commensals in the small intestine.

P2rx7$^{-/-}$ Mice Have Reduced Serum IgM and Are Susceptible to Sepsis

Sensing of commensals profoundly shapes host immune system (Hand et al., 2012; Hooper et al., 2012). In P2rx7$^{-/-}$ mice, reduced IgM serum concentrations were observed in compari- son with WT animals (Figure 7A). In WT mice, long-term dosing of antibiotics resulted in reduction of serum IgM, consistent with a requirement for mucosal colonization in maintaining serum IgM at physiological concentrations (Figure 7A). It was recently found that lack of IgA negatively correlates with serum lipopoly- saccharide (LPS) concentrations (Shulzhenko et al., 2011). Hyper-IgA in P2rx7$^{-/-}$ mice was associated with reduced con- centrations of serum LPS (Figure 7B). In addition, a single low- dose (20 mg) intraperitoneal injection of LPS induced a significant increase in serological IgM in P2rx7$^{-/-}$ mice (Figure 7C), sug- gesting that reduced mucosal colonization and consequently less translocation of bacterial components results in reduced B cell stimulation and IgM secretion.

Because deletion of Icos results in a lack of Tfh cells in both P2X7-proficient and P2X7-deficient mice (Figure S5A), we used Icos$^{-/-}$ and double mutant Icos$^{-/-}$P2rx7$^{-/-}$ mice to address the role of P2X7 in Tfh cells for conditioning serum IgM concentrations through the regulation of mucosal IgA. By comparing the ratio of P2rx7$^{-/-}$ to P2rx7$^{+/+}$ fecal IgA and serum IgM in ICOS-proficient and ICOS-deficient mice, we found that Icos deletion restored the balance between fecal IgA and serum IgM in P2rx7$^{-/-}$ mice (Figure 7D). These results suggest that Tfh cells are responsible for the elevated fecal IgA and reduced serum IgM characteristic of P2rx7$^{-/-}$ mice.

Evaluation of IgM-secreting cells (ISCs) in the spleen by ELISPOT assay revealed the decrease of ISCs in P2rx7$^{-/-}$ mice with respect to their WT counterpart. ISCs reduction in P2rx7$^{-/-}$ mice was Tfh cell dependent since it was restored by Icos deletion (Figure S5B). Further investigation of the spleens of P2rx7$^{-/-}$ mice revealed that follicular and marginal zone B cells were not able to spontaneously secrete IgM (data not shown) whereas B1 cells did and were responsive to stimulation by commensals components. In fact, IgM secretion by these cells was reduced in germ-free mice and was restored in P2rx7$^{-/-}$ mice by LPS injection (Figures 7E and 7F).

Serum from P2rx7$^{-/-}$ mice (1:4 dilution) showed reduced IgM binding to antigens from cecal commensals compared to serum from WT mice (Figure 7G) as well as impaired ability to inhibit cecal bacteria growth in vitro (Figure 7H). Notably, this serolog- ical defect of P2rx7$^{-/-}$ mice was restored by addition of purified IgM from the sera of WT mice (Figure 7H), suggesting that translocation of bacterial antigens from the small intestine results in the priming of B cells to secrete IgM able to inhibit bacterial growth and that this priming is significantly defective in P2rx7$^{-/-}$ mice.

It is known that IgM constitutes a first line of defense against blood-borne microbial infection (Boes et al., 1998), and thus the reduced concentrations of IgM in P2rx7$^{-/-}$ mice should make them more susceptible to polymicrobial sepsis. To test this hy- pothesis, a sublethal terminal cecal ligation and puncture (CLP) was performed. This model has been shown to closely resemble human sepsis (Hubbard et al., 2005; Rittirsch et al., 2009). Strikingly, P2rx7$^{-/-}$ mice displayed severe symptoms in the first hours after surgery and most succumbed within 48 hr (Figures 7I and 7L). Although blood from WT mice was always sterile, both aerobic and anaerobic bacteria could be easily cultured from blood of P2rx7$^{-/-}$ mice (Figure S5C). The same morbidity was observed by CLP in cohoused WT and P2rx7$^{-/-}$ mice, suggest- ing that lack of P2X7 results in the depletion of ''protective'' com- mensals (Figure S5D). Notably, P2rx7$^{-/-}$ mice surviving CLP showed an increase of serological IgM concentrations relative to WT mice, indicating that P2rx7$^{-/-}$ B cells were not intrinsically defective in differentiating to ISCs upon stimulation by bacterial antigens (Figure 7M). Prophylactic intravenous affinity-purified IgM from WT mice was administered to P2rx7$^{-/-}$ mice before CLP. IgM-treated mice were indistinguishable from WT mice and showed comparable survival rates (Figures 7I and 7L), impli- cating reduced IgM as cause of mortality by polymicrobial sepsis in P2rx7$^{-/-}$ mice. Of note, infection with Listeria monocytogenes, in which adaptive immunity is dispensable for containing infection at early time points (Pamer, 2004), did not result in differences for morbidity and bacterial recovery between WT and P2rx7$^{-/-}$ mice (Figures S5E and S5F). To show the causative role of P2rx7$^{-/-}$ Tfh cells in bacterial sepsis after sublethal CLP, the same experiment was repeated in P2rx7$^{-/-}$Icos$^{-/-}$ mice. Strik- ingly, these mice were fully resistant to sublethal CLP, analo- gously to Icos$^{-/-}$ animals (Figure S7N). These results indicate that enhanced adaptive mucosal response in P2rx7$^{-/-}$ mice attenuates systemic sensing and basal IgM response to com- mensals, thus exposing the organism to possible fatal sepsis.

DISCUSSION

The regulated release of ATP influences eukaryotic cell function through ubiquitously expressed purinergic P2 receptors (Burn- stock, 2007). The high concentration of ATP inside cells and its virtual absence extracellularly in healthy tissues renders ATP also an ideal harbinger of cell death; in fact, ATP is sensed as a DAMP by cells of the innate immune system. P2rx7 has been recently shown to be a signature gene of Tfh cells (Choi et al., 2013; Iyer et al., 2013), but its function in this cell subset has not been addressed so far. Interestingly, extracellular ATP con- centrations active on P2X7 are present during an alloreactive response and at site of immunization with complete Freund's

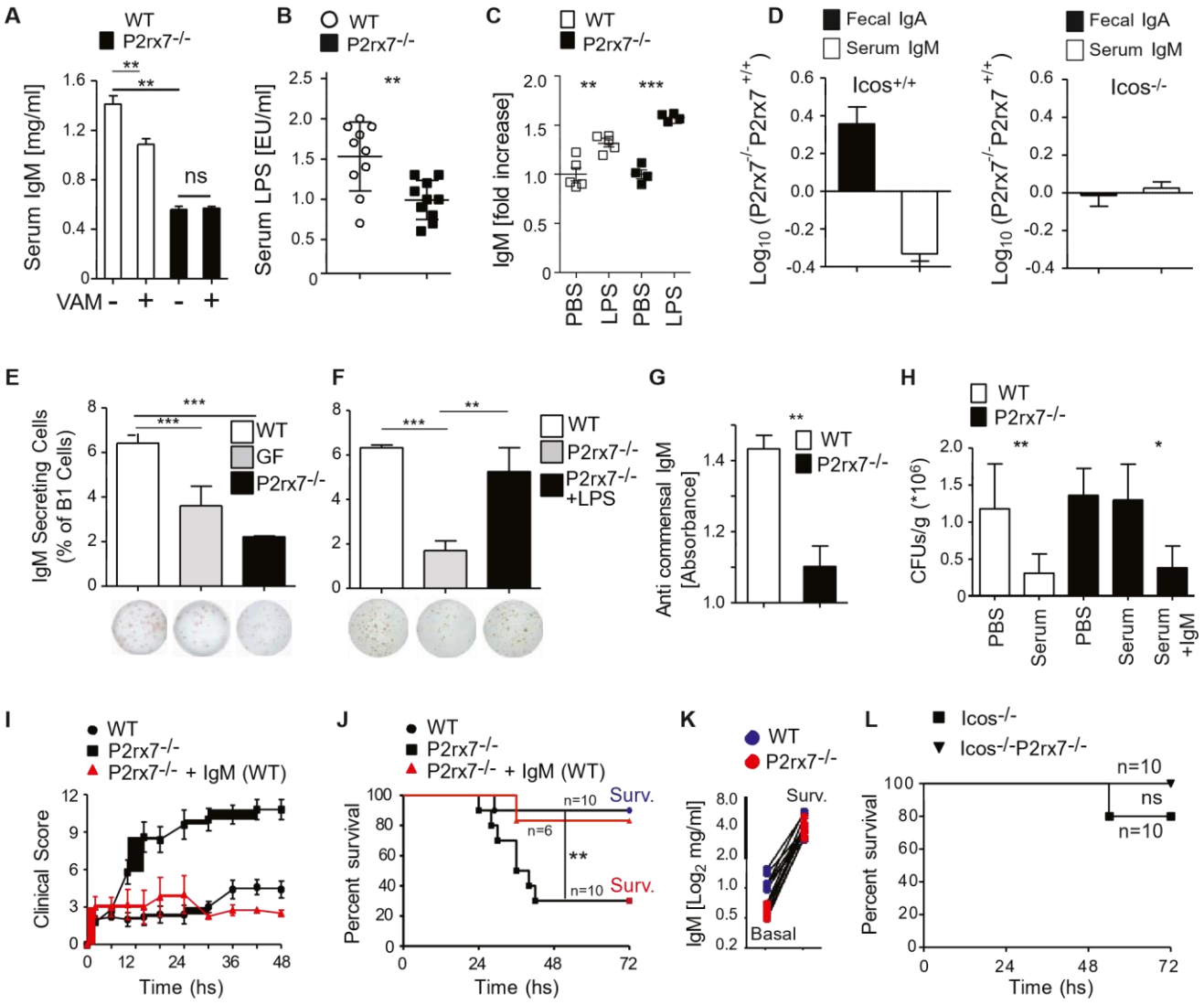

Figure 7. P2X7 Deficiency Is Associated with Reduced IgM-Secreting B1 Cells and Susceptibility to Polymicrobial Sepsis

(A) ELISA quantification of serum IgM in WT and P2rx7$^{-/-}$ mice treated either with PBS (-) or VAM (+) (n = 3).

(B) Serum LPS concentrations in WT and P2rx7$^{-/-}$ mice as determined by Limulus amebocyte lysate (LAL) assay.

(C) ELISA quantification of serum IgM concentrations in WT and P2rx7$^{-/-}$ mice treated either with PBS or LPS.

(D) Ratio of P2rx7$^{-/-}$ to P2rx7$^{+/+}$ fecal IgA (n = 10) and serum IgM (n = 6) in Icos$^{+/+}$ (left) and Icos$^{-/-}$ (right) backgrounds.

(E) Representative picture and mean percentages of IgM-secreting cells by ELISPOT in purified splenic B1 cells from WT, germ-free (GF), and P2rx7$^{-/-}$ mice (WT, 20 mice pooled in group of 5; GF, 10 mice pooled in group of 5; P2rx7$^{-/-}$, 15 mice pooled in group of 5).

(F) Representative picture and mean percentages of IgM-secreting cells by ELISPOT in purified splenic B1 cells from WT, P2rx7$^{-/-}$, and P2rx7$^{-/-}$ mice treated with intraperitoneal injection of LPS (WT, 10 mice pooled in group of 5; p2rx7$^{-/-}$, 10 mice pooled in group of 5; p2rx7$^{-/-}$ treated with LPS, 10 mice pooled in group of 5).

(G) Absorbance in ELISA of antimicrobiota serum IgM in WT and P2rx7$^{-/-}$ mice (n = 5).

(H) CFUs in cecal cultures from WT and P2rx7$^{-/-}$ mice with addition of PBS or autologous serum and IgM from WT mice (serum + IgM) (n = 5). (I and J) Clinical score (I) and survival rate (J) of WT, P2rx7$^{-/-}$, and P2rx7$^{-/-}$ mice treated with IgM purified from WT mice, upon sublethal CLP.

(K) Serum IgM concentrations by ELISA in surviving WT and P2rx7$^{-/-}$ mice before and 1 week after CLP.

(L) Survival rates of Icos$^{-/-}$ and P2rx7$^{-/-}$Icos$^{-/-}$ mice upon sublethal CLP.

Data are representative results of two to five independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001. Error bars represent SD.

adjuvant (Wilhelm et al., 2010), suggesting that signaling through the ATP-P2X7 axis can regulate Tfh cell response in secondary lymphoid organs.

Tfh-cell-dependent high-affinity IgA is critical in maintaining intestinal homeostasis and efficient mucosal defense by limiting translocation of potentially invasive bacteria from the gut lumen into the organism (Wei et al., 2011; Shroff et al., 1995). Conversely, microbiota-independent (''natural'') and T-cell-independent (''primitive'') IgA are sufficient for controlling benign commensal bacteria by promoting their exclusion from the mucus layer (Slack et al., 2012). In an elegant study, Hapfelmeier et al. (2010) have shown that mucosal IgA response in the gut is adapted to actual commensals. In Tfh cells, P2rx7 is robustly downregulated by acute TCR stimulation, thereby sparing actively responding cells from death via P2X7. The sensi- tivity of non-actively stimulated Tfh cells to ATP as a metabolite associated with mucosal colonization might underlie IgA attrition in the gut and allow adapting IgA response to predominant taxa at any given time. Probably this mechanism contributes also to the complexity of developing vaccines that effectively protect the host against intestinal mucosal infections (Slack et al., 2012). Notably, oral immunization of P2rx7$^{-/-}$ mice with E. coli resulted in dramatically enhanced IgA response with respect to WT mice. Because Tfh cell death by extracellular ATP was observed also in human PPs, pharmacological ATP antagonism might constitute a strategy to limit Tfh cells death and promote long-lasting mucosal IgA responses.

Lack of the inhibitory coreceptor PD1 was recently shown to result in expanded Tfh cells in PPs. However, the increased help by PD1-deficient cells interfered with selection of the IgA repertoire and IgA responses were not specific for commensals (Kawamoto et al., 2012). In contrast, Tfh cells expansion by P2rx7 deletion resulted in enhanced IgA responses to commensals and to gavaged E. coli. Further, P2rx7$^{-/-}$ mice showed increased IgA SHMs and reduced mucosal colonization, indicating enhanced antigen-specific Tfh cell help and the fact that P2X7 activity contributes to mucosal commensalism by limiting follicular T cell help.

Commensal-derived signals shape a number of systemic immune responses (Clarke et al., 2010; Hill et al., 2012; Macpherson and Harris, 2004; Noverr and Huffnagle, 2004). Secretory IgA limit access of antigens from the microbiota to the organism (Johansen et al., 1999; Macpherson and Uhr, 2004; Sait et al., 2007; Shulzhenko et al., 2011) and increased IgA are likely responsible of reduced stimulation of B1 cells as well as serum IgM concentrations in P2rx7$^{-/-}$ mice. IgM plays a crucial role in protecting the organism against blood microbial infection (Boes et al., 1998) and contrast lethal dissemination of commen- sals (Kirkland et al., 2012). Sepsis is a major health problem (Hotchkiss and Karl, 2003); in hospitalized patients, antibiotic-induced changes in the intestinal microbiota can enable bacteria to invade the bloodstream (Ubeda et al., 2010). Hypo-IgM in P2rx7$^{-/-}$ mice increases the risk of polymicrobial sepsis and death by otherwise sublethal CLP, an experimental model that closely resembles the human disease (Hubbard et al., 2005; Rittirsch et al., 2009). These results point to P2rx7 in Tfh cells as a master regulator of host-microbiota mutualism. Finally, elevated ATP in the intestine, as may occur during invasion of the microbiota by intestinal pathogens or damage to the epithelium by pathogens, toxins, or injected chemicals, might modify the balance of immune responses toward a more potent innate response. Production of metabolically ''expensive'' and risky adaptive immunity can then be limited to persistent micro- organisms that cannot be cleared by innate immunity alone.

EXPERIMENTAL PROCEDURES

Mice and In Vivo Experiments

All animal experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and authorized by the Animal Studies Committee of Cantonal Veterinary. Mice were on a C57BL/6 genetic background. C57BL/6J, P2rx7$^{-/-}$ (B6.129P2-P2rx7^{tm1Gab}/J), ''Foxp3^{EGFP}'' (B6.Cg-Foxp3^{tm2Tch}/J), Icos$^{-/-}$, and Cd3e$^{-/-}$ mice were bred in specific-pathogen-free (spf) facility at Institute for Research in Biomedicine Switzerland. C57BL/6J germ-free mice were maintained in flexible film isolators at the Clean Animal Facility, University of Bern, Switzerland. For sublethal CLP, the cecum was ligated at 10 mm from the distal pole. Cecal puncture (''through-and-through'') in a mesenteric to antimesenteric direction was carried out after ligation with a 20G needle. Body temperature and clinical score were measured every 3 hr.

Cohousing experiments were performed as follows.

For caesarean delivery and fostering by CD1 adoptive mothers (CoF), WT and P2rx7$^{-/-}$ mice were delivered by caesarean section on gestation day 18.5, fed by CD1 adoptive mother, weaned at 4 weeks, cohoused, and sacri- ficed at 8 weeks of age. For cross-fostering with reciprocal WT and P2rx7$^{-/-}$ mothers (CrF), WT mice were fed by P2rx7$^{-/-}$ mother and P2rx7$^{-/-}$ newborns were fed by WT mother. Cubs were weaned at 4 weeks, cohoused, and sacri- ficed at 8 weeks of age. For cobreeding with WT and P2rx7$^{-/-}$ mothers (CoB), WT and P2rx7$^{-/-}$ newborns were bred together and fed by both WT and P2rx7$^{-/-}$ mothers in the same cage (up to ten cubs with two mothers). Mice were weaned at 4 weeks of age, cohoused, and sacrificed at 8 weeks of age.

Isolation of Human PPs and In Vitro Tfh-Cell-Dependent B Cell Help

Peyer's patches were obtained during endoscopy from the terminal ileum of young adults presenting with chronic abdominal pain and in which subsequent examinations excluded the presence of organic diseases of the bowel. All patients were otherwise healthy and not taking medications. Peyer's patches were identified at endoscopy as small nodules covered by normal mucosa. For CD4 T-B cell coculture, naive B cells were cocultured with sorted CD4^{+} T cells in the presence of endotoxin-reduced SEB. P2X7 selective antagonist A 438079 hydrochloride (Tocris) was added at a final concentration of 10 nM in the coculture. After 9 days of culture, supernatants were collected and IgG content was measured by ELISA.

Statistical Analyses

Student's paired t test was used to determine the significance of differences between mean values. In flow cytometry analyses, mean values ± SD of at least five mice from the same experiment are shown and are representative of at

least three independent experiments. Values of $p < 0.05$ were considered significant.

ACCESSION NUMBERS

The Sequence Read Archive (SRA) accession number for the Ig V_H region sequences reported in this paper is SRP049293.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experi- mental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.10.010.

AUTHOR CONTRIBUTIONS

F.G., M.P., and E.T. designed experiments. M.P. and V.C. performed most experiments. T.R.J., A.R., C.E.F., L.P., R.R., S.P., and E.T. performed experiments. E.R. contributed histolochemical analysis. F.C. contributed human samples. B.B. contributed bioinformatic analysis of IgA sequencing data. M.T., K.D.M., E.S., and E.T. contributed reagents and intellectual input. F.G. and M.P. analyzed data and wrote the paper.

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Immunity, Volume *41*

Supplemental Information

ATP-Gated Ionotropic P2X7 Receptor Controls Follicular T Helper Cell Numbers in Peyer's Patches to Promote Host-Microbiota Mutualism

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Figure S1

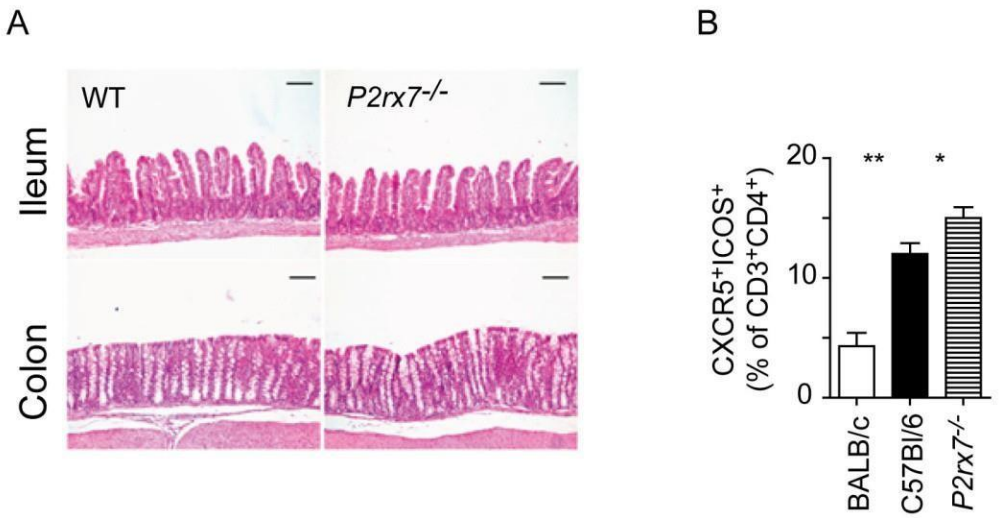

Figure S1. Histochemical analysis of *ileum* and *colon* from WT and *P2rx7*$^{-/-}$ mice. Tfh cells representation in different mouse strains, Related to Figure 1 (A) Representative ileum and colon sections from C57BL/6 (WT) and *P2rx7*$^{-/-}$ mice stained with hematoxylin and eosin (H&E) (bar=100μm). (B) Percentages of $CD3^+CD4^+CXCR5^+ICOS^+$ Tfh cells in PPs of Balb/c, C57BL/6 and *P2rx7*$^{-/-}$. Histograms show mean percentages ± s.d. (n=5). *p<0.05; **p<0.01.

Figure S2

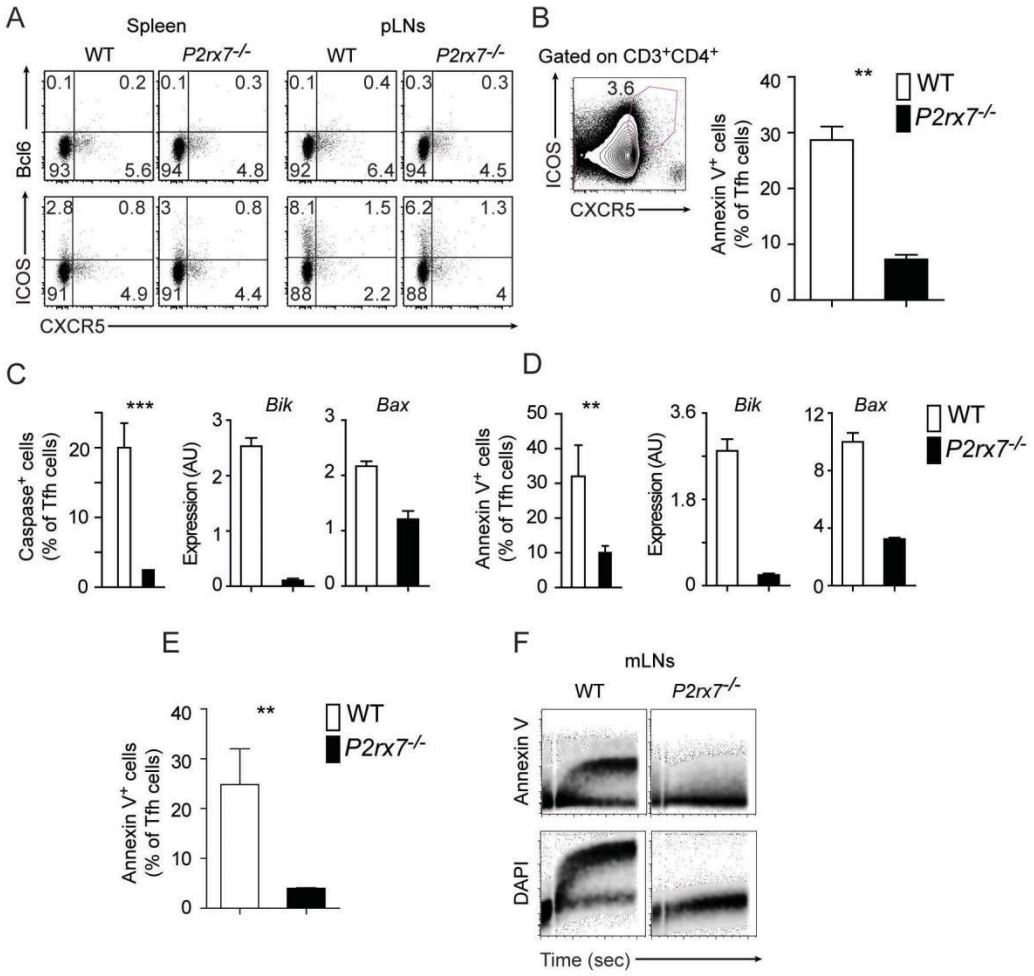

Figure S2. Tfh cells representation in secondary lymphoid organs and PS exposure in mLNs. Tfh cells death in normal and reconstituted mice, Related to Figure 3 (A) Flow cytometry of peripheral LNs (superficial, cervical, axillary, brachial and inguinal) and spleens from WT and *P2rx7*$^{-/-}$ mice for CXCR5, Bcl6 and ICOS on electronically gated CD3^{+}CD4^{+} cells. (B) Statistical analysis of Annexin V^{+} cells in CXCR5^{+}ICOS^{+} Tfh cells gated as shown from mLNs of WT and *P2rx7*$^{-/-}$ mice (n=3). (C) Statistics of VAD-FMK staining (n=5) and quantitative real-time PCR of *Bik* and *Bax* transcripts on Tfh cells from PPs of WT and *P2rx7*$^{-/-}$ mice. (D) Statistical analysis of Annexin V staining (n=5) and real-time PCR of *Bik* and *Bax* transcripts on Tfh cells from PPs of *Cd3e*$^{-/-}$ mice reconstituted with WT or *P2rx7*$^{-/-}$ Foxp3^{EGFP} cells; error bars for technical replicates are shown for *bik* and *bax* real-time PCR values; the results are from a single experiment representative of three with analogous results. (E) Statistical analysis of Annexin V staining on Tfh cells from *Cd3e*$^{-/-}$ mice reconstituted with purified

Tfh cells from PPs of WT or *P2rx7*$^{-/-}$ mice (n=3). (F) Time monitoring of electronically gated Tfh cells from mLNs for PS exposure and DAPI uptake following stimulation with ATP [1.25 mM]. Error bars in all histograms represent s.d.

Figure S3

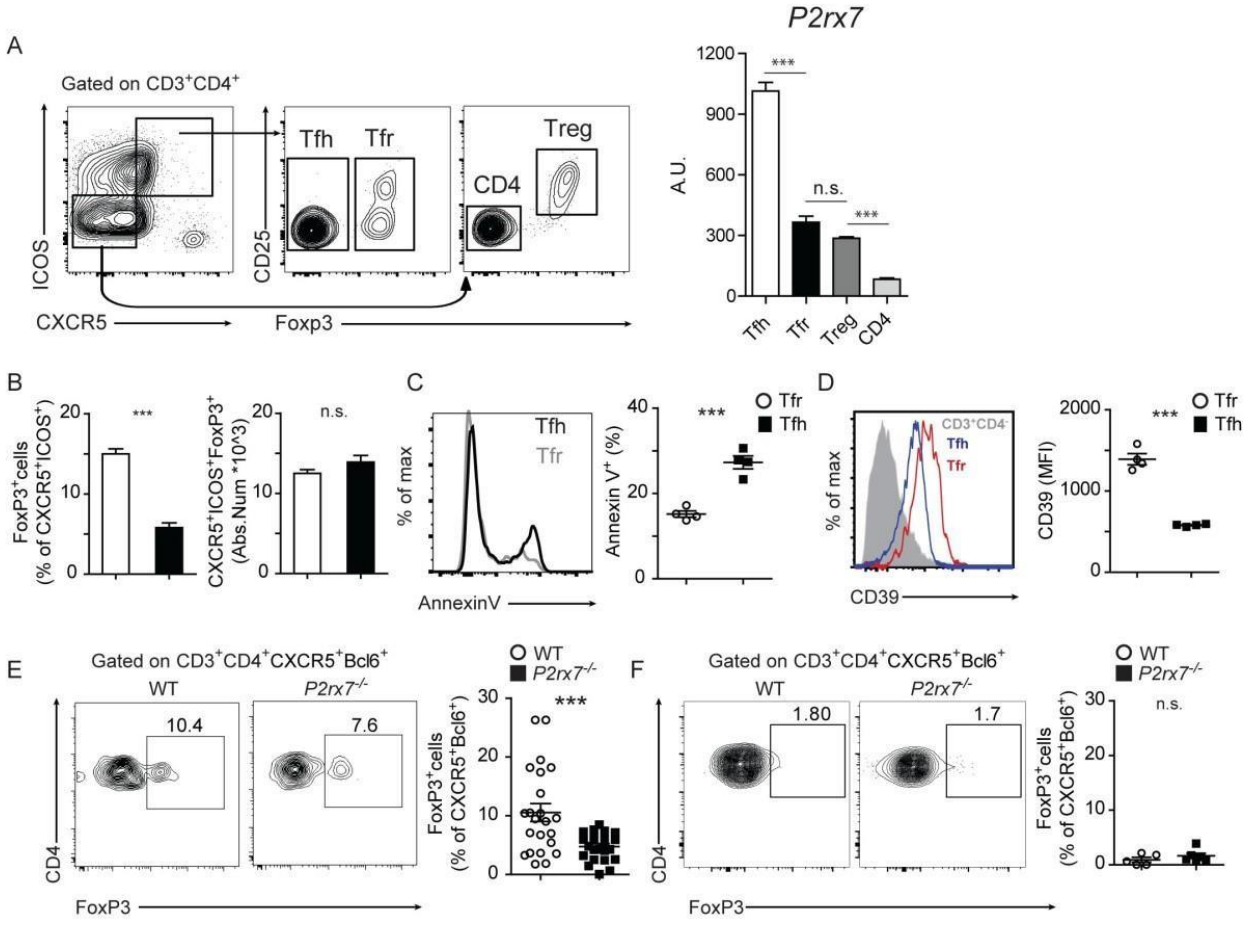

Figure S3. Analysis of *P2rx7* in Tfr cells, Related to figure 4 (A) Real-time PCR of *P2rx7* transcripts in Tfh, Tfr, Treg and CD4$^+$ cells sorted as shown. (B) Statistical analyses of WT and *P2rx7*$^{-/-}$ Tfr cells in PPs; histograms show mean percentages and absolute numbers ± s.d. (n=5). (C) Annexin V staining and corresponding statistical

analysis of WT Tfh and Tfr cells (n=5). (D) Histograms overlay of CD39 staining by flow cytometry in the indicated cell subsets and statistical analysis of MFI. (E-F) Dot plot for CD4 and Foxp3, and statistical analyses of Tfr cells from *Cd3e*$^{-/-}$ mice reconstituted with WT or *P2rx7*$^{-/-}$ Foxp3^{EGFP} cells (E) or with CD4^{+}CXCR5^{+}ICOS^{+} cells (F). Error bars represent s.d.

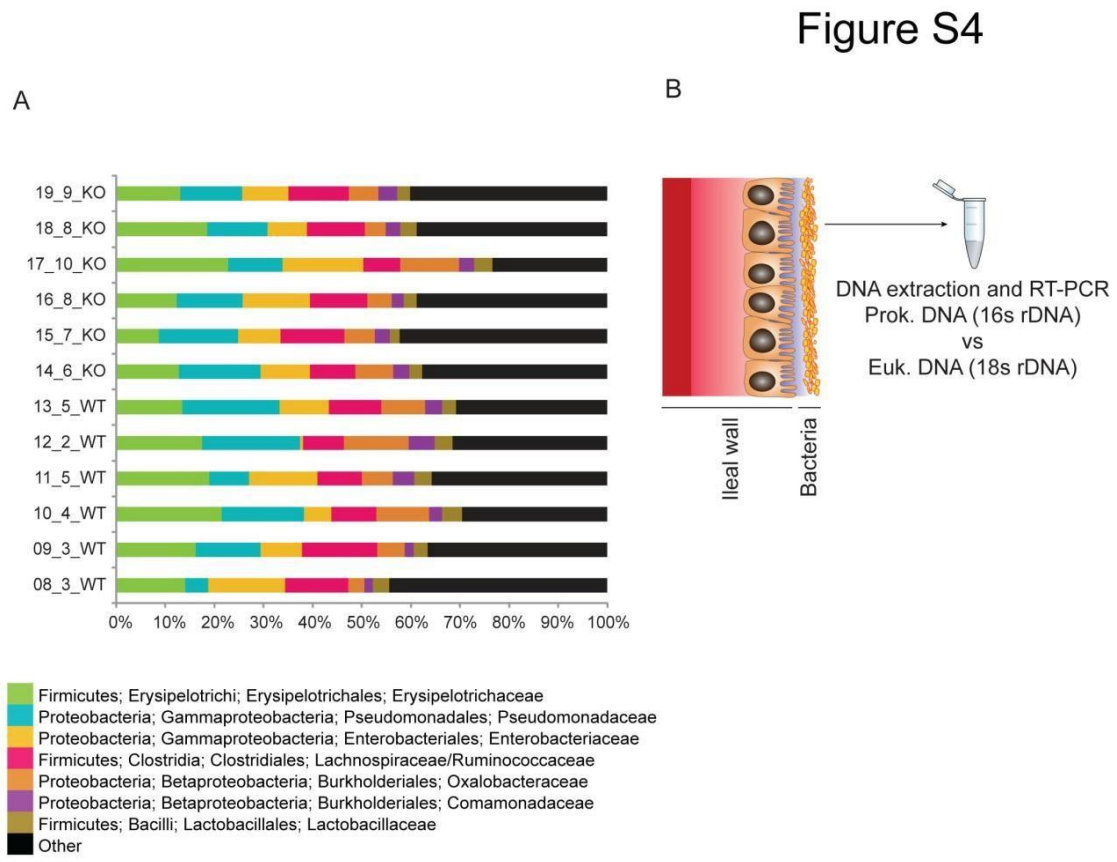

Figure S4. Bacterial phyla distribution in whole intestine of WT and *P2rx7*$^{-/-}$ mice and method used for quantification of mucosal commensals, Related to Figure 6
(A) Bacterial phyla distribution and family richness across individual samples of total gut

content from WT and *P2rx7*$^{-/-}$ (KO) mice. (B) Diagram showing the tissue specimen used for quantification of prokaryotic to eukaryotic DNA ratio in ileal mucosa.

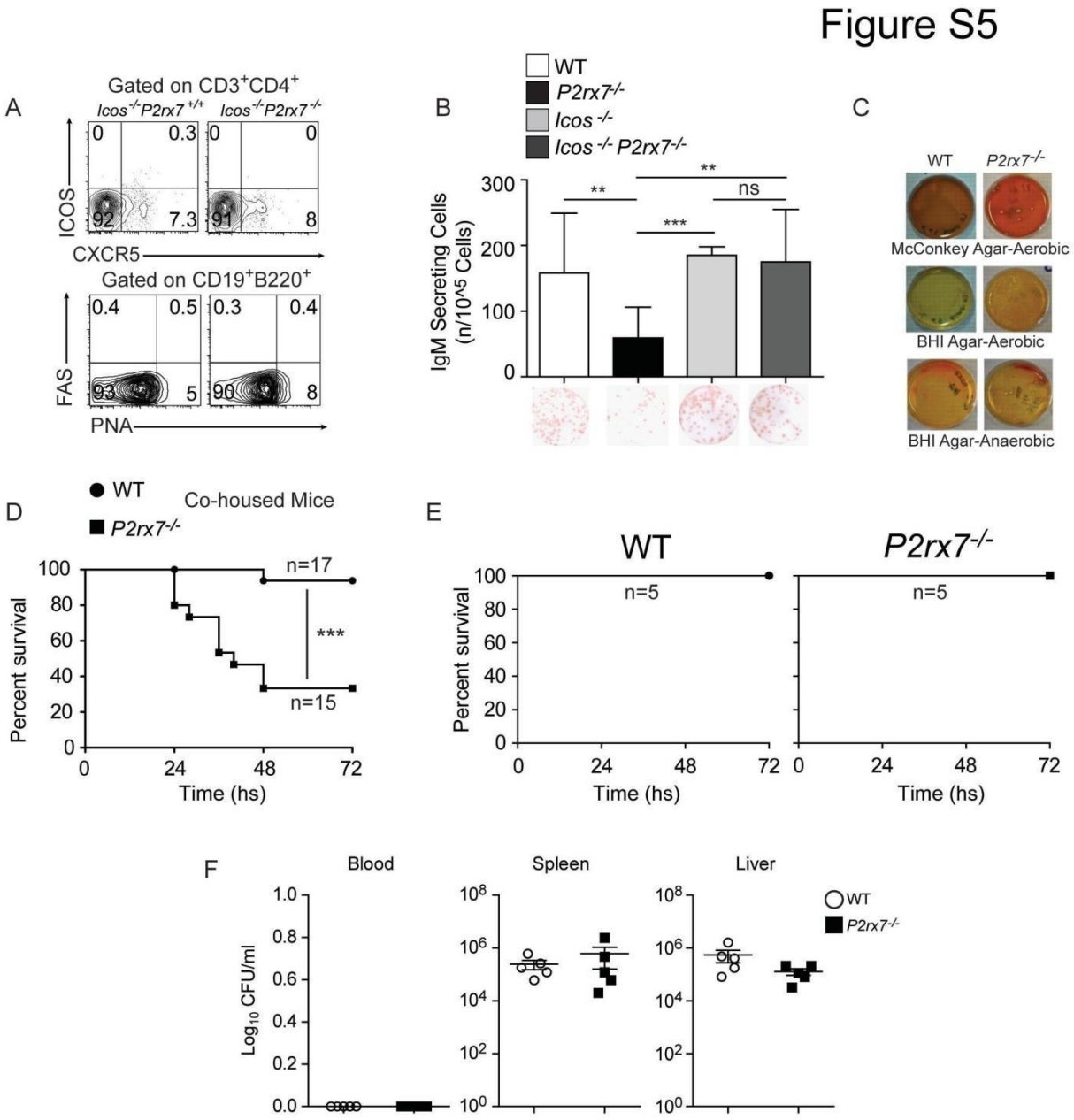

Figure S5. Analysis of Tfh cells and GC B cells in *Icos*$^{-/-}$ mice. IgM secreting splenocytes in different mouse strains. Blood cultures from mice with CLP. CLP in *Icos*$^{-/-}$ and *Icos*$^{-/-}$ *P2rx7*$^{-/-}$ mice. Infection of WT and *P2rx7*$^{-/-}$ mice with *Listeria monocytogenes*, Related to Figure 7 (A) Representative dot plots of PPs from *Icos*$^{-/-}$

and *Icos*$^{-/-}$ *P2rx7*$^{-/-}$ mice stained as indicated to score Tfh and GC B cells. (B) Analysis of IgM secreting cells by ELISPOT in the spleen of the indicated mice. (C) Aerobic and anaerobic blood cultures from WT and *P2rx7*$^{-/-}$ mice 72 h after sublethal CLP. (D) Survival rates of co-housed WT and *P2rx7*$^{-/-}$ mice upon sublethal CLP. (E) Survival rates and (F) CFU measurement in the indicated compartments 3 days after i.v. injection of 3 x 10^3 CFUs of *Listeria monocytogenes*. Error bars represent s.d.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice and *in vivo* experiments

C57BL/6J, *P2rx7*$^{-/-}$ (B6.129P2-*P2rx7*^{tm1Gab}/J), "Foxp3^{EGFP}" (B6.Cg-*Foxp3*^{tm2Tch}/J), *Icos*$^{-/-}$ and *Cd3e*$^{-/-}$ (Malissen et al., 1995) mice were bred in specific pathogen-free (spf) facility at Institute for Research in Biomedicine, Bellinzona, Switzerland. *Foxp3*^{EGFP} *P2rx7*$^{-/-}$ mice were generated by crossing *P2rx7*$^{-/-}$ with *Foxp3*^{EGFP} mice. *Icos*$^{-/-}$ *P2rx7*$^{-/-}$ mice were generated by crossing *P2rx7*$^{-/-}$ with *Icos*$^{-/-}$ mice. C57BL/6J germ free mice were maintained in flexible film isolators at the Clean

Animal Facility, University of Bern, Switzerland. For adoptive transfer of Foxp3 expressing cells, EGFP$^+$ CD4 cells were sorted at FACSAria (BD Biosciences) from pooled cell suspensions of spleen, inguinal, axillary, brachial, cervical and mesenteric LNs of *Foxp3*^{EGFP} or *Foxp3*^{EGFP} *P2rx7*$^{-/-}$ mice. Eight wk old *Cd3e*$^{-/-}$ mice were injected with 1x10^5 EGFP$^+$CD4$^+$ T cells. Recipient mice were sacrificed 4 wk after reconstitution. For adoptive transfer of Tfh cells, CD4$^+$CD8$^-$CXCR5$^+$ICOS$^+$ cells were sorted at FACSAria from pooled PPs of C57BL/6J or *P2rx7*$^{-/-}$ mice. Eight wk old *Cd3e*$^{-/-}$ mice were injected with 1x10^5 Tfh cells. Recipient mice were sacrificed 4 wk after reconstitution. For analysis of *P2rx7* transcription upon antigen stimulation, CD45.2 OT-II TCR transgenic mice were fed with 2 % ovalbumin in the drinking water for 4 d. At day 7, CD4$^+$CD8$^-$CXCR5$^+$ICOS$^+$ cells were sorted from PPs and either processed for real-time PCR or co-cultured with OVA peptide pulsed splenic APCs (CD11c$^+$B220$^-$ cells) from CD45.1 mice. After 16 h, CD45.2$^+$CD4$^+$CD11c$^-$ were sorted and processed for real-time PCR.

Cecal puncture ('through-and-through') from mesenteric toward anti-mesenteric direction was carried out after ligation with a 20G needle. Body temperature and clinical score were measured every 3 h. The following clinical parameters were monitored: motility (0 to 4), fur (0 to 2), temperature (0 to 4),

hunched posture (0 to 3). For IgM treatment, 0.5 mg/mouse of affinity purified IgM from the serum of WT mice were injected intravenously 2 h before CLP. Cohousing experiments were performed as follows. Caesarean delivery and fostering by CD1 adoptive mothers (CoF): WT and *P2rx7*$^{-/-}$ mice were delivered by caesarean section on gestation day 18.5, fed by CD1 adoptive mother, weaned at 4 wk, co-housed and sacrificed at 8 wk of age. Cross-fostering with reciprocal WT and *P2rx7*$^{-/-}$ mothers (CrF): WT mice were fed by *P2rx7*$^{-/-}$ mother and *P2rx7*$^{-/-}$ newborns were fed by WT mother. Cubs were weaned at 4 wk, co-housed and sacrificed at 8 wk of age. Co-Breeding with WT and *P2rx7*$^{-/-}$ mothers (CoB): WT and *P2rx7*$^{-/-}$ newborns were bred together and fed by both WT and *P2rx7*$^{-/-}$ mothers in the same cage (up to 10 cubs with 2 mothers). Mice were weaned at 4 wk of age, co-housed and sacrificed at 8 wk of age. In all of the above breeding conditions male mouse (father) was moved from the cage at the delivery. For experiments with one dose of LPS, 20 µg/mouse of LPS were injected i.p. The dose of LPS used was much lower than those used in studies to induce septic shock (Wang et al., 2003). We could not detect evidences of LPS toxicity such as weight loss, increased body temperature or behavioural change after injection. Mice were sacrificed 1 wk after treatment. All animal experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and authorized by the Cantonal Veterinary.

Antibodies and flow cytometry

The following mAbs were purchased from BD Biosciences (http://www.bdbiosciences.com/eu/index.jsp): biotin conjugated anti-CXCR5 (clone: 2G8, Cat.#: 551960, working dilution: 1:50), PE conjugated anti-Fas (clone: Jo2 Cat.#: 554258, working dilution: 1:300), PE conjugated anti-bcl6 (clone:K112-91, Cat.#: 561522, working dilution: 1:50), PE conjugated anti-ICOS (clone: 7E.17G9, Cat.#: 552146, working dilution: 1:300). The following mAbs were purchased from Biolegend (http://www.biolegend.com/): APC conjugated anti-PD-1 (Clone: RMPI-30, Cat.#: 109111, working dilution 1:200), APC conjugated anti-B220 (clone: RA3-6B2, Cat.#: 103212, working dilution: 1:400), PE-Cy7 conjugated anti-CD4 (Clone: GK1.5, Cat.# 100422, working dilution: 1:300), APC-Cy7 conjugated anti-CD19 (clone: 6D5,

Cat.#: 115530, working dilution: 1:300). The following mAbs were purchased from eBioscience (http://eu.ebioscience.com/): Percp-eFluor710 conjugated anti-CD3 (Clone: 17A2, Cat.#: 46- 0032-80 , working dilution: 1:200), APC conjugated anti-CD11c (Clone: N418, Cat.# 17-0114, working dilution: 1:200), APC-eFluor780 conjugated CD45.2 (Clone: 104, Cat.#: 47-0454-82, working dilution: 1:200), Pe-Cy7 conjugated anti-CD45.1 (Clone: A20, Cat.#: 25-0453-82, working dilution: 1:200). APC conjugated streptavidin was purchased from Biolegend (Cat.#: 405207) and efluo405 conjugated streptavidin from eBioscience (Cat.#: 48-4317-82). Fluorescein labelled Peanut Agglutinin (PNA) (Cat.#: FL-10-71, working dilution: 1:500) was purchased from Vectorlabs (http://www.vectorlabs.com/). Samples were acquired on a FACSCanto II or LSRFortessa (BD Biosciences) flow cytometer. Data were analysed using FlowJo software (TreeStar, Ashland, OR) or FACS Diva software (BD Biosciences).

Real-time PCR

Total RNA from FACS sorted cells was precipitated in Trizol (Invitrogen) and reverse transcribed to cDNA using Random hexamers (Roche, Cat.#: R 15504) and M-MLV reverse-transcriptase (Invitrogen, Cat.#: 28025-013). For quantification of transcripts, mRNA samples were treated with 2 U/sample of DNase (Applied Biosystems). Transcripts were quantified by real-time PCR on an ABI PRISM 7700 Sequence Detector with predesigned TaqMan Gene Expression Assays and reagents according to the manufacturer's instructions (https://www.lifetechnologies.com). The following probes were used: *Ifng* (Mm00801778_m1), *Il17a* (Mm00439618_m1), *Aicda* (Mm00507774_m1), *Bcl6* (Mn00477633_m1), *Gapdh* (Mm99999915_g1), *Prdm1* (Mm00476128_m1), *Rorc* (Mm01261022_m1), *Tbx21* (Mm00450960_m1), *Hprt1* (Mm00446968_m1), *P2rx1* (Mm00435460_m1), *P2rx2* (Mm01202368_g1) p2rx3(Mm00523699_m1), *P2rx4* (Mm00501787_m1), *P2rx5* (Mm00473677_m1) p2rx6(Mm00440591_m1), *P2rx7* (Mm01199500_m1). The following probes for human purinergic receptors were used: *P2rx1* (Hs00175686_m1), *P2rx2* (Hs04176268_g1), *P2rx3* (Hs01125554_m1), *P2rx4* (Hs00602442_m1), *P2rx5* (Hs01112471_m1), *P2rx6*

(Hs01003997_m1), *P2rx7* (Hs00175721_m1). All reactions were performed in triplicate. The relative amounts of mRNAs were calculated by the ΔΔCT method. *18S*, *Hprt* or *Gapdh* were used as internal housekeeping genes.

TUNEL assay, Annexin V and VAD-FMK staining

PPs were fixed in Neutral Buffered Formalin (16 h at 4°C). After fixation samples were dehydrated, embedded in paraffin and then cut at microtome to the desired thickness (6 µm). In situ DNA fragmentation was performed with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method using the Trevigen TACS® 2 TdT-Fluor *In Situ* Apoptosis Detection Kit (http://www.trevigen.com/ Cat. #: 4812-30-K) according to the manufacturer's protocol. Counterstain with Alexa-Fluor 647 conjugated rat anti-mouse CD19 antibody (http://www.biolegend.com/ Clone: 6D5, Cat.#: 115525, working dilution: 1:200) was performed after permeabilization with Cytonin IHC (Trevigen). Incubation was performed (16 h at 4°C) with antibody diluted 1:200 in Cytonin IHC. For detection of PS exposure $1x10^6$ cells were freshly

harvested from PPs and stained with appropriate antibodies, labeled with fluorochrome conjugated Annexin V and analyzed on LSRFortessa flow cytometer. Annexin V staining was performed in Biolegend Annexin V binding buffer (#422201) ($1x10^6$ cells/ml) following the manufacturer's protocol. For detection of activated caspases PPs cells were incubated for 30-45 min at 37 °C with FITC-VAD-FMK (CaspGlow Fluorescein Active Caspase Staining Kit; BioVision) in RPMI medium according to the manufacturer's protocol and then stained with the appropriate antibodies.

***In vitro* time monitoring of DAPI uptake and PS exposure**

Cellular suspensions were prepared from freshly isolated PPs, stained with the appropriate antibodies, washed, resuspended in Annexin V binding buffer for Annexin V staining ($1x10^6$/ml) and loaded with DAPI (1µg/ml). Phosphatidylserine exposure and DAPI uptake following cell stimulation with different doses of Bz-ATP or ATP were time monitored at LSRFortessa and their kinetics analyzed using FlowJo software.

Oral immunization with *E. coli* and flow cytometry for detection of anti-*E. coli* IgA

Single colonies of *E. coli* were aseptically inoculated into LB medium and incubated at 37°C for 18 h. Bacteria were harvested by centrifugation, washed in sterile PBS and concentrated to a density of 2×10^{10} CFUs/ml in PBS. Bacterial suspensions (10^{10} CFUs in 300 µl) were gavaged into the stomach. The procedure was repeated every 3 day for 3 weeks and mice were sacrificed at day 28. Intestinal contents were collected by lavages with 5 ml of intestinal wash buffer (PBS, 0.5M EDTA, Soybean trypsin inhibitor, PMSF), spun at 14'000 rpm in a sterile tube and filtered (0.22 µm) to remove any bacteria-sized contaminants (Hapfelmeier et al., 2010). For flow cytometry analysis of anti-*E. coli* IgA, 3ml of LB broth were inoculated with single colonies and cultured overnight at 37°C. Cultures were subsequently centrifuged (3 min at 7'000 rpm), washed 3 times with sterile-filtered PBS, 2% BSA, 0.005% NaN_3 and resuspended at a density of approximately 10^7 bacteria per ml. Intestinal contents and bacteria were then mixed and

incubated at 4°C for 1h. Bacteria were washed twice, before being resuspended in monoclonal FITC-anti-mouse IgA (Southern Biotech http://www.southernbiotech.com/ Cat.#: 1040-02, working dilution 1:200). After 1 h incubation bacteria were washed twice and resuspended in 2% paraformaldehyde in PBS for acquisition on a FACSCanto using FSC and SSC parameters in logarithmic mode. For each animal analyzed, ELISA was used to determine the total IgA concentration in an undiluted aliquot of the same intestinal wash sample used for surface staining of *E. coli*. This value was used to calculate the total IgA concentration at each dilution of intestinal wash used for flow cytometry of *E. coli* and was plotted against the geometric mean fluorescence obtained in flow cytometry.

Isolation of human PPs and in vitro Tfh dependent B cell help

Peyer's patches were obtained during endoscopy from the terminal ileum of young adults presenting with chronic abdominal pain, and in which subsequent examinations excluded the presence of organic diseases of the bowel. All patients were otherwise healthy and not taking medications. Peyer's patches were identified at endoscopy as small nodules covered by normal mucosa.

PBMCs purified from apheresis blood samples obtained from adult healthy volunteers were used in the experiments. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation. In order to isolate naïve B lymphocytes, $CD19^+$ B cells were selected by CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) according to the manufacturer's instructions. Following staining for lineage (lin) (CD3, clone UCHT1; CD14, clone M5E2, both from Biolegend; and CD56, clone b159, BD BIosciences), CD19 (clone HIB19BD, BD Biosciences), CD27 (clone O323, eBioscience), surface IgG and IgA (Jackson ImmunoResearch), naïve B cells were sorted as $lin^-CD19^+CD27^-IgG^-IgA^-$. The CD19 negative fraction was used to purify T cells. Briefly, $CD4^+$ T cells enriched by negative selection with EasySep human CD4 negative isolation kit (STEMCELL Technologies) were stained with anti-CD4 FITC (clone RPA T4, BD Biosciences), anti-CXCR5 PE (clone MU5UBEE) and anti- CD45RA PE-Cy5 (clone HI100), both from eBioscience. The purity of the sorted B and T cell subsets was > 95%. For CD4 T-B cell co-culture naive B cells were co-cultured with sorted $CD4^+$ T cells (5×10^4 cells/well) in the presence of endotoxin-reduced SEB (1 mg/ml) in RPMI 1640

complete medium supplemented with 10% heat-inactivated FBS in 96 well round bottom plates. P2X7 selective antagonist A 438079 hydrochloride (Tocris) was added at a final concentration of 10 nM in the co-culture. After 9 days of culture supernatants were collected and IgG content was measured by ELISA. Briefly, 96-well flat-bottomed plates (Greiner Bio-One, Frickenhausen, Germany, http://www.gbo. com/en) were coated with isotype-specific goat anti-human IgG antibodies (SouthernBiotech, http://www.southernbiotech.com) diluted in 0.2 M Na_2HPO_4 pH 9.6 and incubated for 16 h at 4 °C. Plates were washed and blocked with PBS 10% FCS for 2 h at room temperature. After washing, serial dilutions of culture supernatants were added and incubated for 2 h at room temperature. Plates were washed again, and alkaline phosphatase- conjugated goat anti-human IgG was added and incubated for 1 h at room temperature. The reaction was developed with Sigma-Aldrich 104 substrate (Sigma-Aldrich).

Next generation sequencing of IgA from PPs B cells

$CD19^+$ cells from PPs were sorted and total RNA extracted with E.Z.N.A. HP Total RNA kit (OMEGA Biotech, Cat.#: R6912-02) according to the manufacturer's instructions. The arm PCR Technology (amplicon rescued multiplex PCR), a patented multiplex amplification strategy (patent No. 7,999,092) developed by iRepertoire was used to amplify murine Ig Heavy chain (http://www.irepertoire.com/#!mbhim/c14vs). High throughput sequencing was performed by iRepertoire and sequences obtained downloaded in a fastq format and analysed with HMMER3.1.

Quantification of IgA and IgM secreting plasma cells by ELISPOT

Plasma cells secreting IgM, or IgA were detected using ELISPOT assay. Briefly, 96-well plates (Millipore) were coated with 10 µg/ml purified goat anti-mouse IgA or IgM (Southern Biotechnologies). After washing and blocking with 1% BSA in PBS for 30 min, serial dilutions of cultured B cells were added and incubated at 37 ºC for 16 h. The plates were washed and incubated with isotype-specific, biotin-conjugated secondary antibodies, followed by streptavidin- HRP (Sigma-Aldrich). The assay was developed with AEC (Sigma-Aldrich). For quantification of gut IgA secreting plasma cells, lamina propria lymphocytes from all small intestine or colon, were isolated as described in "Current Protocols of Immunology", Wiley (unit 3.19). For quantification of total splenic IgM secreting cells, spleens were collected and the obtained cellular suspension, after RBCs lysis in ACK solution, counted and then plated. Quantification of IgM secreting splenic B1 cells was performed on purified $CD19^+B220^-CD23^-IgD^+CD43^+$ cells.

Serum anti-commensals IgM quantification by ELISA

Fresh caecal content was collected and carefully resuspended in PBS (0.01 g/ml). The obtained suspension was centrifuged at 400 x g for 5 min to remove larger particle from bacteria. Bacterial suspensions were then lysed by physical disruption through sonication. The protein concentration of the lysate was quantified with Bradford protein assay. ELISA plates (CORNING half-area 96 well plate, Cat.#: 3690) were coated for 16 h at 4°C with 100 µl of protein

suspension (5 µg/ml). Sera were diluted 1:4 and incubated on coated plates (16 h at 4°C). After 4 washes in PBS 0.025 % Tween 20, 50 µl of alkaline-phosphatase conjugated goat anti-mouse IgM (Southern Biotech http://www.southernbiotech.com/ Cat.#: 1021-04) (1:500 in PBS 10 %FCS) were incubated for 2 h at room temperature. The assay was developed with Sigma 104 phosphatase substrate.

In vitro inhibition of bacterial growth by sera and IgM

Fresh caecal content was diluted to 0.01 g/ml in PBS and serial dilutions of the obtained suspension plated in 24 well plates containing 0.4 ml/well of BHI agar. Undiluted sera were added in some wells. 200 µl of affinity purified IgM from sera of WT mice (0.5 mg/ml) were added to *P2rx7*$^{-/-}$ sera.

Flow cytometry of IgA and IgG$_3$ coated bacteria

For analysis of IgA or IgG$_3$ coated bacteria in flow cytometry, intestinal contents were collected by lavages with 5 ml of PBS. Lavages were centrifuged at 400 x g for 5 min to remove larger particle from bacteria. Supernatants were centrifuged at 8'000 x g for 10 min to remove unbound Igs.

Bacterial suspensions were resuspended at a bacterial density of approximately 10^8 bacteria per ml (OD = 0.125) and then diluted 1 to 10 for staining with APC conjugated rabbit anti-mouse IgA antibodies (http://www.brookwoodbiomedical.com/ Cat.#: SAB1186, working dilution 1:400) or biotin conjugated goat anti-mouse IgG$_3$ F(ab')2 (Southern Biotech http://www.southernbiotech.com/ Cat.#: 1102-08, working dilution 1:200). After 1 h incubation bacteria were washed twice and resuspended in 2% paraformaldehyde in PBS for acquisition at FACSCanto using FSC and SSC parameters in logarithmic mode. SYBR Green was added to the bacterial suspension in order to identify bacteria-sized particles containing nucleic acids.

Scanning electron microscopy (SEM)

The last 1 cm of the terminal ileum was cut, fixed by immersion in fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 6 hours and

post-fixed with 1% osmium tetroxide for 1.5 hours at room temperature. Samples were then routinely processed and critical point dried for scanning electron microscopy (SEM).

Quantification of mucosal adherent bacteria by RT-PCR.

After removal of faecal pellet the last centimeter of ileum was recovered and directly transferred in 500 µl of Lysis Buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5 % SDS) containing 0.4 mg/ml of proteinase K. Samples were incubated 1 h at 60 °C, DNA precipitated in ethanol and resuspended in nuclease free water. 20 ng of DNA from each sample were used to perform RT-PCR. Microbial load was measured by RT-PCR using microbial 16S (small ribosomal subunit gene) broad-range (universal) primers: 5′-GTG STG CAY GGY TGT CGT CA-3′ (forward) and 5′-ACG TCR TCC MCA CCT TCC TC-3′ (reverse) with SYBR Green. 18S rDNA was quantified using a probe from Applied Biosystem (cat. # 4333760F).

Quantification of serum IgM by ELISA

ELISA plates were coated (16 h at 4°C) with 75 µl of unlabeled goat anti-murine IgM (Southern Biotech http://www.southernbiotech.com/ Cat.#: 1021-01) at 5 µg/ml in PBS, washed 4 times with PBS 0.025 % Tween 20, saturated with 200 µl of PBS 10% FCS for 2 h at room temperature. Fifty µl of serial dilutions of sera were incubated 16 h at 4°C. After 4 washes in PBS 0.025 % Tween 20, 50 µl of alkaline-phosphatase conjugated goat anti-mouse IgM (Southern Biotech http://www.southernbiotech.com/ Cat.#: 1021-04) (1/500 in PBS 10 %FCS) were added and plates incubated for 2 h at room temperature. The assay was developed with Sigma 104 phosphatase substrate.

Microbial profiling of intestinal microflora

Fecal DNA was extracted from stool and mucus of 8 wk old mice using the QIAamp DNA Stool Mini Kit (Qiagen). To guarantee the withdrawal of adherent bacteria, mucus was removed by curettage. The entire *16S rRNA* gene was amplified from DNA extracts. The entire procedure was performed by Second Genome through the Signature Discovery platform. Briefly, to calculate the summary intensity for each feature on each array, the central 9 pixels of individual features were ranked.

Fluorescence intensity for sets of probes complementing an operational taxonomic unit (OTU) was averaged after discarding the highest and lowest value and the mean was log2 transformed then ranging from 0 to 16. The hybridization score (HybScore) for an OTU was calculated as the mean fluorescence intensity of the perfectly matching probes exclusive of the maximum and minimum.

Statistical analyses

Student's paired *t* test was used to determine the significance of differences between mean values. In flow cytometry analyses, mean values ± s.d. of at least 5 mice from the same experiment are shown and are representative of at least three independent experiments. Values of $P < 0.05$ were considered significant.

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CD73 reveals novel layers of circulating human memory B cell

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Abstract

The cellular basis of lifetime antibody production rests on long lived plasma cells and memory B cells. The latter have been first described in mouse as cells carrying surface switched isotype, somatically hyper-mutated BCRs and able to respond faster upon antigenic stimulation compared to the naïve counterpart. In human, a common feature of the somatically mutated B cell subsets, both IgM and class switched, is the surface expression of the CD27 antigen which therefore has been used as a general marker of memory B cells. Nonetheless, over the last decades a population of switched memory B cells which do not express CD27 has been identified, implying the existence of antigen-experienced B cells not captured by the CD27 marker. The present study shows that in human ecto-5'-nucleotidase (5'-NT) CD73 is differentially expressed in peripheral naïve and memory B cells and it can be used as a marker to discriminate multiple layers of circulating memory B cells. Notably, we identified a novel B cell subset in the CD27 negative compartment, which lacks CD73 expression and carries somatically mutated IgM BCRs, resembling features and properties of IgM memory B cells. Additionally, we showed that CD73 expression, in the CD27 positive IgG-switched memory compartment defines a subset of cells with higher ability to expand and differentiate to plasma cells in a germinal center fashion and characterized by higher frequency of poly-reactive BCRs. These data suggest the presence of distinct levels of selection within the humoral memory compartment, which could have potential implications in vaccines design as well as in the pathogenesis of autoimmune diseases.

Introduction

Hallmark of adaptive immune responses is the generation of long-lived protection after primary exposure to a pathogen. This mechanism is defined as immunological memory and emerges from a range of changes that evolves over time, involving both T and B lymphocytes. Among T cells multiple types of long lived memory cells are generated, including those that circulate in secondary lymphoid organs, those that reside within tissues and those that are poised towards effector function (Sallusto and Lanzavecchia 1994, Hamann *et al.* 1997, Sallusto *et al.* 2004, Jaigirdar and MacLeod 2015). In humoral responses, this protection stems from a combination of sustained antibody titers, deriving from long-lived plasma cells (LLPCs) and memory B cells. Both types of cells are thought to primarily derive from the germinal center (GC) reaction (Shlomchik and Weisel 2012). LLPCs have been extensively investigated both in human and mouse and have been defined as the reservoir of persistent serum antibody production (Manz *et al.* 1997, Amanna *et al.* 2007, Halliley *et al.* 2015), whereas more controversial is the definition of memory B cells and of their role in sustained serum antibody production. Historically, the latter have been defined, both in mouse and human, as cells carrying isotype-switched BCRs and mutated Ig-V genes, implying a higher affinity for the antigen and higher ability to respond faster upon antigenic re- challenge (McHeyzer-Williams *et al.* 1991). Indeed, in a more general and agnostic terms memory B cells can be defined as a subset that has been primed by a specific antigen and has returned to a quiescent state, persisting for a life time, being able to respond faster and more prompt to a second challenge with that specific antigen. Initial studies, in mouse and human, were focused on IgG^+ memory B cells, as these can be easily identified and isolated. However, the detection of somatically mutated IgM^+ B cells has led to a new definition of memory which finally consider the existence of memory B cells carrying IgM BCRs. Klein et al described for the first time a member of tumor necrosis factor receptor superfamily, CD27, as a surface marker to discriminate naïve from somatically mutated B cells (Klein *et al.* 1998). Since then CD27 has been widely used to identify circulating human memory B cells. Nonetheless, in the last decades, several studies in the field have revealed distinct levels of complexity and heterogeneity. Indeed, within IgG^+ and IgA^+ circulating human B cells there is a fraction lacking CD27 expression, implying the presence of antigen-experienced isotype- switched B cells not captured by CD27 expression. These cells express an array of inhibitory receptors, homologous to Fc receptors, called Fc receptor like (FCRL) molecules which have been found expanded in some pathological conditions, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (Ehrhardt *et al.* 2005, Fecteau *et al.* 2006, Wei *et al.* 2007).

Recent advances in characterizing memory B cells have been achieved using transgenic- mouse model, in which relative numbers of antigen-specific memory B cells are elevated. This strategy enabled the identification of memory B cell surface markers, which were then confirmed in non-transgenic mice. In this regard CD80, PDL-2 and CD73 have been defined as murine memory B cells markers (Anderson *et al.* 2007, Tomayko *et al.* 2010). If mouse study can be used to predict human B cell responses and whether there is a correlation between mouse and human B cell markers are still debating points. Nevertheless, it has been showed that ecto-5'-nucleotidase (5'-NT) CD73 is highly expressed in circulating human B cells and its expression correlates with higher capacity to perform class switch recombination (CSR) and plasma cells differentiation (Schena *et al.* 2013). In the present study, we analyzed CD73 expression in circulating human B cells and investigated the functional properties, BCR mutations, and poly-reactivity against self- and pathogen-antigens of naïve and memory B cell subsets isolated according to CD73 expression, aiming to detect potential novel layers of human memory B cells.

Results CD73 positive cells are significantly decreased in circulating memory B cell subsets

The expression and activity of CD73 undergo dynamic changes in accordance to the environmental context, contributing to the outcome of several cellular responses (Antonioli *et al.* 2013). In human B cells, CD73-dependent adenosine generation favors CSR, endowing cells with an intrinsic control of differentiation toward immunoglobulin isotypeswitched plasma cells (Schena *et al.* 2013). To gain insight into the role of CD73 in the B cell physiology, we sought to fully characterize its expression by flow cytometry on peripheral circulating B cells in adult healthy donors. Bone marrow emigrants transitional B cells represent the most immature circulating B cell subset, originally identified by surface expression of CD24 and CD38 and defined as $CD19^{+}CD27^{-}CD24^{high}CD38^{high}$ (Carsetti *et al.* 1995, Carsetti 2004, Carsetti *et al.* 2004). Additionally, bright co-expression of CD24 and CD38 have been previously associated with CD10 expression (Cuss *et al.* 2006). In fact, we observed higher expression level of CD10 in transitional B cells compare to the naïve subset
(Figure 1A). A detailed analysis of human $CD27^{-}$ compartment in 8 healthy donors revealed that among circulating B cells CD73 is expressed at the highest level on transitional and naïve B cells, which showed respectively 88% and 82% of $CD73^{+}CD39^{+}$ cells (Figure 1A). In healthy adults ~ 40% of peripheral B cells are $CD27^{+}$ and defined as memory B cells. Within this

compartment, the percentages of different circulating memory B cell subsets showed comparable frequency in all the analyzed healthy donors: ~ 25% IgM^+D^+, ~ 10% IgM only, ~ 10% IgD only, ~ 30% IgG. The observed $IgM^-IgD^-IgG^-$ population can be reasonably assumed as the circulating IgA-isotype-switched memory B cells (~ 25%) (Figure 1B). A dynamic regulation of CD73 in the $CD27^+$ compartment was observed. Specifically, higher expression in the IgG and IgA class-switched B cells memory (60% and 64% respectively) and a moderate reduction in the IgM^+IgD^+ memory cells (45%) (Figure 1A). Collectively, our results suggest that CD73 is highly expressed on antigen inexperienced B cells, namely naïve and transitional subsets, whereas it is bimodal-expressed in the $CD27^+$ compartment, with isotype-switched memory B cells expressing higher level compared to IgM memory counterpart.

CD73 expression in human naïve B cells correlates with more efficient cognate interaction with T follicular helper cells

In mice, CD73 is expressed primarily on B cells that have undergone class-switch recombination (Yamashita *et al.* 1998), and is used as marker of memory (Anderson *et al.* 2007, Tomayko *et al.* 2010). Furthermore, recent evidences showed that mouse GC- independent memory is enriched in $CD73^-$ IgM B cells, while the GC-dependent counterpart is mainly generates isotype-switched B cells characterized by surface expression of CD73 (Taylor *et al.* 2012). In the present study, we aimed to investigate whether CD73 could identify a population of circulating human B cells more prone to generate a GC reaction and therefore prone to interact with T follicular helper cells (Tfh). Human blood $CD4^+$ $CD45RA^-CXCR5^+$ T cells have been defined as a circulating memory counterpart of Tfh able to support antibody secretion (Morita *et al.* 2011). $CD4^+CD45RA^-CXCR5^+$ were sorted from peripheral blood of 3 healthy donors and co-cultured with autologous peripheral naïve B cells ($CD19^+CD27^-IgM^+IgD^+$) separated based on CD73 expression (Figure 2A). To mimic the antigen-specific interaction between T and B cells staphylococcus enterotoxin B (SEB) was added to the culture. Notably, it has been previously reported that the aforementioned culture condition ensures a T cell-dependent immunoglobulin secretion (Morita *et al.* 2011). After 7 days of co-culture, the IgG-secreting plasma cells were measured by ELISPOT assay and a significant increase was observed when Tfh cells where co-cultured with naïve $CD73^+$ B cells respect to the $CD73^-$ counterpart (Figure 2B). The number of viable cells was similar between the tested culture conditions (data not shown) indicating that the less ability of $CD73^-$ B cells to produce IgG was not due to an impaired survival. Collectively, these data provide evidence that

expression of CD73 in human naïve B cells correlates with more efficient cognate interaction with Tfh cells, which results in higher T cell-dependent immunoglobulin secretion.

Single human B cell expansion confirmed the higher potential of naïve CD73$^+$ to class switch to IgG

To further understand the role of CD73 activity in shaping the outcome of B cell responses, we used a high-throughput strategy to screen a broader range of peripheral blood B cells and to analyze functional characteristics of their BCRs at the single cell level. Naïve and IgM memory B cells were sorted based on CD73 expression. Transitional B cells were included in the screening but due to their low peripheral blood representation, no further discrimination for CD73 expression was done. Cells were plated at a density of 1 cell per well, expanded *in vitro* with the addition of feeder cells and conditioned medium. After 12 days the B cell efficiency response and the IgG-CSR efficiency were evaluated (Suppl. Figure 1). The culture supernatants were screened for IgM production and the analysis showed an overall comparable IgM differentiation efficiency between naïve and IgM memory subsets, either positive or negative for CD73 (Figure 3A). Instead, a significant difference was observed in the response efficiency of transitional B cells, which showed a decrease in the percentage of IgM expanding cells compare to naïve CD73$^+$ B cells (Figure 3B). Notably, in line with the results collected in a bulk B cell stimulation system, we observed higher IgG CSR efficiency in the naïve CD73$^+$ compartment compare to CD73$^-$ counterpart, which intriguingly showed a comparable efficiency to IgM memory subsets. Collectively, the results obtained at the single cell level provided additional evidence that CD73 expression endows naïve B cells with intrinsic plasticity to perform CSR. Analogous CSR efficiency was observed in transitional and naïve CD73$^+$ B cells, indicating that the high CD73 expression of the former could already be crucial in favoring *in vitro* CSR and plasma cells differentiation. Furthermore, the diminished potential of naïve CD73$^-$ B cells to differentiate into antibody-secreting cells was like what observed in the IgM memory B cell compartment.

Predominant poly-reactivity in naïve CD73$^+$ B cells

The development of a proper adaptive immune response is based on the random generation of antigen receptors. A stringent selection process, aiming to remove the undesired BCRs specificities, is necessary in the bone marrow as well as in the periphery. Indeed, peripheral human B cell selection has been proposed to be regulated by two different check points, which allow the decrease of poly-reactive BCRs frequency (Wardemann *et al.* 2003). We reasoned that CD73 expression could identify B cell subsets with different degree of poly-reactivity. Circulating naïve and IgM memory B cells were isolated from 6 healthy donors, according to CD73 expression and clonal expanded for 12 days in a 384 well plate (Suppl. Figure 1). The culture supernatants were screened by ELISA assay for binding to a defined set of antigens, which included self-antigens (insulin, IL-2 and IL-17AF) and pathogen-antigens (influenza, tetanus toxoid). The frequency of B cells expressing poly-reactive BCRs was determined considering as poly-reactive a clone which bound to at least two different antigens in the screening. Overall, the analysis demonstrated a significantly reduced poly-reactive BCRs fraction in the naïve CD73$^-$ B cell subset, which showed Igs-reactivity profile similar to IgM memory B cells (Suppl. Figure 2). Notably, the poly-reactivity was significantly decreased in the naïve CD73$^-$and IgM memory CD73$^-$ B cells (Figure 4). Taken together, these results demonstrate that low expression of CD73 in naïve and IgM memory compartments defines an advanced differentiation step, characterized by a restricted BCR repertoire compare to the CD73$^+$ counterparts.

IgM$^+$CD27$^-$CD73$^-$ B cells are enriched in mutated sequences

Given the different functional properties observed in the IgM compartment (either CD27$^+$ or CD27$^-$) according to CD73 expression, we set out to assess whether the expression of the marker correlates with different BCR-genetic properties. The presence of mutations in the Ig heavy chain V gene segments is generally considered as an indication of previous antigenic exposure and thus is generally considered as a hallmark of memory B cells. Ig heavy chain V gene sequences were amplified from cDNA extracted from a single B cell clone expanded *in vitro* for 12 days. Clones containing unique VH sequences were analyzed for gene segment usage, germline identity, and CDR3 composition, using the IgBlast algorithm. From 3 healthy donors, a total of 301 sequences were analyzed and the germline identity was evaluated. In the IgM$^+$CD27$^-$CD73$^+$ a total of 183 sequences showed a 4.3% of sequences with < 95% of germline identity. Notably, in the IgM$^+$CD27$^-$CD73$^-$ a total of 136 sequences were analyzed and 30.1% was found mutated,

with < 95% of germline identity (Figure 5A). The difference in the mutation status between the IgM^+CD27^- subsets was surprisingly remarkable, as the

$CD73^-$ cells were enriched in mutated sequences. Furthermore the mutations showed the tendency to accumulate in the CDR1 and CDR2 gene fragments. These results demonstrate for the first time that the $IgM^+CD27^-CD73^-$ subset represent a population of IgM-bearing B cells that express somatically mutated V genes. Importantly, usage of CD73 as a surface marker allows the identification of antigen-experienced B cells within the IgM^+CD27^- population, which until now has been referred as a naïve population.

We next compared the percentage of mutated VH sequences in the IgM^+CD27^- and IgM^+CD27^+ compartments. A total of 71 sequences of IgM^+CD27^+ B cells from 1 healthy donor were included in the analysis. Remarkably, $IgM^+CD27^-CD73^-$ showed comparable mutations frequency to the one observed in the IgM^+CD27^+ memory B cells and distant from the highly germlined $IgM^+CD27^-CD73^+$ naïve B cells (Figure 5B). Taken together, these results demonstrate for the first time that low expression level of CD73 in the IgM^+CD27^- compartment defines an antigen-experienced subset enriched in mutated sequences and detached from the highly germlined $CD73^+$ counterpart.

CD73 defines a subset of human reactive memory B cell

The bimodal expression of CD73 observed in the IgG+CD27+ memory compartment (Figure 1), suggest that this marker might define a novel subset of switch memory B cells. To test this hypothesis, peripheral IgG^+CD27^+ B cells from 7 healthy donors were sorted according to CD73 expression and expanded *in vitro* at the density of 1 cell/well (Suppl. Figure 1). After 12, days the culture supernatants were screened by ELISA assay for IgG secretion and the analysis showed a significantly increased B cell response efficiency in the $CD73^+$ subset compare to the $CD73^-$ counterpart (Figure 6A). These data suggest that CD73 expression defines a subset of isotype class-switched memory B cells more prone to secrete IgG antibodies upon in vitro stimulation. We therefore aimed to evaluate the kinetic of IgG secretion in the IgG $CD27^+$ $CD73^+$ and $CD73^-$ memory compartment at 6 different time points (Figure 6B). This was done by quantifying in the culture supernatants the IgG secretion using an ELISA assay, at different ime points: 2-4-6-8-10 and 12 days. Our results showed that over the time, the immunoglobulin concentration was delayed and reduced in $CD73^-$ memorycompartment. Indeed, at 4 days $CD73^+$ cells showed ~ 40% of response efficiency compare to the ~ 10% showed by $CD73^-$, which correlates with a concentration of IgG in the culture supernatants of roughly 1 ng/ml. In the latest

time points, $CD73^+$ B cells showed higher fraction of IgG secreting cells reaching higher immunoglobulin concentration in the supernatants (Figure 6B). Collectively, these data suggest that CD73 can be used as a marker to define a subset of reactive IgG $CD27^+$ memory B cells, which are faster and more efficient in immunoglobulin secretion upon *in vitro* stimulation.

Predominant poly-reactivity in the $CD73^+$ IgG class switched memory B cells

Poly-reactivity was evaluated in the IgG isotype-switched memory B cell compartment. After clonal expansion, culture supernatants were screened for binding to a defined set of antigens which included self-antigens (insulin, IL-2 and IL-17AF) and pathogen-antigens (influenza, tetanus toxoid). As previously described, the poly-reactive BCRs fraction was defined considering the clones which bound to at least two different antigens in the ELISA screening. The analysis of 5 healthy donors showed that class-switched memory $CD73^+$ B cells are enriched in poly-reactivity compare to the $CD73^-$ counterpart (Figure 7A). To determine whether this finding correlates with specific Ig sequence features, heavy chain VH sequences were amplified from cDNA extracted from single clones of B cell expanded *in vitro* for 12 days. A total of 431 clones from 2 healthy donors were analyzed for germline identity and CDR3 composition using the IgBlast algorithm. The CDR3 amino acid length comparison showed significant longer sequences in the $CD73^+$ memory B cells compare to the $CD73^-$ counterpart and no differences in the amount of mutated sequences were observed (Figure 7B-
C). Thus, these data show that the enrichment of poly-reactive BCRs in the $CD73^+$ IgG memory compartment is associated to longer CDR3s sequences.

Discussion

Immunological humoral memory is the result of a successful immune response, which leads to generation of long-lived protection after a primary exposure to a pathogen. This protection stems from long-lived plasma cells (PCs) and long-lived memory B cells. Both types of cells are thought to primarily derive from a germinal center (GC) reaction (Shlomchik and Weisel 2012). Understanding how these cells are generated from naïve compartment has important implication not only in vaccine design, but also for our understanding of human memory B cells development and their contribution in the pathogenesis and progression of autoimmune diseases. A major challenge for the detection of memory B cells is the lack of a unique phenotype that distinguishes them from naïve B cells. Despite the importance of studying human B cell memory response, much information in the field is coming from mouse experiments. Indeed, using a transgenic

mouse model the co-stimulatory molecule CD80 has been identified as a memory B cell marker (Anderson *et al.* 2007). In later studies this molecule has been combined with CD73 and PD- L2 to identify at least five different populations arising in response to immunization (Tomayko *et al.* 2010). In the present study we focused on the analysis of peripheral human B cells, aiming to characterize the expression and the role of CD73 in cell differentiation and memory generation. We observed that CD73 is highly expressed on circulating antigen-inexperienced B cells, namely transitional and naïve cells and significantly decreased in circulating memory subsets defined according to the expression of CD27. We observed enrichment in $CD73^-$ B cells in the $IgM^+IgD^+CD27^+$ subset. This finding is in line also with recent evidence in mice showing that memory B cells can differentiate through two independent pathways: a GC-independent which generates memory B cells mainly IgM^+CD73^-, and GC-dependent which leads to the formation of classswitched memory B cells and are characterized by CD73 surface expression (Takemori *et al.* 2014). Interestingly, we observed that CD73 expression endowed human naïve B cells with higher capacity to cognate interaction with T follicular helper cells (Tfh). Our finding suggests that the higher class-switch recombination (CSR) efficiency observed in naïve $CD73^+$ B cells (Schena *et al.* 2013) is linked to their intrinsic capacity to respond to a T-cell dependent stimulation. Indeed, these functional properties are linked to the enzymatic function of CD73, since treatment of naïve and memory B cells $CD73^-$ with adenosine significantly increased the differentiation of the latter to class switched B cells (Schena *et al.* 2013). We confirmed this data using an enzymatic antagonistic antibody which dampens significantly both CSR and plasma cell differentiation (data not shown). Notably therapeutic interventions targeting the adenosinergic pathway have recently gained much attention (Young *et al.* 2016) and open up the possibility to interfere with GC-dependent B cell responses by targeting CD73.

The adenosine receptor expressed by human B cells is the A2A, which is a Gs-protein coupled receptor linked to activation of adenylyl cyclase enzyme. This results in the conversion of ATP into c-AMP, which in turn acts as a second-messenger activating c-AMP dependentkinase, PKA, responsible for the phosphorylation of specific target proteins (Yang *et al.* 2015). It has been shown that AID physically interacts with PKA in the cytoplasm and is phosphorylated by PKA catalytic subunit at specific residues (Pasqualucci *et al.* 2006). We speculate that the higher CSR efficiency observed in the $CD73^+$ compartment, in both naïve and IgG memory B cells, is linked to the PKA activation A2A-dependent, with the consequent phosphorylation of AID, which is required for CSR. Whether naïve B cells are intrinsically programed for recruitment into the GC-independent or GC-dependent pathway, or can enter either pathway depending on signals

received upon activation, remains to be explored. In this study we showed that CD73 expression might represent an early decision check point in the development of human B cell memory, which drives towards a GC-dependent response. Unravelling the signal driving B cells towards T-dependent response may also improve our understanding of the transforming mechanisms driving the development of GC-derived B cell malignancies.

To better understand the origin and function of B cell populations expressing CD73, we investigated the composition of the functional BCR repertoire in naïve and memory B cell subsets isolated according to CD73 expression. Recently, the need to develop an efficient method to solve the complexity of BCRs repertoire in normal as well pathological situation, such as autoimmune disorders, immunodeficiencies and infection diseases has emerged (DiLillo *et al.* 2011). One limitation in studying B cell physiology has been the unlikelihood to generate long-term B cell lines from mature B cells. Indeed, stimulation of naïve and memory B cells causes their terminal differentiation to PCs along with cell cycle arrest. Traggiai et al described the first published improved method for the Epstein Barr Virus-mediated transformation of memory B cells, which increased the efficiency of B cell immortalization to a level that has allowed the isolation of naturally generated monoclonal antibodies in many diseases (Traggiai *et al.* 2004). The drawback of this approach is that, due to the low clonal efficiency, it involves the screening of large numbers of B cells to obtain monoclonal antibodies of the desired specificity. Recent advances in the isolation, culture and expansion of human B cells are enabling the isolation of the cognate immunoglobulin heavy and light chain variable genes from a single B cell. These genes can then be cloned and expressed in eukaryotic cell lines. Wardemann et al were the first to apply this approach to examine the development and silencing of autoreactive B cells (Wardemann *et al.* 2003) and subsequently it has been used to understand the human B cell selection process in healthy and diseased individuals (Wardemann and Nussenzweig 2007, Meffre and Wardemann 2008, Isnardi *et al.* 2010, Meffre 2011). Despite the possibility to clone and express antibodies from single human B cells, this method does not provide the functional analysis of the B cell clone of interest. To overcome this issue, we optimized a published method (Huang *et al.* 2013), with the aim to functionally screen at a single cell level distinct subset of peripheral B cells and thereafter investigate their genetic Ig sequence information. In this study, we used CD73 as a surface marker to further discriminate naïve and memory B cells. Cells were seeded at a density of 1 cell/well and expanded *in vitro* for 12 days in the presence of feeder cells and conditioned medium. We observed that adding a TLR9 agonist, transferrin, BAFF at the cocktail stimulation and using an anti-CD40 antibody instead of the CD40-ligand determined a

significant improvement of the single cell expansion efficiency compare to the published method (Lindner J.M et al, manuscript in preparation). The culture supernatants were screened for IgM and IgG secretion to evaluate respectively IgM response and CSR efficiency. Importantly, our results confirmed the intrinsic role of CD73 in shaping the outcome of the B cell response, endowing naïve B cells with higher intrinsic plasticity to perform CSR. Indeed, we showed that low expression of CD73 resulted in a significant decrease in the IgG-CSR efficiency compared to the positive counterpart, but a comparable IgM response efficiency. The decision between extrafollicular response and GC migration represents the main branching point during a T-dependent B cell response and the mechanisms which direct B cells down one pathway versus the other are so far unknown (MacLennan *et al.* 2003). Remarkably, our results point to CD73 in naïve compartment as a master regulator of B cell response, which drives towards a GC B cell differentiation.

Transitional B cells represent newly bone marrow (BM) emigrants and the most immature peripheral B cell subset. Here we observed for the first time that high expression of CD73 in human transitional B cells is associated to high IgG CSR efficiency. We speculate that the high CD73 expression could already be crucial in promoting CSR of the immature transitional compartment. Notably CD73 expression is regulated by Hypoxia Inducible Factor 1 (HIF-1), which is likely involved in CD73 modulation in the BM niche. In this context, it is important to consider that CD73 has been shown to be highly expressed by BM stromal cells, also termed mesenchymal stem cells (MSCs) (Boxall and Jones 2012) and to be involved in BM stromal interactions (Barry *et al.* 2001) MSC migration (Ode *et al.* 2011), and, potentially MSC modulation of adaptive immunity (Eckle *et al.* 2007).

A historic definition of B cell memory has been generally focused on class switched IgG^+ B cells, whereas IgM^+IgD^+ were assumed to represent antigen-inexperienced expressing variable region genes without somatic mutations. However, it's now well established that two distinct populations in human IgM memory can be identified: IgM only, with a GC derivation (Weill *et al.* 2009) and IgM^+IgD^+ memory B cells with a doubtful origin. The latter has been defined as human marginal zone (MZ) B cells. Nevertheless, although they share many properties with their mouse counterpart, they also display striking differences (Spencer *et al.* 1998, Dammers *et al.* 2000). Indeed, the presence of somatic mutations in the IgM receptor of human MZ B cells has caused confusion about their definition, leading these cells to be called IgM memory by most authors. Splenic $IgM^+IgD^+CD27^+$ B cells share with mouse MZ B cells localization at the

periphery of B cell follicles. However in contrast to mouse MZ B cells previously described as a splenic-resident subset with un-mutated B-cell receptors, human $IgM^+IgD^+CD27^+$ harbor mutated Ig variable genes and recirculate (Weller *et al.* 2004). Mutations are an indication of prior activation, from which most of memory B cells are assumed to originate. In our study, we showed for the first time that low expression of CD73 in the naïve $IgM^+IgD^+CD27^-$ compartment defines an antigen-experienced subset enriched in mutated sequences, distant from the highly germlined $CD73^+$ counterpart. Furthermore, the level of mutation in the $IgM^+IgD^+CD27^-CD73^-$ was similar to the one observed in $IgM^+IgD^+CD27^+$ memory B cells. Overall our study pointed out that CD27 is not an appropriate un-equivocal marker of memory B cells, as already reported after the discovery of IgG^+CD27^- population (Fecteau *et al.* 2006, Wu *et al.* 2011). Remarkably, from our data it emerges that human memory B cells progressively acquire phenotypes and properties that distinguish them from not experienced naïve B cells, with CD73 playing an important role in the early differentiation steps. We speculate that $IgM^+IgD^+CD27^-CD73^-$ B cells might represent early precursors of $IgM^+IgD^+CD27^+$ B cells, which have already encountered the antigen and consequently mutated Ig variable genes. Further experiments are necessary to confirm this hypothesis and to gain insights into biological pathways and molecular mechanisms that regulate cell fate and development.

The healthy immune system constantly produces B cells able to recognize specifically only one antigen and a significant fraction of poly-reactive B cell clones. The latter are defined as BCRs able to bind multiple structurally unrelated antigens (Casali and Notkins 1989, Zhou and Notkins 2004). Besides the possible role of antibody poly-reactivity in magnifying the antigen detection power and in regulating immune system own functions, is still questionable whether these "promiscuous" interactions bear any physiological meaning. Indeed, definition of poly-reactivity would be certainly strengthened if correlated to a functional consequence (Dimitrov *et al.* 2013). Wardemann et al suggested that in healthy individuals the negative selection during B cell ontogeny is responsible for the significant reduction of poly-reactivity. In their model nearly 90% of the poly-reactive antibodies are counterselected in the immature B cell stage and only ~ 4% of circulating naïve B cells express poly-reactive BCRs (Wardemann *et al.* 2003). The set of antigens used in the aforementioned study to define polyreactive antibodies includes: double-strand DNA (dsDNA), single-strand DNA (ssDNA) insulin and LPS. In our study we aimed to amplify the panel of antigens and to specifically introduce self-molecule, like insulin, IL-2 and IL-17 and non-self-(pathogen)-antigens like influenza and tetanus toxoid. In line with the hypothesis that CD73 represent a B cell

differentiation marker, we observed that reduced CD73 expression in naïve and IgM^+IgD^+ memory compartment defines a subset characterized by a restricted BCR repertoire compare to the $CD73^+$ counterpart. Thus we identified a novel B cell subset in the CD27 naïve compartment which lacks CD73 expression and carry somatically mutated IgM BCRs resembling features and properties of IgM memory B cells.

CD73 indeed is not confined only in the IgM compartment but we could also detect in the IgG^+ memory cell subsets. Our results clearly show that CD73 can be used as a marker to define a subset of GC-reactive IgG memory B cells, which is faster and more efficient in Igs secretion upon *in vitro* stimulation. The higher IgG secretion efficiency observed in $CD73^+$ memory B cells might reflect their need to further recirculate the GC and receive more cognate T cell help. In fact, upon reactivation memory B cells proliferate and eventually reenter the GC reaction, to improve immunity versus modified pathogen or infection conditions by downstream CSR and new rounds of affinity maturation (Dogan *et al.* 2009). The $CD73^-$ cells might instead represent the result of a successful GC reaction, which shutdown CD73 expression to escape further rounds of affinity maturation and to instead contribute to the long-lived PCs pool. In line with our hypothesis, in a mouse study it has been shown that CD73 is expressed on GC B cells upon immunization, but is absent among PCs and plasma blasts (Conter *et al.* 2014). In addition, it has been observed that CD73 expression is crucial for the adhesive interaction between follicular dendritic cells and GC B cells (Airas 1998). It is intriguing to consider that hypoxia-inducible factor 1 (HIF-1alpha) is a master regulator in promoting CD73 expression (Synnestvedt *et al.* 2002), impling a dinamic regulation in the GC microenvironment, due to the hypoxia condition and the high rate of cell proliferation and cell death.

In conclusion, taken together our data suggest a novel role for CD73 and adenosine signaling in regulating human B cell response and memory generation. Adenosine is generally referred as an inhibitory molecule, able to counter balance the activation effect of the ATP on innate immune system, from which adenosine is generated. Our findings provide new insights in the activation effect of adenosine on human B cells and in the CD73-depentent immunomodulation. Lastly, our data showed a novel subset of antigen-experienced $CD27^-$ characterized by low expression of CD73 and carrying somatically mutated IgM BCRs.

Experimental procedures Human blood samples

Buffy coat samples were collected from healthy adult donors with approved informed consent (Local Swiss Ethics Committee) in the University Hospital Insespital Bern, Switzerland.
Peripheral blood mononuclear cells (PBMCs) were freshly isolated by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. For cryopreservation of human cells, freshly isolated PBMCs were aliquoted at a concentration of 10^7 cells per aliquot in 1 ml of fetal bovine serum (FBS) 10% dimethyl sulfoxide (DMSO). Cryovial tubes were placed in a prechilled freezing container (Nalgene Mr. Frosty, Sigma), stored at −80°C for 24 h and then stored at −150°C until thawing.

mAbs and flow cytometry

The following monoclonal antibodies were used in this study for flow cytometry and cell sorting: PC5.5 labeled anti-human CD3 (clone # UCHT1), PC5.5 labeled anti-human CD14 (clone # RMO52), PC5.5 labeled anti-human CD16 (clone # 3G8) and PC5.5 labeled anti- human CD56 (clone # N901) (all purchased from Beckman Coulter), BV785 labeled anti- human CD19 (clone # HIB19), BV650 labeled anti-human CD27 (clone # O323), BV510 labeled anti-human CD24 (clone # ML5), Alexa Fluor 700 labeled anti-human IgM (clone # MHM-88), PC7 labeled anti-human IgD (# clone IA6), PerCP-eFluor 710 labeled anti-human CD73 (clone # AD2), PE labeled anti-human CD10 (clone # HI10a), FITC labeled anti-human CD39 (clone # A1), PC5.5 labeled anti-human CD38 (clone # LS198-4-3) and APC labeled anti-human IgG (polyclonal).

For flow cytometer analysis, cells were incubated with fluorochrome-conjugated mAbs specific for human surface antigens described above and diluted in PBS 2% FBS prior to analysis. Labeled cells were analyzed on an LSRFortessa (BD Biosciences) flow cytometer or sorted on a BD FACSAria using Diva software (BD Biosciences). Data were analyzed using FlowJo software.

Human B cell enrichment

To perform functional assay human peripheral B cells were isolated from PBMCs by negative selection with the EasySep Human B Cell Enrichment Kit, according to the manufacturer's instructions (STEMCELL Technologies).

Media and reagents for cell culture

The medium used for B cell culture was RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific), 2 mM GlutaMAX (GIBCO), 10 mg/ml nonessential amino acids (GIBCO), 1mM sodium pyruvate (GIBCO), 50 U/ml penicillin (Sigma), 50 U/ml streptomycin (Sigma), 50 U/ml kanamycin (GIBCO), and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich).

***In vitro* Tfh-dependent B cell help**

To isolate peripheral naïve B lymphocytes, $CD19^+$ B cells were selected by CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Total B cells were stained with the following mAbs: anti-human CD3, anti-human CD14, anti-human CD16, anti-human CD56, anti-human CD19, anti-human CD27, anti-human IgG/A and anti-human CD73. Naïve B cells ($lin^-CD19^+CD27^-IgG^-IgA^-$) were sorted according to CD73 expression by the cell sorter FACSAria (BD Biosciences). From the CD19 negative fraction, $CD4^+$ T cells were purified by negative selection with EasySep isolation kit (STEMCELL Technologies) and stained with following monoclonal antibodies: anti-human CD4 (clone # RPA T4), anti-human CD8 (clone # RPA-T8) anti-human CXCR5 (clone # MU5UBEE) and anti-human CD45RA (clone # JS-83). Tfh cells were sorted as $CD4^+CD8^-CD45RA^-CXCR5^+$. Equal number ($5\times10^4$ cells/well) of Tfh and naïve B cells were cocultured in 96-well U-bottom plates in the presence of 1 μg/ml endotoxin-reduced SEB (Toxin Technology).

Quantification of IgG secreting plasma cells by ELISPOT assay

For IgG secreting plasma cells quantification, 96-well ELISPOT plates (Millipore MSIPS4510 Sterile, hydrophobic high protein binding immobilon-P membrane) were coated with 10 μg/ml purified goat anti-human IgG (Southern Biotechnologies) for 2 h at room temperature. After washing with PBS solution the plates were blocked with 1% BSA in PBS and incubated for 30 min at 37°C. Serial dilution of cultured B cells were added in a final volume of 200 μl of B cell medium (RPMI 10% Hyclone, complete medium) and incubated overnight at 37°C. Next the plates were washed 3 times with PBS 0.25% Tween20, 4 times with PBS and incubated 2 h at room temperature with isotype-specific, biotin-conjugated secondary antibody (Southern Biotechnologies). After washing, avidin-peroxidase (horseradish peroxidase, HRP, Sigma-Aldrich) was added and left 1 h at room temperature. The assay was developed with AEC

(Sigma-Aldrich). For quantification of antibody secreting cells, plates were acquired, counted and quality controlled using an ELISPOT reader and ImmunoSpot 5.1 software (CTL, Europe GmbH).

Human single-cell expansion

Transitional, naïve, IgM memory and IgG memory were sorted according to CD73 expression and seeded into 384-well plates at a density of 4 cells per well in a final volume of 50 µl of Bcell medium containing: 2.5 µg/ml CpG oligodeoxynucleotide 2006 (50-tcgtcgttttgtcgttttgtcgtt30; TIB Molbiol), 1 µg/ml anti-CD40 (Novartis clone 626.1), 30 µg/ml transferrin (CalBioChem), 4 µg/ml IL-2 (Boehringer Mannheim), 10 ng/ml IL-21 (Novartis internal production human recombinant protein), 0.1 µg/ml BAFF (R&D Systems) and 2.5×10^5 freshly irradiated (50 Gy) PBMCs/ml. After 12 days of incubation at 37°C, culture supernatants were harvested, diluted in PBS+1% BSA and used for ELISA screening. B cells in each well were lysed with 20 µl lysis buffer containing: 100 mM Tris-HCl, pH 7.5 (Sigma- Aldrich #T2944) 500 mM LiCl (lithium chloride, Sigma-Aldrich #62476) 10 mM EDTA, pH 8.0 (ethylenediaminetetraacetic acid, Sigma-Aldrich #03690) 1% LiDS (lithium dodecyl sulfate, e.g. Sigma-Aldrich #L9781) 5 mM DTT (dithiothreitol, e.g. Sigma-Aldrich #43815). The plates were stored at −80°C for future use in Ig genes amplification and cloning.

Quantification of culture supernatant IgM or IgG by ELISA assay

384-well ELISA plates were coated with unlabeled goat anti-human IgM or IgG (Southern Biotech) at 1 µg/ml in PBS and incubated 16 h at 4°C. After washing 4 times with PBS 0.025 % Tween 20 the plates were blocked with PBS 0.5% BSA and incubated for 2 h at room temperature. Serial dilutions of culture supernatants, or undiluted supernatants were incubated 16 h at 4°C. After 4 washes in PBS 0.025 % Tween 20, isotype specific HRP conjugated secondary antibodies (Southern Biotech) were added and plates incubated for 2 h at room temperature. After final washing with PBS 0.025 % Tween 20 the assay was developed with TMB (3,3',5,5'-tetramethylbenzidine) soluble substrates and the reaction stopped with sulfuric acid. Absorbance (OD_{405}) was measured using Gen5 2.01 software (BioTek). For all the donors the expansion efficiency was defined using with the following formula:

$$E = 1 - \left(\frac{W_t - W_p}{W_t}\right)^{1/n(1-e^{-n})}$$

E = expansion efficiency
W_t = total wells seeded
W_n = number of Ig-positive wells
n = average cells seeded per well

For the IgM B cell populations, namely transitional, naïve and IgM memory the class switch recombination efficiency was calculated as the fraction of IgG-positive ELISA wells of the total IgM responders.

Detection of antigen-specific immunoglobulin antibodies by ELISA assay

After *in vitro* expansion of human B cell subsets, culture supernatants were screened to detect antigen-specific immunoglobulin secretion. 384-well ELISA plates were coated and incubated 16 h at 4°C with self and pathogen antigens, namely: insulin (SIGMA-ALDRICH), IL-2 (Boehringer Mannheim), IL-17AF (Novartis internal human recombinant protein production), influenza (Thermofisher) and tetanus toxoid recombinant protein. As a control, plates coated with PBS were included in the screening. After washing with PBS 0.025 % Tween 20 the plates were blocked with PBS 0.5% BSA and incubated for 16 h at 4°C. Culture supernatants harvested at day 12 were diluted in PBS+1% BSA and screened for the reactivity against selected antigens. After incubation of 16 h at 4°C, the plates were washed with PBS 0.025 % Tween 20 and incubated 16 h at 4°C with isotype specific HRP conjugated secondary antibodies (Southern Biotech). A final washing with PBS 0.025 % Tween 20 was performed then the assay was developed with 3,3',5,5'-tetramethylbenzidine TMB soluble substrates (Seramun, Diagnostica GmbH) and the reaction stopped with sulfuric acid 1M (Sigma-
Aldrich). Absorbance (OD_{405}) was measured using Gen5 2.01 software (BioTek). The cut-off OD at which a signal was considered positive was determined for each antigen based on the overall OD_{405} values distribution. The poly-reactivity was defined as a positive OD_{405} in at least two antigens.

RT-PCR and Ig gene amplification/ BCR cloning

Heavy chain VH sequences were amplified from cDNA using PCR reactions with either or both of the following approaches: 1) a two-round PCR using nested primer sequences in the VH leader peptide and, subsequently, framework 1 regions with their respective nested reverse primers in

the IgG or IgM CH1 domain, or 2) a single-round PCR reaction using primer sequences spanning the VH ATG start codon and a respective CH1 domain reverse primer. All forward primers consisted of a mix of oligonucleotide sequences designed to amplify any possible human VH sequence from the IMGT database. In either strategy, the primers used in the final PCR round contained flanking sequences homologous to expression vectors containing a CMV promoter, (in the case of the two-round PCR, a Kozak sequence, start codon, and canonical VH leader peptide), and the remaining CH domain open reading frame. In this way, the PCR product could be annealed to the plasmid and extended using polymerase (a so-called "overlap extension" reaction) to directly generate an expression vector for the given heavy chain, which could also be Sanger sequenced to cover the VH ORF and recover the amplified VH sequences.

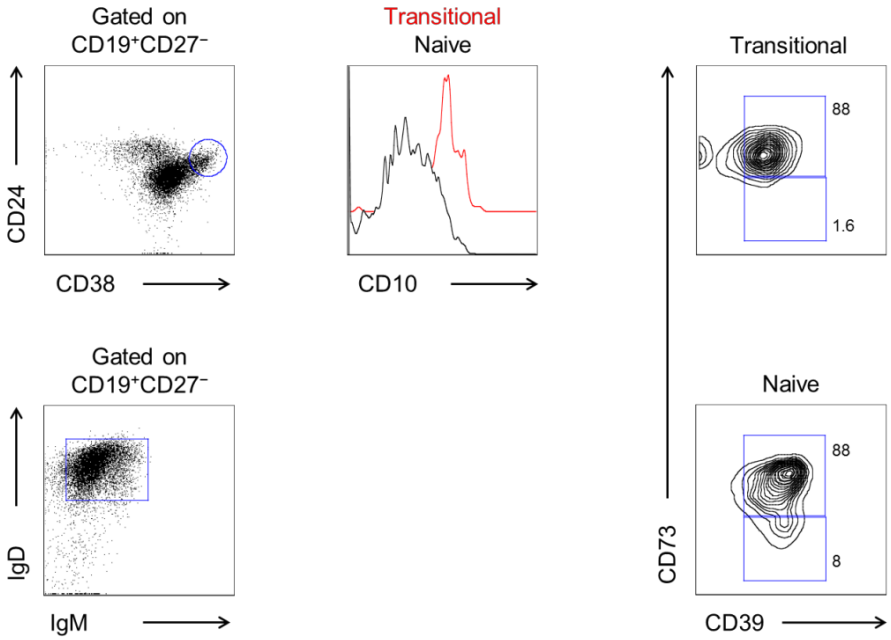
Clones containing unique VH sequences were analyzed for gene segment usage, germline identity, and CDR3 composition using the IgBlast algorithm (https://www.ncbi.nlm.nih.gov/igblast/). IgBlast output was parsed using an internally generated Perl script and statistically analyzed with the GraphPad Prism software package.

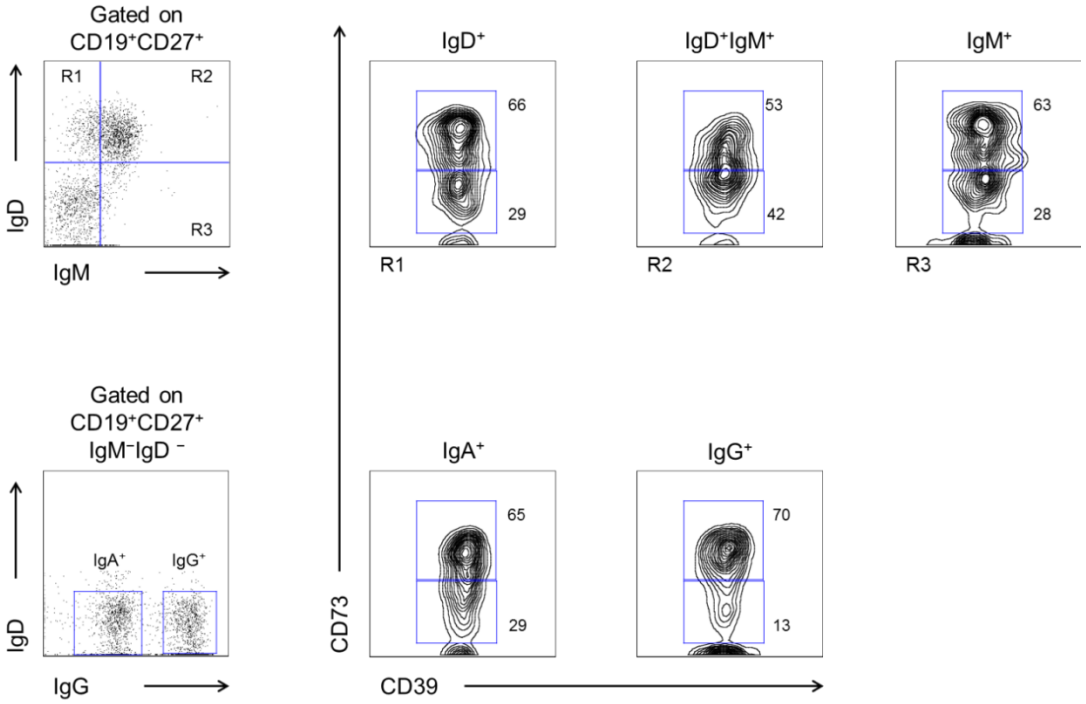
Author contributions

VC and ET design experiments. VC and JML performed all experiments. TP and JH contributed intellectual inputs. VC and JML analyzed data. VC and ET wrote the manuscript.

Figure 1

A


Gated on
CD19+CD27−
CD24
CD38
Transitional
Naive
CD10
Transitional
88
1.6
Gated on
CD19+CD27−
IgD
IgM
Naive
88
8
CD73
CD39


Gated on
CD19+CD27+
R1
R2
R3
IgD
IgM
IgD+
IgD+IgM+
IgM+
66
29
53
42
63
28
R1
R2
R3
Gated on
CD19+CD27+
IgM-IgD -
IgA+
IgG+
IgD
IgG
IgA+
IgG+
65
29
70
13
CD73
CD39

B

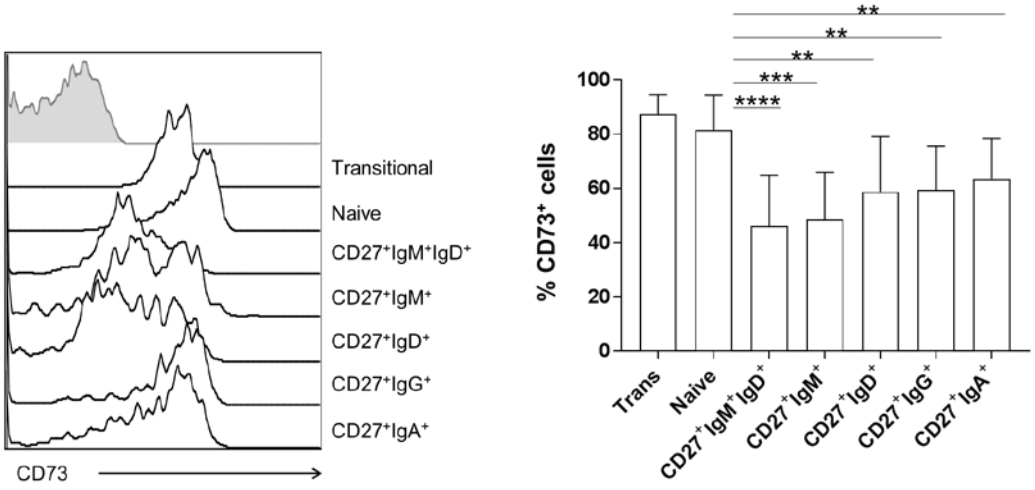
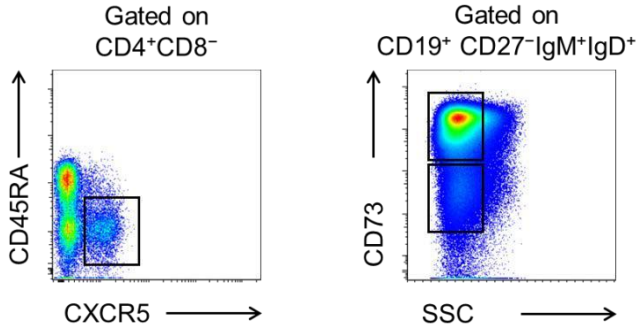

Transitional
Naive
CD27+IgM+IgD+
CD27+IgM+
CD27+IgD+
CD27+IgG+
CD27+IgA+
CD73
% CD73+ cells
100
80
60
40
20
0
**
**
**
***
****
Trans
Naive
CD27+IgM+IgD+
CD27+IgM+
CD27+IgD+
CD27+IgG+
CD27+IgA+

Figure 1. CD73 positive cells are significantly decreased in circulating memory B cell subsets.

PBMCs were isolated from 8 healthy donors and analyzed by flow cytometry with a combination of specific monoclonal antibodies. CD73 expression was evaluated in circulating human B cells at different stage of maturation. (A) Representative flow cytometry plots showing peripheral cell suspension analyzed for the expression of CD19, CD24, CD38, IgM, IgD, CD10, CD27, IgG, CD39 and CD73. Briefly, transitional B cells were defined as $CD19^{+}CD10^{+}CD27^{-}CD24^{high}CD38^{high}$ and naïve B cells as $CD19^{+}CD27^{-}CD10^{-}IgM^{+}IgD^{+}$. In the $CD19^{+}CD27^{+}$ compartment memory B cell subsets were defined, namely as IgD^{+}, IgM^{+}, $IgD^{+}IgM^{+}$, IgG^{+} and IgA^{+} ($IgM^{-}D^{-}G^{-}$). CD73 expression was analysed in all subsets and it is showed in combination with the pan-B cells marker CD39. Numbers reported in dot plots indicate the percentage of cells within the quadrant. (B) CD73 expression in all indicated peripheral B cell subsets (left panel). Bars graph (right panel) shows percentages of CD73 positive cells within the indicated B cell populations. Data are represented as mean ± SD (n=8). Statistic was performed using unpaired t-test. **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 2

A



B

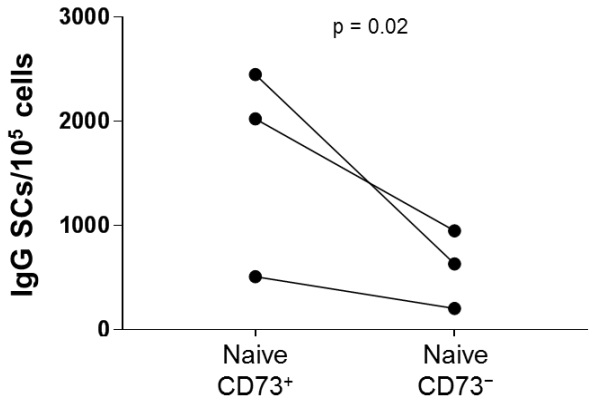


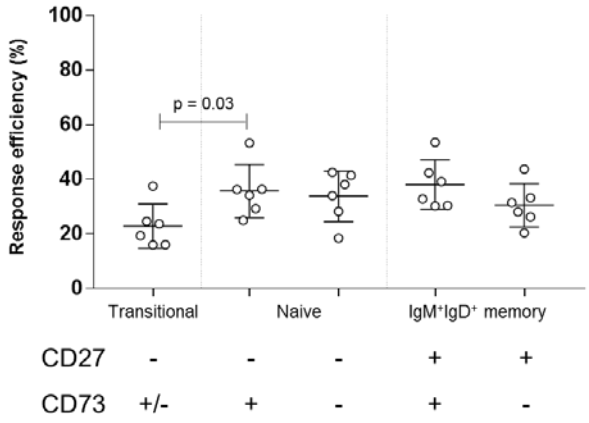
Figure 2. T follicular helper cells stimulation of naïve CD73$^+$ B cells induces higher class switch recombination.

(A) Circulating T follicular helper cells were isolated according to the expression of CD45RA and CXCR5 as CD4$^+$CD8$^-$CD45RA$^-$CXCR5$^+$. Representative dot plots of sorting strategy are shown (left panel). Naïve B cells were isolated as CD19$^+$CD27$^-$IgM$^+$IgD$^+$ from healthy donors PBMCs and sorted according to CD73 expression (right panel). (B) Circulating Tfh cells were co-cultured with autologous naïve B cells, either CD73$^+$ or CD73$^-$ in the presence of SEB. IgG secreting cells were measured at day 7 by ELISPOT assay. Data are representative results of three independent experiments (n=3). Statistic was performed using ratio paired t-test. *p = 0.02.

Figure

3

A



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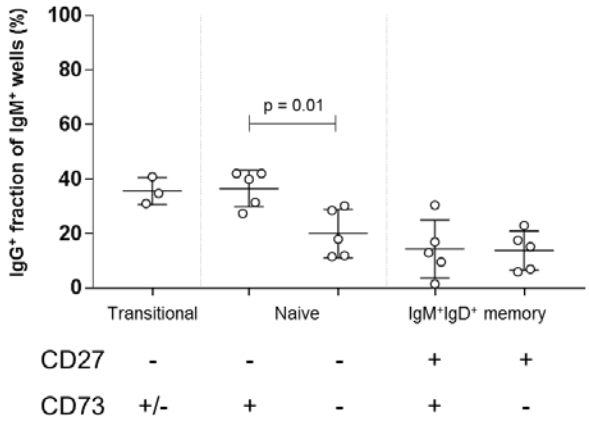


Figure 3. Single human B cell expansion confirmed higher potential of naïve CD73$^+$ to class switch recombine to IgG.

Transitional, naïve CD73$^+$, naïve CD73$^-$, IgM memory CD73$^+$ and IgM memory CD73$^-$ B cells were seeded 1 cell/well in 384 well plates and stimulated in vitro with CpG 2006, anti-CD40, IL-2, IL-21, BAFF and tranferrin for 12 days. (A) IgM secretion in the culture supernatants was measured by ELISA assay and the B cell response efficiency was calculated according to a formula which takes into account the

average of cells seeded per well, the total number of seeded wells and the number of IgM positive wells (Supplementary figure 1). Statistic was performed using unpaired t-test. *p = 0.03. (B) IgG secretion in culture supernatants was measured by ELISA assay and the class switch recombination efficiency expressed as a fraction of the OD_{450} IgG^+ wells of the total OD_{450} IgM^+ wells and expressed as percentage. Statistic was performed using unpaired t-test. *p = 0.01

Figure 4

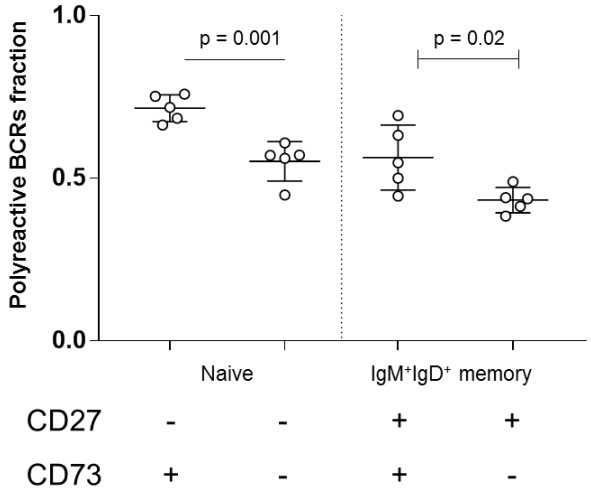


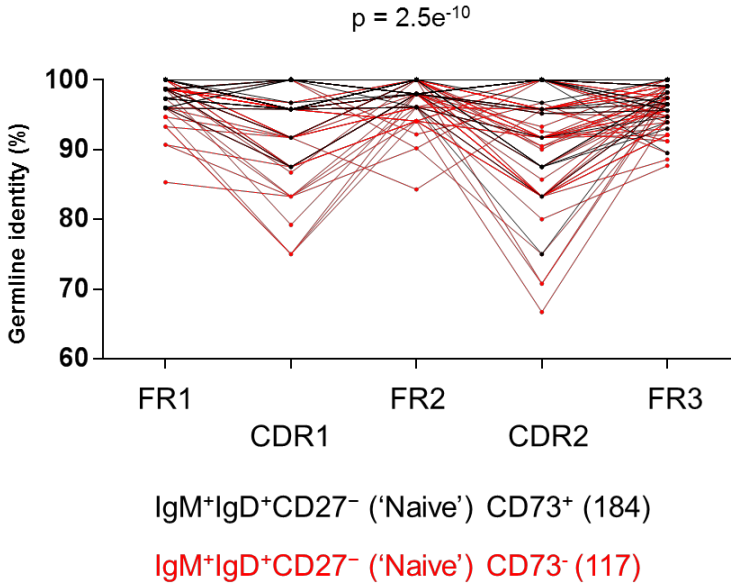
Figure 4. Decreased frequency of poly-reactive B cell clones in $CD73^-$ B cell subsets.

Culture supernatants from sorted and in vitro expanded naïve and IgM memory B cell subsets were analysed by ELISA assays for the presence of IgM antibodies reactive against: IL-2, IL-17AF, insulin, influenza and tetanus toxoid (Supplementary Figure 1). Poly-reactivity was defined by the recognition of at least two different antigens in the screening and expressed as the fraction of poly-reactive wells of the total IgM binders. Statistic was performed using unpaired t-test. **p = 0.001; *p = 0.02.

5

A

Figure



	CD73+		CD73-	
Germline Identity	> 95% (not mutated)	< 95% (mutated)	> 95% (not mutated)	< 95% (mutated)
Donor 1	34	2	9	4
Donor 2	37	0	22	9
Donor 3	105	6	45	28
Total sequences	176	8	76	41

B

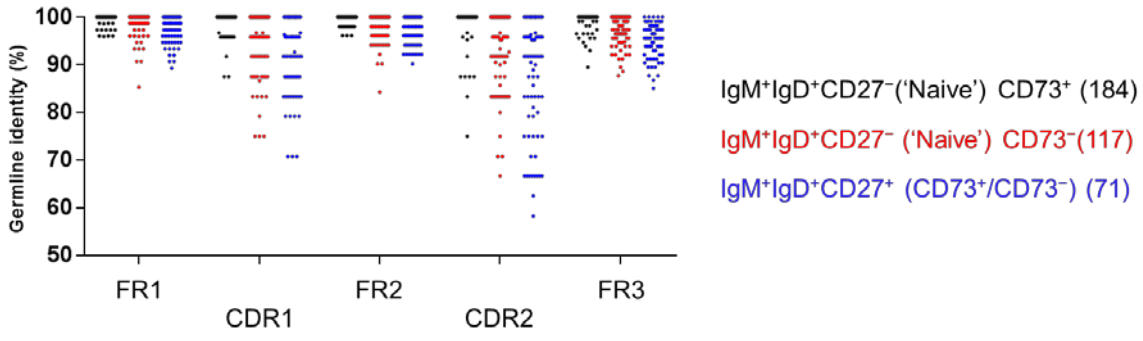
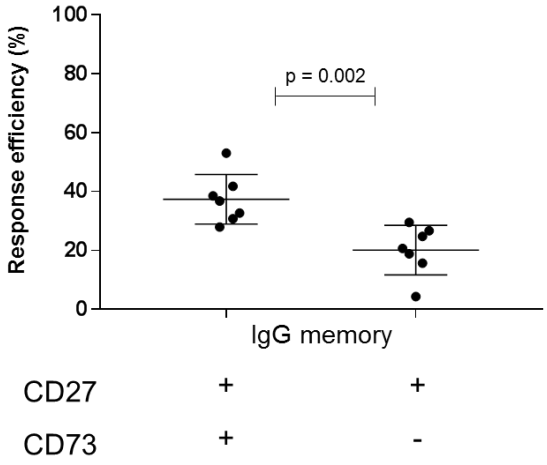


Figure 5. Enrichment of mutated sequences in IgM$^+$IgD$^+$CD27$^-$CD73$^-$ B cells.

Heavy chain VH sequences were amplified from cDNA extracted from monoclonal naïve B cell expanded in vitro for 12 days. Clones containing unique VH sequences were analyzed for gene segment usage, germline identity, and CDR3 composition using the IgBlast algorithm. Analysis was performed on 301 sequences extracted from 3 different healthy donors. (A) The percentage of germline identity of FR1, FR2 and FR3 as well as of CDR1 and CDR2 gene segments is expressed for naïve $CD73^+$ (black line, n=184) and $CD73^-$ (red line, n=117). Sequences showing a germline identity > 95% were defined not mutated; whereas sequences with a germline identity < 95% were defined mutated. The table shows the number of mutated versus not mutated sequences in $CD73^+$ and $CD73^-$ naïve B cells. Statistic was performed using Fisher exact test. p = 2.5e-10. (B) 71 heavy chain VH sequences were amplified from cDNA extracted from IgM memory B cell clones isolated from one healthy donor and expanded for 12 days in culture. The percentage of germline identity of FR1, FR2 and FR3 as well as of CDR1 and CDR2 gene segments is expressed for naïve $CD73^+$ (black dots, n = 184) and $CD73^-$ (red dots, n = 117) and IgM memory B cells (blue dots, n = 71).

6

A



B

Figure

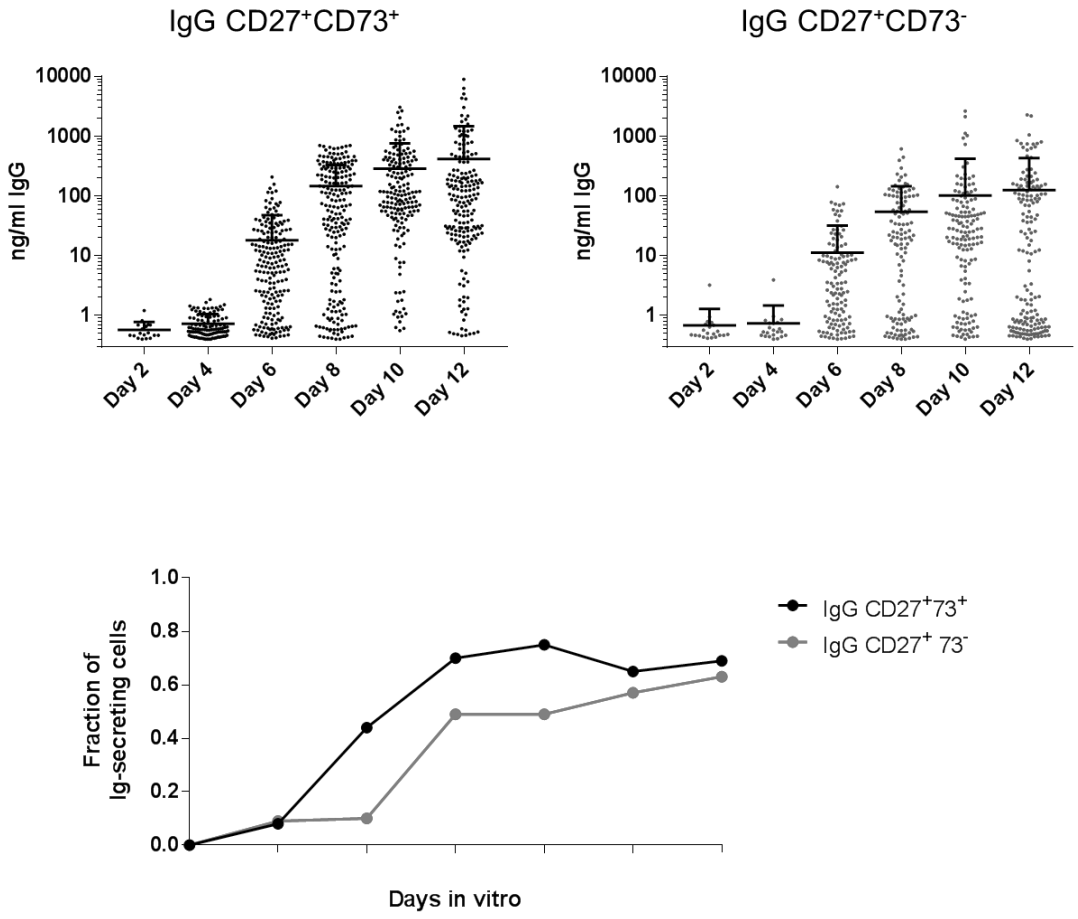


Figure 6. CD73 defines a subset of human germinal center reactive memory B cell.

IgG^+CD27^+ memory B cells were sorted according to CD73 expression, seeded at the density of 1cell/well in 384 well plates and stimulated in vitro with CpG 2006, anti-CD40, IL-2, IL-21, BAFF and tranferrin for 12 days. (A) IgG secretion in the culture supernatant was measured by ELISA assay and the response efficiency was calculated according to a formula which considers the average of cells seeded per well, the total number of wells seeded and the number of IgG positive wells (Supplementary figure 1). Statistic was performed using unpaired t-test. **p = 0.002. (B) The IgG secretion kinetic analysis in the $IgG^+CD27^+CD73^+$ and $IgG^+CD27^+CD73^-$ was performed calculating the amount of IgG produced by single-B cell cultures in the indicated time points. The expansion efficiency of IgG differentiation was calculated with the formula mentioned above.

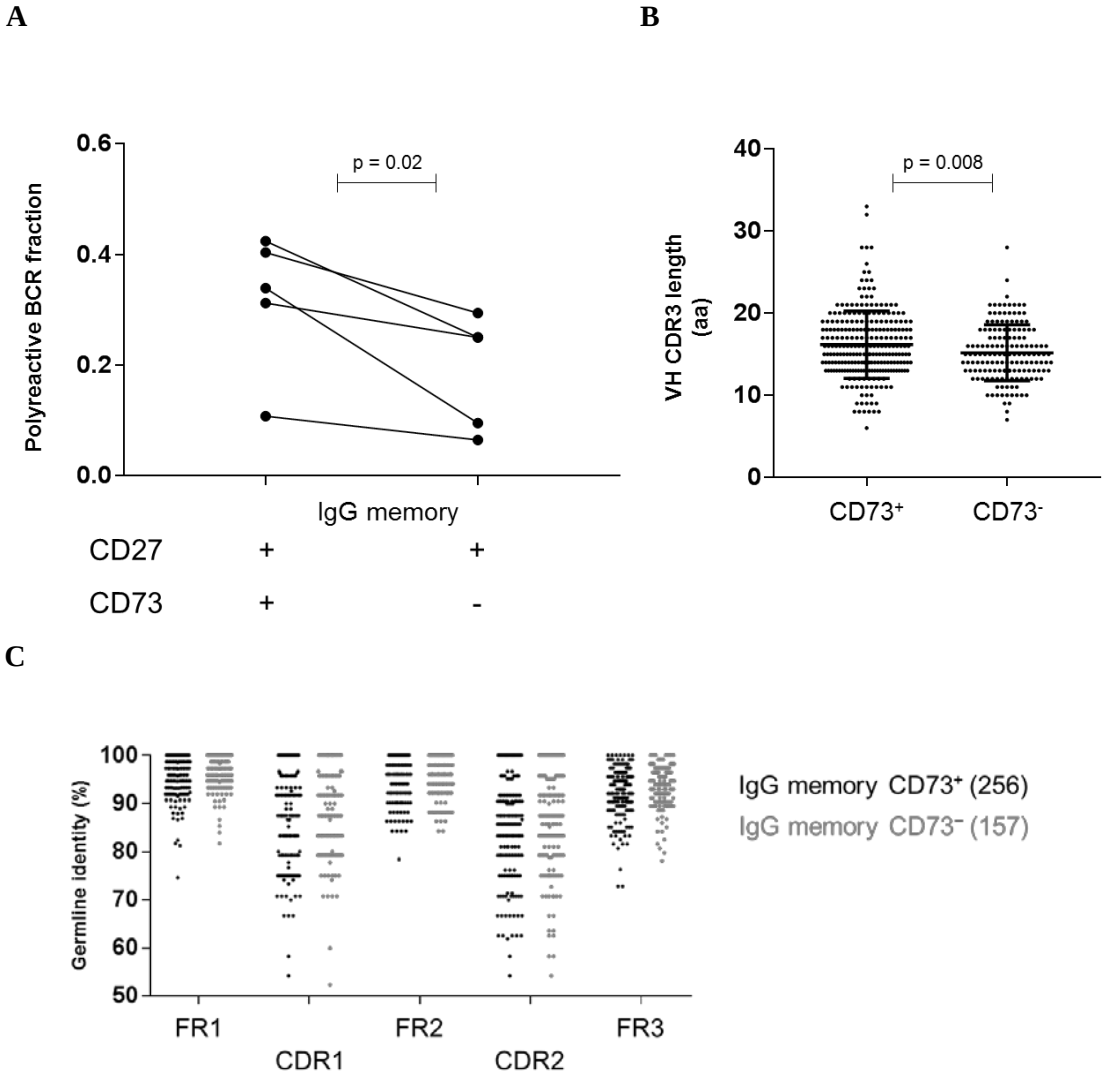


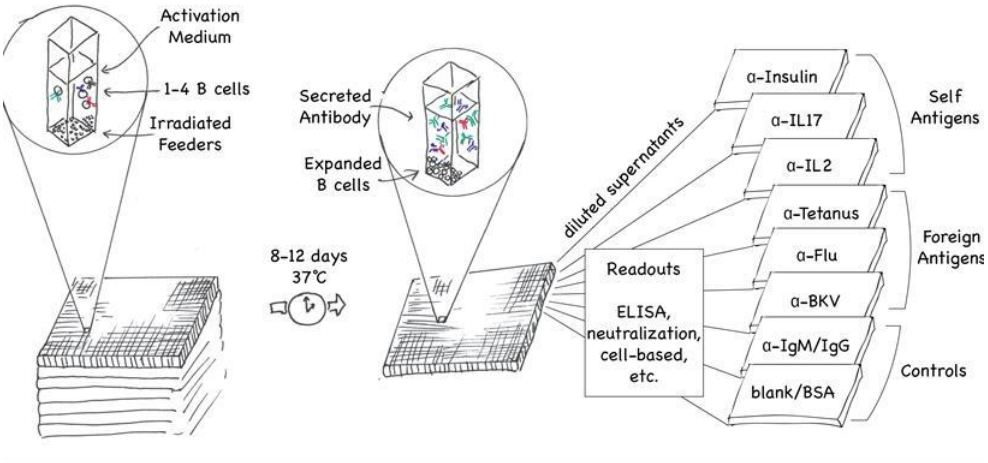
Figure 7. Decreased BCR poly-reactivity in CD73^{-} IgG class switched memory B cells.

Figure

(A) After clonal expansion of IgG memory B cell $CD73^+$ and $CD73^-$ subsets, culture supernatants were screened by ELISA assays for the presence of IgG antibodies reactive against: IL-2, IL-17AF, insulin, influenza and tetanus toxoid (Supplementary Figure 1). Poly-reactivity was defined by the recognition of at least two diverse antigens and expressed as a fraction of the total number of IgG secreting wells (total binders). Statistic was performed using unpaired t-test. *p = 0.03. (B-C) 431 clones from 2 healthy donors containing unique VH sequences were analyzed for CDR3 amino acid length (B) and germline identity (C) using the IgBlast algorithm. (B) Statistic was performed using ratio paired t-test with Welch's correction. **p = 0.008. (C) No differences in the mutated sequences were observed between the switch $CD73^+$ and $CD73^-$ compartment.

Supplementary Figure 1

A



B

$$E = 1 - \left(\frac{W_t - W_p}{W_t}\right)^{1/n(1-e^{-n})}$$

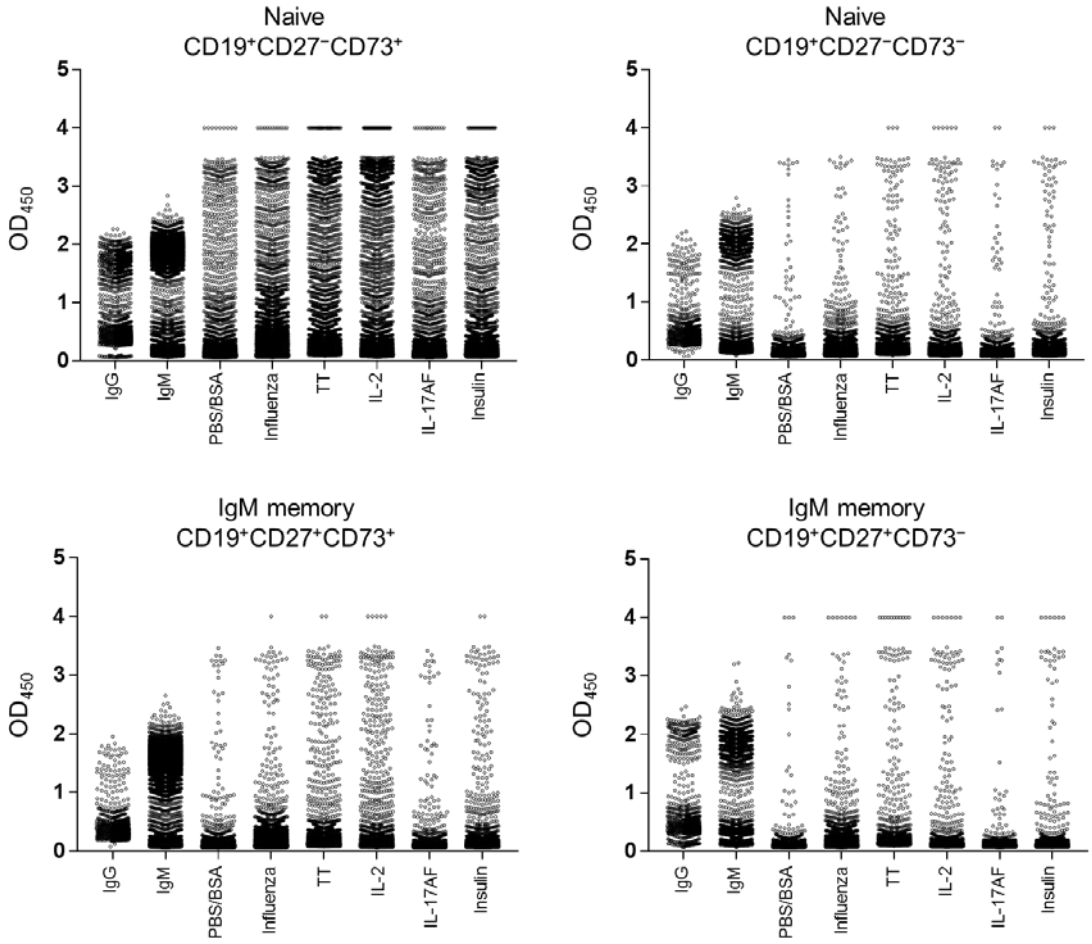
E = expansion efficiency
W_t = total wells seeded
W_n = number of Ig-positive wells
n = average cells seeded per well

Subset	IgM+CD27-CD10+	IgM+CD27-CD73+	IgM+CD27-CD73-	IgM+CD27+CD73+	IgM+CD27+CD73-
# of Plates	5	5	5	10	5
# cells/well	4	4	4	4	4
Total wells seeded	1880	1880	1880	3760	1880
B cells seeded	7520	7520	7520	15040	7520
IgG negative wells	1450	1351	1253	2160	1701
Fraction IgG negative wells	0.771	0.719	0.666	0.574	0.905
IgG Stimulation efficiency	0.064	0.081	0.098	0.132	0.025
IgG BCRs observed	481	607	738	1980	189
IgM negative wells	963	774	359	405	925
Fraction IgM negative wells	0.512	0.412	0.191	0.108	0.492
IgM Stimulation efficiency	0.157	0.202	0.344	0.433	0.165
IgM BCRs observed	1178	1521	2587	6513	1243
Switching Efficiency	0.408566734	0.398966519	0.285337786	0.304019511	0.152251155

Suppl. Figure 1. Functional BCRs repertoire by human B cell single-cell expansion

(A) Workflow for the single-cell expansion. After flow cytometry-based sorting, cells are plated in a 384 well plate at the density of 1 cell/well and stimulated with conditioned medium. On day 12, supernatants are collected and screened by ELISA assay to test the reactivity against a defined set of antigens. Other functional assays are compatible, for instance neutralization assay. Meanwhile, the cell pellet is lysed and can be stored at -80° for future Ig genes amplification and cloning. (B) Human B cell clonal expansion efficiency's calculation.

Supplementary Figure 2



Suppl. Figure 2. ELISA screening on single B cell culture supernatants

After *in vitro* single B cell-expansion, culture supernatants were screened to detect antigen-specific immunoglobulin secretion. Self-antigens (insulin, IL-2, IL-17AF), pathogen-antigens (influenza and tetanus toxoid) and control plates coated with PBS were included in the screening.

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3. Discussion

The concept of *'instructive immunology'* stems from the functional interconnection between innate and adaptive immune systems. Following the historical model predicted by Janeway, germline-encoded pattern-recognition receptors (PPRs) expressed by cells of the innate immune system, like dendritic cells (DCs) and macrophages, recognize conserved microbial molecular signatures and therefore instruct cells of the adaptive immune system to mount protective responses (Janeway 1989). Recently, our understanding of the interplay between the two systems has improved, most essential being the evidence that PPRs are expressed also by B and T lymphocytes (Bernasconi *et al.* 2003, Imanishi *et al.* 2007). The main targets recognized by PPRs include the invariant molecular structures shared by large groups of pathogen, called pathogen-associated molecular patterns (PAMPs) and molecules released by stressed cells undergoing necrosis that act as endogenous danger signals and so called damageassociated molecular patterns (DAMPs).

ATP released during inflammatory processes acts as a DAMP for cells of the innate immune system through stimulation of purinergic P2 receptors, which results in the activation of the NLRP3 inflammasome, with consequent release of the pro-inflammatory cytokines IL-1 and IL-18 (Di Virgilio and Vuerich 2015). Purinergic receptors are widely expressed also by adaptive immune cells, where extracellular ATP has been shown to regulate many cell responses. Among P2 receptors, P2X7 is the most investigated receptor in regulating immune cell physiology. Grassi and colleagues have shown that extracellular ATP delivers a costimulatory signal to mouse T cells through stimulation of P2X7 receptor (Schenk *et al.* 2008); moreover few years later they have shown that P2X7 activity regulates the immunosuppressive function of regulatory T cells (Schenk *et al.* 2011). In our study we focused on the role of P2X7 receptor in regulating the adaptive IgA response in the small intestine. IgA is the dominant immunoglobulin isotype at the mucosal surface of the intestine and has a central role in holding homeostatic relationship between host and resident microbiota. Tfh-cell-dependent IgA responses occur in GCs of Peyer's patches (PPs) and are responsible for the production of high-affinity antibodies, critical in limiting the translocation of potentially invasive bacteria from the gut lumen to the organism (Proietti *et al.* 2014). We identified a novel role of P2X7 receptor as regulator of the adaptive IgA response in the small intestine. Deletion of *P2rx7* resulted in the expansion of Tfh located in the PPs with consequent enhanced production of high affinity IgA GC-derived. *P2rx7*$^{-/-}$ mice showed increased SHM of IgA repertoire and a reduced mucosal colonization, indicating enhanced antigen specific Tfh cell help. Serum IgM plays a crucial role in protecting the

organism against blood microbial infection and its production has been proposed to be driven by PRRs agonists which support the differentiation of marginal zone as well as innate B cells into IgM secreting cells (Cerutti *et al.* 2013). The increased high-affinity anti-bacterial IgA antibodies and the reduced translocation of microbial components observed in *P2rx7*$^{-/-}$ mice, resulted in lower IgM serum levels and an increased susceptibility to polymicrobial sepsis. These results point to *P2rx7* as a master regulator of host-microbiota mutualism. P2X7 receptor activity is conserved in human, gaining more attention considering sepsis as one of the major health problems (Hotchkiss and Karl 2003). Furthermore, a general antibiotic treatment in human patients might induce changes in the intestinal microbiota eventually enabling bacteria to invade the bloodstream (Ubeda *et al.* 2010).

The gut-microbiota can affect many aspects of host-metabolism, including energy harvesting from nutrients, hepatic lipogenesis and adipose tissue development (Backhed *et al.* 2004, Turnbaugh *et al.* 2006, Cox and Blaser 2013). Interestingly, it has been shown that Tfh cell activity is important for generating a diverse bacterial community in the gut and that sensing of microbiota-derived ATP via P2X7 promotes the generation of proficient ecosystem (Perruzza *et al.* 2017). Taken together these data suggest a central role for the purinergic signaling pathway in the reciprocal regulation of the adaptive immune system and microbiota, ensuring the maintenance of a healthy microbial community. In comparison to many other exocrine signaling systems, the purinergic network has many layers of complexity and displays a great deal of plasticity. Increase in the extracellular ATP is a key danger signal in innate inflammatory processes. Here we showed for the first time that extracellular ATP is able to counterbalance the activation of the innate harm by down-regulating effector functions of Tfh cells to promote host-microbiota mutualism.

Most messengers based signaling systems function only in an on/off mode, marked by the presence or absence of the ligand. By contrast, in the purinergic network, hydrolysis of the ligand does not necessarily terminate the signal, but generates a new metabolite with the capacity to signal on its own at a different receptor, initiating a distinct signaling cascade. Indeed, an additional layer in the regulation of extracellular ATP is due to its hydrolysis to adenosine. After the release in the extracellular space, ATP is hydrolyzed by ectonucleotidases in a two-step enzymatic process, which is important for calibrating the duration, magnitude and composition of the *purinergic halo* surrounding the immune cells. The first step consists in conversion of ATP to ADP and AMP and is orchestrated by CD39, while the second step brings to the generation of adenosine from extracellular AMP and is mediated by ecto-5'nucleotidase (5'-NT) CD73. The expression and activity of both CD39 and CD73 is dynamically regulated in

accordance with the environment context, contributing to the outcome of many adenosine-mediated cellular responses. Under physiologic conditions, adenosine, similarly to ATP, is present at low level in the extra cellular space. The role of adenosine in favoring CSR has been already observed (Schena *et al.* 2013) and in our study we linked this phenomenon to higher capacity of $CD73^+$ naive B cells to cognate interact with circulating Tfh cells compared to the CD73 negative counterpart. The first step in the GC reaction is the activation of naive B cells by the exogenous antigen followed by interactions with the antigenspecific T cells, in order to become fully active. The intrinsic role of CD73 in shaping the outcome of the B cell response was confirmed at a single cell level, where the clonal expansion of $CD73^+$ naive B cells resulted in higher plasticity to perform IgG CSR.

The extracellular adenosine concentration can rapidly increase in response to hypoxia, ischaemia, inflammation or trauma. In GCs, antigen-activated B cells proliferate, express high affinity antibodies, promote antibody class switching, and yield B cell memory and long-lived plasma cells (LLPCs). Significant rounds of apoptosis and cell proliferation occur within the GC niche, rendering this microenvironment highly hypoxic. Interestingly, hypoxia–inducible factor 1 (HIF-1alpha) is a master regulator in promoting CD73 expression (Synnestvedt *et al.* 2002). Consistent with this notion, CD73 expression might be dynamically regulated in the GC microenvironment. The adenosine receptor expressed by human B cells is a Gs-protein coupled receptor linked to activation of adenylyl cyclase enzyme, namely the A2A receptor. Activation of A2A receptor induce an increase in cytosolic c-AMP which then acts as a second-messenger activating c- AMP dependent-kinase, such as PKA, which phosphorylate specific target proteins (). Intriguingly, it has been shown that AID physically interacts with PKA in the cytoplasm and is phosphorylated by PKA catalytic subunit at specific residues (Pasqualucci *et al.* 2006). We speculate that the higher CSR efficiency observed in the $CD73^+$ compartment, in both naive and IgG memory B cells, is linked to the PKA activation A2A-dependent, with the consequent phosphorylation of AID, which is required for CSR.

Analysis of CSR in naïve and memory human B cells separated on CD73 expression, showed a comparable level of CSR efficiency in $IgM^+IgD^+CD27^+$ memory cells, both CD73 positive and negative, and $IgM^+IgD^+CD27^-CD73^-$ B cells defined as naive B cells. This prompted us to investigate on eventual relation between the aforementioned compartments. Human $IgM^+IgD^+CD27^+$ memory B cells are generally defined as marginal zone like B cells (MZ). Nevertheless, in contrast to the mouse counterpart, human MZ B cells recirculate and harbor mutated Ig variable genes (Weller *et al.* 2004, Weill *et al.* 2009). The involvement of this population in a T-dependent or -independent response is still under debate. In our study we

identified a novel subset of antigen-experienced B cell in the classical defined naïve $IgM^+IgD^+CD27^-$ compartment, which is characterized by lower expression of CD73. Analysis of the mutations in the Ig variable genes in the $CD27^-$ compartment showed an enrichment in the CD73 negative population, which appears distant to the fully germlined CD73 positive counterpart. Interestingly, the level of mutations observed in the $IgM^+IgD^+CD27^-CD73^-$ cells was comparable to the $IgM^+IgD^+CD27^+$ memory compartment, with a preferential accumulation in the CDR1 and CDR2 segments. Remarkably, from our data it is emerging that human memory B cells progressively acquire markers and properties that distinguish them from naive B cells, with CD73 playing an important role in the early differentiation steps. Indeed we speculate that $IgM^+IgD^+CD27^-CD73^-$ B cells might represent early precursors of $IgM^+IgD^+CD27^+$ B cells, which have already encountered the antigen and consequently mutated Ig variable genes. Further experiments are necessary to confirm this hypothesis and to gain insights into biological pathways and molecular mechanisms that regulate cell fate and development.

The GC reaction has the unique potential to harmoniously generate plasma cells, which confer immediate protection by the secretion of specific antibodies, and memory B cells which confer rapid and enhanced response to secondary challenge. The overall concept of humoral immunological memory stems from the possibility to generate the long-lived cell populations. In this study, we identified CD73 as a marker in the main developmental check point existing between LLPCs and memory B cells generation in the course of a GC response. Our results clearly show that CD73 defines a subset of GC-reactive memory B cells, which is faster and more efficient in Igs secretion upon in vitro stimulation. The higher IgG secretion efficiency observed in the $IgG^+CD27^+CD73^+$ memory B cells might reflect their need to further recirculate the GC and receive more cognate T cell help. The $CD73^-$ cells might instead represent the result of a successful GC reaction, which shutdown CD73 expression to escape further rounds of affinity maturation and to instead contribute to the long-lived PCs pool.

In conclusion, our studies suggest a new checkpoint role for the purinergic halo surrounding immune cells which can either enhance or inhibit adaptive immune cell activation. Several questions remain to be answered before we can fully understand the several roles of autocrine and paracrine purinergic signaling in immune cell regulation. Nevertheless, our studies have contributed in the field raising better appreciation on the role of innate signals, like extracellular ATP and adenosine, in tightly shaping the adaptive immune responses in both T and B cell compartment. Our findings could be important for developing new immunomodulatory therapies

that target purinergic receptors, enabling a modulation of germinal center responses, which has been never been proposed before.

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