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Regulatory functions of B cells and regulatory plasma cells

short title: regulatory B cells

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Abstract

B cells critically contribute to health through the production of antibodies that provide a vital line of defence against infectious agents. In addition, B cells are known to play an integrative role in immunity, acting as crucial antigen-presenting cells for T cells, and being an important source of cytokines that can target multiple cell types including stromal cells, innate cells, and adaptive lymphocytes. This review focuses on the role of B cells as negative regulators of immunity through the production of interleukin-10 (IL-10) in autoimmune, infectious, and malignant diseases. It discusses the phenotypes of the B cell subsets most competent to produce IL-10 *in vitro* and to exert suppressive functions *in vivo* upon adoptive transfer in recipient mice, the signals and transcription factors regulating IL-10 expression in B cells, and the recent identification of plasmocytes, including short-lived plasmablasts and long-lived plasma cells, as an important source of IL-10 in secondary lymphoid organs and inflamed tissues *in vivo* during mouse and human diseases. With our increasing knowledge of this non-canonical B cell function a coherent framework starts emerging that will help monitoring and targeting this B cell function in health and disease.

Keywords: B cells, interleukin-10, immune regulation

Introduction

The suppressive function of B cells was initially identified in autoimmune and inflammatory disease models, with data showing that B cells could attenuate the progression of these pathologies through their production of interleukin (IL)-10 [1-3]. Such immunoregulatory function provided a possible explanation for previous reports describing the exacerbation of T cell-mediated inflammatory disorders in B cell-deficient mice [4, 5]. Of particular interest, B cell-driven immunosuppression could stop an already established autoimmune disease and induce an almost complete remission from symptoms, suggesting the possibility of a curative application. For example, in experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS), mice with wild-type B cells recovered rapidly after a short episode of paralysis from the disease induced by immunization with myelin oligodendrocyte glycoprotein (MOG), while mice with an *Il10* gene deficiency restricted to B cells developed a severe chronic disorder [1]. Thus, B cell-derived IL-10 interrupted an ongoing disease.

The discovery of the immunosuppressive activities of B cells prompted the search for the B cell subset(s) mediating this regulatory function, with the goal of identifying a regulatory B cell subset that could be harnessed therapeutically. Splenic B cells administered into mice with an *Il10*-deficiency restricted to B cells restored the process of recovery from EAE [1], indicating that the spleen contained such B cells. The spleen contains several B cell subsets including transitional cells originating from bone marrow, follicular and marginal zone B cells, as well as B1 cells, and some plasmocytes (short-lived plasmablasts and long-lived plasma cells), suggesting that at least one of these sub-populations, which are distinguishable by their cell surface

receptor expression profiles, has suppressive functions. This article reviews our current knowledge on the B cell populations that have so far been shown to perform such suppressive activity.

Main text

- B cell subsets with suppressive functions in adoptive transfer experiments:

Several splenic B cell subsets suppressed disease in recipient mice after adoptive transfer. CD1d^{hi}CD21^{hi}CD23⁺IgM^{bright}C1qRp^{int} T2-marginal zone precursor-like B cells isolated from mice in remission from collagen-induced arthritis (CIA) prevented the development of arthritis in recipient mice upon transfer, limiting both the incidence and the severity of clinical signs [6]. Cells with a similar phenotype protected recipient mice from systemic lupus erythematosus (SLE) if they were previously activated via CD40 *in vitro* before transfer [7]. Human B cells expressing CD24 and CD38 at high levels, which include transitional cells, also have a high competence to produce IL-10 *in vitro*, compared to other subsets [8]. Thus, mouse and human B cells with phenotypic features of transitional T2 cells produce IL-10 upon *in vitro* stimulation, and can mediate suppressive function in recipient mice upon adoptive transfer.

CD19⁺CD1d^{hi}CD5⁺ cells represent another B cell subset found in the spleen of naïve mice, and capable of IL-10-mediated regulatory function upon adoptive transfer, as shown in models of contact hypersensitivity [9], EAE [10], and intestinal inflammation [11]. These cells differ from T2-marginal zone precursor like B cells by their CD23^{lo} phenotype, which instead makes them look like marginal zone B cells that were described as one of the B cell subsets expressing high levels of CD1d [12]. CD1d^{hi} B

cells from the mesenteric lymph nodes of mice with ulcerative colitis also suppressed immunopathology in a model of spontaneous T cell-mediated intestinal inflammation [2], indicating that B cells with suppressive function can be found outside the spleen. CD1d might contribute directly to IL-10 expression in B cells because its ligation induced IL-10 production by intestinal epithelial cells [13]. Considering its presence on distinct subsets shown to have suppressive functions, CD1d can be considered as a marker frequently found on B cells competent to produce IL-10 upon stimulation *in vitro*, and to exert a suppressive activity in recipient mice after adoptive transfer. However, only a small fraction of CD1d^{hi} B cells expressed IL-10 upon activation *in vitro*, even after strong stimulation with pharmacological agents such as phorbol 12-myristate 13-acetate (PMA) and ionomycin in these experiments, and other B cell subsets such as B1 B cells displayed suppressive function, while expressing CD1d at low level [14-16]. CD1d should therefore not be considered as a universal marker of “regulatory B cells”. In addition, CD1d^{hi} B cells can also produce elevated amounts of the pro-inflammatory cytokine IL-6 [17], a mediator of the pathogenic functions of B cells in EAE and MS [18].

Other surface receptors expressed on B cells, and up-regulated upon their activation, have been associated with immunosuppressive B cells. The tetraspanin CD9, which is expressed by marginal zone B cells, transitional B cells, B1 cells, and plasma cells, is such a marker [19-21]. CD9 expression can be acquired by B cells upon activation [19]. In *in vitro* stimulated cultures, CD9-positive B cells contain the majority of IL-10-expressors (about 88%) with only few IL-10-producing B cells present in the CD9-negative fraction [22], consistent with the fact that this marker identifies the most competent subpopulations for IL-10 production. CD9-positive B cells were found to

be the main source of splenic B cell-derived IL-10 in foot-and-mouth disease virus-infected mice [23]. CD9 might be functionally relevant because its engagement increased IL-10 production in macrophages [24]. Another marker found on some B cells, which is up-regulated upon B cell activation, and has been associated with B cell regulatory function is T cell Ig domain and mucin domain protein 1 (TIM-1), a member of the TIM gene family that consists of eight and three members, respectively, in mouse and human [25]. Polymorphisms in the TIM-1 gene are associated with an increased susceptibility for asthma and allergy [26]. The TIM-1 protein is found mainly intracellularly in resting B cells, and translocates to the cell surface upon cell activation [27]. Surface TIM-1 expression is markedly induced on mouse B cells upon B cell Receptor for antigen (BCR) engagement [28], and on human B cells (particularly transitional, naïve, and memory B cells) upon BCR and Toll-like receptor (TLR) 9 stimulation [29]. The expression of TIM-1 correlated with that of IL-10 in activated human B cells [29], and mouse TIM-1-positive B cells ameliorated pancreatic islet allografts acceptance in an IL-10-dependent manner upon adoptive transfer in recipient animals [28]. Conversely, B cells deficient in TIM-1 failed to suppress encephalitogenic T cell responses and disease progression in EAE in transfer experiments [30]. TIM-1 directly increases the production of IL-10 by B cells in synergy with the co-engagement of the BCR [30]. This function involves the TIM-1 extra-cellular mucin domain, whose deletion impairs IL-10 production by B cells [31]. TIM-1 is a pattern recognition receptor recognizing phosphatidylserine [32], a phospholipid normally localized in the inner leaflet of the plasma membrane of healthy cells that translocates to the outer plasma membrane as cells enter apoptosis [33]. TIM-1 can interact with apoptotic cells, and mediate their uptake [34]. Upon co-culture with apoptotic cells wild-type but not *Tim-1*-deficient B cells display an

increased IL-10 expression [30]. Thus, receptors expressed by activated B cells can promote their IL-10 expression. For human B cells, tumor necrosis factor receptor 2 (TNFR2) is another receptor up-regulated on activated B cells and associated with IL-10-production [35]. Indeed, TNFR2 is up-regulated on a fraction of B cells after TLR9 stimulation, and TNFR2-positive B cells are the major IL-10 producers in these cultures [35]. The stimulation of TNFR2 on these cells increases their release of IL-10, but also their secretion of IL-6 [35]. TNFR2 engagement might thus not unequivocally promote the regulatory function of B cells. Nonetheless, TNFR2 signalling contributes to the suppressive function of CD4⁺CD25⁺ T regulatory cells [36]. Among TNFR2-positive B cells, IL-10 expression is particularly enriched within the IgM⁺CD27⁺ memory B cell subset [35]. Remarkably, the differentiation of IL-10-expressing B cells in these cultures coincides with their development into antibody-secreting cells (ASC), as observed in another study [35, 37]. Thus, ASC might represent one type of IL-10-producing B cell.

- Signals and transcription factors controlling the expression of IL-10 in B cells:

Resting B cells do not secrete IL-10 unless appropriately stimulated. The signals driving their suppressive function *in vivo* have been investigated with genetically modified mice. Multiple stimulatory signals were found to be required in a non-redundant manner for B cells to achieve a suppressive effect, implicating the BCR for antigen, CD40, TLR, and receptors for cytokines such as IL-1 β , IL-6, and IL-21 [1, 38-40]. These signals might act in a sequential manner [38, 41]. Thus, naïve mouse B cells do not secrete any IL-10 after activation via the BCR, unless they have previously been stimulated via TLR4 [38]. This IL-10 production requires the

endoplasmic reticulum calcium sensor stromal interaction molecules 1 and 2 (STIM1 and STIM2) [42]. TLR4 signalling must modulate the response of naïve B cells to BCR engagement so that the latter gains the competence to elicit IL-10 expression. This might be related to the transcription factor Nuclear Factor of Activated T-cells (NFAT) c1/ α A [43], a short isoform of NFATc1 that has a particular N-terminal peptide differing markedly from those found in other NFATc proteins, including NFATc1/ β [44]. NFATc1/ α is induced in mouse B cells upon BCR signalling, and inhibits *Il10* transcription by binding together with the transcriptional repressor histone deacetylase 1 (HDAC1) to an intronic site of the *Il10* gene [43]. Accordingly, mice lacking the *Nfatc1* gene in B cells developed a milder form of psoriasis than controls following the cutaneous application of Aldara cream containing the TLR7 agonist imiquimod, with this effect reflecting an increased accumulation of IL-10-producing B cells and plasmacytes [43]. As expected, these mice also developed a milder form of EAE than controls [45]. The role of NFAT transcription factors in the control of IL-10 expression in B cells is however more complex, because some reports have shown that NFAT transcription factors can also promote IL-10 expression in B cells. This is for instance the case in the B cell response to the M2 protein of the murine gammaherpesvirus 68 (MHV68). M2 is important for the re-activation of this virus in latently infected B cells, and it can drive on its own the secretion of high amounts of IL-10 by primary B cells as well as their differentiation towards a pre-plasma cell phenotype [46]. This process is dependent on NFAT, which induces the transcription factor interferon regulatory factor 4 (IRF4) that is key for B cell differentiation into both ASC and IL-10-producers [46]. This finding is consistent with the fact that B cells lacking STIM-1 and STIM-2 are defective in both NFAT activation as well as IL-10 expression [42]. Of note, IRF4 has been reported to be a weak DNA binding protein

that needs to cooperate with NFAT to activate the *Il10* gene promoter in T cells [47]. Distinct NFAT family members might therefore have opposite effects on *Il10* expression in B cells.

The suppressive function of B cells is also controlled by signals associated with inflammatory microenvironments such as hypoxia. Hypoxia induces specific gene-expression programs including the hypoxia-inducible factors (HIF) that are heterodimeric transcription factors comprising an oxygen-labile α subunit (HIF- α) and a constitutively stable β subunit (HIF- β) [48]. Hypoxia results in the stabilization of the α subunit, and the induction of HIF target genes. B cells stimulated via the BCR or TLR4 in an environment with normal oxygen concentration up-regulate HIF-1 α expression, thereby becoming prepared to function in the hypoxic conditions of inflammatory environments [49]. Remarkably, B cells cultured under hypoxic conditions displayed a strongly increased transcription of the *Il10* gene compared to cells exposed to normoxic conditions [49]. The deletion of *Hifa* in B cells impaired their capacity to produce IL-10. Mice lacking *Hif1a* in B cells had a reduced number of IL-10 producing B cells, and suffered a worsened EAE compared to controls, which was corrected upon the adoptive transfer of *Hif1a*-deficient CD1d^{hi}CD5⁺ B cells engineered to constitutively express IL-10 by lentiviral transduction [49, 50]. In keeping with this, mice lacking *Hif1a* in B cells also developed an exacerbated CIA, both in terms of disease incidence and severity. This correlated with reduced numbers of IL-10-producing B cells in the spleen and lymph nodes of immunized mice, linking the exacerbated disease to a reduced immunosuppression mediated by B cell-derived IL-10 [49]. These mice displayed normal antibody titers after immunization with T cell-dependent or T cell-independent type II antigens [49],

suggesting that HIF specifically controlled the antibody-independent IL-10-mediated regulatory functions of B cells rather than their global activation.

Cellular damage can also lead to the release of molecules promoting the suppressive function of B cells. For instance, the alarmin IL-33, which is normally kept within intracellular stores in healthy cells, and is liberated into the extracellular space upon cell damage [51], increases the production of IL-10 by B cells. The injection of IL-33 into the peritoneal cavity of mice resulted in the differentiation of IL-10-producing B cells with a CD19⁺CD25⁺CD1d^{hi}IgM^{hi}CD5⁻CD23⁻Tim-1⁻ phenotype. Upon adoptive transfer, these B cells protected *Il10*-deficient recipient mice from inflammatory bowel disease in an IL-10-dependent manner [52].

- Plasmacytes as mediators of the regulatory function of B cells *in vivo*:

In naive mice the B cell subsets with the highest competence for IL-10 production have in common a distinctively strong propensity to differentiate into ASC upon stimulation [53-56]. Some signals and transcription factors implicated in the regulatory function of B cells, such as IL-21 and IRF4, contribute to plasmacyte differentiation [40, 46]. Further supporting a link between IL-10 production by B cells and their differentiation into ASC, several studies documented that mouse and human B cells acquired a plasmacyte phenotype when differentiating into IL-10 producers *in vitro*. Accordingly, the B cells expressing the suppressive cytokines IL-10 and IL-35 in a detectable manner *in vivo* were plasmacytes in autoimmune, infectious, and malignant diseases [57].

The observation of IL-10 expression exclusively in CD138^{hi} plasmacytes in a context in which B cell-derived IL-10 had suppressive function was initially made in a model of infection by the Gram-negative bacterial pathogen *Salmonella* Typhimurium [58]. The identification of ASC as IL-10 expressers was made using B-Green reporter mice that carried an eGFP reporter sequence inserted after the STOP codon of the *Il10* gene, followed by the intact 3'UTR of the endogenous gene, so that the eGFP reported IL-10 protein expression rather than *Il10* gene transcription in this strain [58]. IL-10-expressing CD138^{hi} plasmacytes were absent from the spleen of naïve mice, while they represented about 20-50% of the CD138^{hi} cells on day 1 post-infection [58]. Consistently, the expression of *Il10* mRNA was up-regulated by more than 100-fold in splenic CD138^{hi} cells at 24 hours post-infection, while CD19⁺CD138⁻ cells did not display any *Il10* mRNA up-regulation compared to naïve B cells, underlying IL-10 expression as a unique feature of CD19⁺CD138^{hi} plasmacytes *in vivo* [58]. The IL-10 from these plasmacytes was suppressive since mice lacking IL-10 expression selectively in B lineage cells displayed an increased number of pro-inflammatory CD4⁺ T cells producing IFN- γ and TNF, an improved control of the bacteria, and an enhanced resistance to the infection, compared to controls [58]. Importantly, these plasmacytes did not secrete any IL-6 upon re-stimulation *in vitro* [17], further underlining their specialization for immune regulation.

A similar suppressive circuit between IL-10-producing plasmacytes and pro-inflammatory T cells of T_H1 and T_H17 types was observed in EAE [17, 59]. Plasmacytes were identified as the main source of B cell-derived IL-10 in the spleen and lymph nodes of EAE mice [17, 59]. Using IL-10-eYFP Venus reporter mice, Baba and colleagues found that the induction of EAE led to the accumulation of

proliferating IL-10-expressing plasmablasts in draining lymph nodes (dLN) that peaked on day 14 post-immunization [59]. These cells displayed a CD138⁺CD44^{hi}CD43^{hi}CXCR4^{hi}MHC-II^{hi}B220^{lo}CD38^{lo}CXCR5^{lo}Blimp-1^{lo} phenotype, and expressed mostly switched IgG isotypes (particularly IgG1 and IgG2c) [59]. Their accumulation in the dLN was necessary for protection from disease because mice with a B cell-specific deficiency in L-selectin (*SELL*; CD62), in which only B lineage cells could not enter the LN, developed an exacerbated EAE compared to controls [59]. Furthermore, mice with B cell-specific deficiencies in *Prdm1* or *Irf4*, which both lacked plasmocytes due to the requirement of these transcription factors for ASC differentiation, developed an exacerbated EAE compared to controls [59]. Noteworthy, these two transcription factors are also needed for the optimal expression of *Il10* in B cells [59-61] and several other cell types (29724515, 26765224, 25073792, 21378976, 18962896). Since no B cell has been identified so far that expresses these two transcription factors and is not a plasmocyte, these data establish a molecular link between B cell differentiation into plasmocyte and their IL-10 production, thereby emphasizing the role of plasmocytes as IL-10-producing suppressive cells. The development of these regulatory plasmocytes occurred independently of the germinal centre reaction since *Bcl6*-deficient mice, which do not make germinal centre, showed a normal EAE course [59]. In dLN, plasmocytes accumulated in areas enriched in dendritic cells expressing the IL-10 receptor. IL-10 from plasmablasts suppressed the production of IL-6 and IL-12 by DC *in vitro* [59], confirming the previous finding that IL-10 from stimulated B cells suppressed the capacity of DC to secrete IL-6, IL-12, IL-23, and TNF, as well as to stimulate CD4 T cell proliferation [38].

Plasmocytes might also inhibit the development of EAE locally from within the inflamed central nervous system (CNS). In MS patients, plasmocytes, but not B cells, were the major source of IL-10 in CNS lesions, together with astrocytes [62]. In healthy individuals plasmocytes are not found in this tissue, but they accumulate in the CNS as a result of local autoimmune inflammation, at least in part independently of their antigen specificity [63, 64]. In MS, this non-specific response might account for the accumulation of plasma cells reacting against irrelevant pathogens such as measles, zoster, and rubella, which defines the MRZ reaction typical of this disease and is used as a diagnostic criterion for MS in some clinics [65]. The plasmocytes found in the CNS of MS patients include non-proliferating plasma cells, in agreement with the fact that in these patients immunoglobulin oligoclonal bands are not affected by B cell-depletion therapy with anti-CD20 [63]. Similarly, during EAE non-proliferating plasma cells can accumulate in the mouse CNS, where they preferentially reside in areas expressing BAFF, APRIL, and CXCL12 [63] that resemble their physiological survival niches of the bone marrow [66].

Some of the plasmocytes found in the CNS during EAE derive from the small intestine lamina propria [64]. This tissue contains IL-10-producing plasma cells of the IgA isotype [64]. After EAE induction, IgA plasma cells accumulate in brain and spinal cord as BLIMP-1⁺B220^{lo} cells having low level of surface CD138 expression. Their accumulation in the CNS is paralleled by a decrease of the plasma cell number in the small intestinal lamina propria, suggesting that this involves the migration of cells from the intestine into the inflamed CNS. This notion is supported by the finding of microbiota-reactive plasma cells in the CNS of mice with EAE [64]. Furthermore, mice orally challenged with rotavirus, and subsequently immunized with MOG to

induce EAE had rotavirus-specific IgA⁺ plasmocytes in their CNS as well as bone marrow [64]. This suggests that intestinal plasmocytes can regulate CNS inflammation intrathecally because the adoptive transfer of intestinal plasmocytes into plasma cell-deficient mice ameliorates the course of EAE in recipient mice while intestinal B cells have no effect [64]. This protection involves IL-10 but not IgA [64]. A similar mobilization of intestinal IgA plasma cells outside of the gut might occur in MS patients, because acute relapses are associated with a reduced binding of faecal bacteria by IgA [64].

IgA-expressing plasmocytes have also been shown to have immunosuppressive effects in prostate cancer models [67]. In mice small prostate cancers can be treated with oxaliplatin, an immunogenic chemotherapeutic agent used for patients with severe prostate cancer [68]. This drug induces the immunogenic death of cancer cells and thereby promotes the tumoricidal activity of cytotoxic CD8 T cells [69]. However, this treatment fails to control the growth of large tumours in mice, because it also induces in parallel the development of immunosuppressive IgA⁺CD19⁺CD138⁺B220^{lo} plasma cells expressing IL-10, PD-L1, and FasL, which progressively inhibit the anti-tumour CD8 T cell response via IL-10 and PD-L1 [67]. Remarkably, the removal of these plasmocytes is sufficient to enable the control of large tumours by oxaliplatin treatment [67].

An immunosuppressive role for IgA-expressing plasmocytes was also found in hepatocellular carcinoma (HCC) models, in which these cells inhibited the natural control of the tumour by endogenous CD8 T cells [70]. Mice expressing high amounts of urokinase plasminogen activator specifically in hepatocytes spontaneously

undergo endoplasmic reticulum (ER) stress (*MUP-upa* mice) leading to HCC development upon chronic feeding with a high fat diet (HFD) over several months [71]. Large tumours become detectable around 7 months after the initiation of the HFD [70]. At earlier time points, the tumour development is controlled by CD8 T cells [70]. In parallel to this protective CD8 T cell response a B cell-mediated immunosuppressive response progressively develops. Indeed, at 3 months after the beginning of the HFD starts the accumulation in the liver of IgA plasmocytes expressing both IL-10 and PD-L1. These IgA plasmocytes might differentiate locally in the liver, or in secondary lymphoid tissues, but most likely do not originate from the intestine, because their intrahepatic accumulation was not associated with a reduction of the intestinal pool of IgA⁺ cells [70]. These plasma cells progressively inhibit the anti-tumour CD8 T cell response, which ultimately leads to the development of large tumours. The regulatory plasma cell response involves IgA itself: *MUP-upa* mice deficient for IgA develop less tumour than control mice at 11 months of age. The deficiency in IgA was associated with the absence of suppressive IgA⁺PD-L1⁺IL-10⁺ cells in the liver, the increased accumulation of effector CD8 T cells, and a lower percentage of intra-tumoral CD8 T cells showing an exhausted PD-1⁺TIM3⁺ phenotype [70]. The accumulation of IgA⁺IL-10⁺ PC in the liver also depended on the PD-1/PD-L1 interaction [70], suggesting that their differentiation involved a cognate interaction between B cells and T follicular helper cells and thus the formation of germinal centres [72]. In this case, the regulatory plasmocyte response might thus be T cell-dependent and antigen-specific.

The studies described above show that plasmocytes can express immunosuppressive molecules, and subsequently regulate immunity in autoimmune,

infectious, and malignant diseases, by acting either in secondary lymphoid organs or in the targeted inflamed tissue. Some of these plasma cells are likely to derive from the B cell subsets showing suppressive functions in adoptive transfer experiments.

- Identification of a subset of natural LAG-3⁺CD200⁺PD-L1⁺PD-L2⁺CD19⁺CD138^{hi} regulatory plasma cells in mice

A distinct origin was found for the IL-10-expressing plasmocytes that developed in the spleen of mice 24 hours post-infection with *Salmonella* Typhimurium [58]. These cells suppressed the early innate immune response mediated by neutrophils and NK cells, consequently limiting the control of the bacteria in infected hosts [58]. At this time point post-infection, only a fraction of the splenic plasmocytes expressed IL-10 [58]. Remarkably, IL-10⁺ and IL-10⁻ plasmocytes differed by their transcriptomes, epigenomes, and BCR repertoires [73]. IL-10⁺ plasmocytes distinctively expressed the inhibitory receptor lymphocyte activated gene 3 (LAG-3), and showed the lowest level of DNA CpG motifs methylation at the *Il10* gene compared to all other B cell subsets [73]. LAG-3 was uniformly expressed on IL-10⁺CD138^{hi} cells, while almost no LAG-3⁻CD138^{hi} cells expressed IL-10, highlighting LAG-3 as a specific marker for IL-10-expressing plasmocytes [73]. Of particular significance, LAG-3 was previously identified as a marker for IL-10-producing regulatory Tr1 cells in mouse and human [74]. LAG-3⁻CD138^{hi} cells also distinctively expressed the immune inhibitory receptors PD-L2 and CD200. They also showed surface expression of PD-L1, alike all other plasmocytes, suggesting that they could employ multiple molecular mechanisms to suppress immunity.

IL-10⁺LAG-3⁺CD138^{hi} plasmacytes were in a non-proliferative state in spleen at 24h post-infection. They additionally displayed other features of terminally differentiated plasma cells including the elevated expression of the transcription factor BLIMP-1, and a typical plasmacytoid morphology [73]. The terminally differentiated status of these cells suggested that they developed from pre-existing plasma cells present in naïve mice, because it normally takes B cell proliferation over several days for them to differentiate into plasma cells. Indeed, LAG-3⁺CD19⁺CD138^{hi} plasma cells were present in the spleen, bone marrow, and mesenteric lymph nodes of naïve mice, including germ-free mice, indicating that they developed naturally and independently of any pathological condition or external microbial challenge [73].

LAG-3⁺CD138^{hi} cells developed normally at steady state in naïve TCRαβ⁺ T cell-deficient mice, CD40-deficient mice, as well as *Myd88*^{-/-} *Trif*^{-/-} mice, implicating that they formed independently of classical T-B cell interaction, germinal centre, and TLR signalling [73]. In contrast, the formation of these cells was markedly affected in mice with altered BCR signalling. They were almost absent in *Cd19*^{-/-} and Bruton tyrosine kinase (Btk)-deficient mice, which are both important for effective BCR signalling, while they were more abundant in mice deficient for *Cd72* that encodes for a negative regulator of BCR signalling [73]. Furthermore, LAG-3⁺CD138^{hi} cells displayed a unique BCR repertoire compared to LAG-3⁻CD138^{hi} cells, indicating that their development was probably driven in an antigen-specific manner [73]. Taken together with the normal abundance of these cells in naïve germ-free mice, this suggests that LAG-3⁺CD138^{hi} cells form at steady state upon B cell activation by endogenous T cell-independent type II antigens, which require neither T:B cell cognate interaction nor TLR signalling to elicit B cell differentiation into plasmacytes.

Their generation must however involve a particular mode of B cell stimulation because immunizations with classical type II antigens, type I antigens, or T cell-dependent antigens induce antigen-specific LAG-3⁺CD138^{hi} cells but not LAG-3⁺CD138^{hi} cells [73].

LAG-3⁺CD138^{hi} cells do not produce IL-10 in spleen at steady state. However, after *Salmonella* infection, these cells show detectable IL-10 expression within hours, in the absence of cell division [73]. A similar IL-10 induction can be triggered by the administration of LPS into mice, but not with agonists of BCR or CD40, suggesting that this suppressive response reflects an innate process independent of the antigen specificity of these plasma cells [73]. Accordingly, the BCR repertoire of splenic IL-10⁺LAG-3⁺CD138^{hi} cells found on day 1 post-infection was similar to the one of IL-10⁺LAG-3⁺CD138^{hi} cells from naïve mice [73]. This concept predicts that it is the size of the initial pool of pre-existing natural regulatory LAG-3⁺CD138^{hi} plasma cells that determines the strength of this regulatory circuit. Naïve *Cd72*^{-/-} mice have an elevated number of LAG-3⁺CD138^{hi} cells at steady state compared to wild-type mice. After challenge they display higher numbers of IL-10⁺LAG-3⁺CD138^{hi} cells, and are more susceptible to the disease than control mice both during primary challenge as well as after secondary infection post-vaccination [73].

The importance of the initial number of LAG-3⁺CD138^{hi} cells in naïve mice raises two important questions concerning the establishment of this regulatory circuit: 1) which antigens drive the generation of these cells at steady state? 2) which B cell subsets give rise to these cells *in vivo*? Some features of the BCR repertoire of these cells provided some element of response to these two questions. A proportion of LAG-

3⁺CD138^{hi} cells (about 1% in spleen, and 25% in bone marrow) expresses a VH11⁺Vk14⁺ BCR known to react against phosphatidylcholine [73]. This immunoglobulin subsequently recognizes apoptotic cells as well as damaged red blood cells [75], suggesting that the latter are a source of self-antigens driving the development of LAG-3⁺CD138^{hi} cells at steady state. Apoptotic bodies are another important source of auto-antigens [76] that could trigger this response. LAG-3⁺CD138^{hi} cells also differentially express other molecules implicated in the handling of apoptotic cells including Sirp1 α and Nr4a1 (also called Nur77) [77-79], underlining a particular relationship between LAG-3⁺CD138^{hi} cells and damaged self. The presence of the VH11⁺Vk14⁺ BCR on some LAG-3⁺CD138^{hi} cells also gives some indication about their origin because this BCR is known to be expressed exclusively on B1a cells among the different B cell subsets found in naïve mice. B1a cells might thus be a source of LAG-3⁺CD138^{hi} cells. LAG-3⁺CD138^{hi} cells and some B1a cells also share a differentially higher expression of PD-L2. Indeed, about 50-70% of B1a cells express PD-L2 in the peritoneal cavity of naïve mice [80]. The PD-L2-expressing B1a cells contain the majority of the phosphatidylcholine-reactive B1a cells, and produce more IL-10 than their PD-L2⁻ counterpart [80]. PD-L2⁻ B1a cells do not up-regulate PD-L2 upon activation *in vitro*, indicating that this molecule identifies a cell subset rather than an activation stage [80]. Thus, PD-L2⁺ B1a cells might be part of the progenitors of LAG-3⁺CD138^{hi} plasma cells. Importantly, fate mapping studies showed that other B cell subsets gave rise to LAG-3⁺CD138^{hi} cells, including B1b and B2 cells, in addition to B1a cells. Among B2 cells, CD1d^{hi}CD5⁺ B cells might be particularly relevant because they share with LAG-3⁺CD138^{hi} cells an elevated sensitivity to Btk signalling inhibition compared to other B cells [81].

Collectively, these observations show that LAG-3⁺CD138^{hi} cells represent a natural regulatory plasma cell subset with a unique BCR repertoire and an epigenome specialized for *Il10* expression.

Conclusion

The IL-10-mediated regulatory activity of the B cell compartment involves, at least in mice, three complementary layers. The first layer involves the natural differentiation at steady state of B cells into plasma cells expressing the inhibitory receptor LAG-3, which have a unique epigenome, and reside primarily in spleen, bone marrow as well as mesenteric lymph nodes. These cells develop in a BCR-dependent manner independently of T cells, TLR signalling, and microbial exposure, suggesting that they arise in response to endogenous T-independent type II antigens released during cell damage. The second pathway involves intestinal IgA plasma cells, possibly generated at steady state in response to the intestinal microbiota, which can be mobilized to inflamed tissues during local inflammation. The third pathway involves particular B cell subsets with a higher competence for IL-10 production upon stimulation *in vitro*, and the capacity to suppress immune responses in an IL-10-dependent manner in recipient mice upon adoptive transfer. This pathway might implicate B cells specific for the antigens driving the regulated immune reaction. The B cell subsets most competent to produce IL-10 are the most prone to ASC differentiation. The notion that the differentiation of B cells into “regulatory B cells” follows a trajectory aligned with their maturation into ASC is supported by the fact that the signals and transcription factors contributing to the differentiation of suppressive B cells are also necessary for ASC formation. Consistent with this, plasmacytes have been identified as the major source of B cell-derived IL-10 in

autoimmune, infectious, and malignant diseases *in vivo*. There are some cases suggesting an association between the establishment of immune tolerance and the formation of plasmocytes in human. In MS patients, plasmocytes (but not B cells) are the major source of IL-10 at lesions sites, together with astrocytes [62]. Several studies on human allergy have reported a correlation between the increase of circulating antigen-specific plasmablasts and the response to antigen-induced immunotherapy. For instance, the number of Ara h2-specific plasmablasts increased during peanut oral immunotherapy [82]. Along the same line, intralymphatic immunotherapy induced grass pollen antigen-specific plasmablasts in pilot study [83]. Noteworthy, B cells can produce IL-10 without becoming fully differentiated ASC, as shown for instance *in vitro* using B cells deficient in the *Prdm1* gene coding for BLIMP-1 [59]. However, B cells deficient in *Prdm1* did not suppress the progression of EAE *in vivo* [59]. A possible explanation for this apparent paradox could be that B cells secrete quantitatively less IL-10 than plasmocytes, which display a unique specialization for protein synthesis and secretion. Thus, B cells lacking BLIMP-1 might not produce enough IL-10 to mediate immune suppression in this disease. It is likely that the regulatory power of individual B cells/plasmocytes depends on the amount of IL-10 it secretes. In addition, the overall level of B cell-mediated immune regulation also likely depends on the number of such cells, their localization, and the sensitivity of their microenvironment to the suppressive mechanisms (e.g. IL-10). It will be important to acquire a more precise quantitative knowledge about these different aspects in order to envision the therapeutic utilization or targeting of the relevant suppressive B cells or plasmocytes in individual diseases.

An important issue with respect to the utilization of such cells in adoptive cell therapy concerns the stability of their regulatory phenotype and the biological role of the antibody they produce. The example of B cells reacting against phosphatidylcholine might be relevant to illustrate this point. A significant proportion of healthy humans possess serum antibodies that recognize phosphatidylcholine, and are exclusively of the IgM isotype [84]. This is striking because most natural antibodies are of IgG isotype in human [85]. Patients with autoimmune or infectious diseases also have IgM antibodies reacting against phosphatidylcholine in their serum [86-88]. For instance, the presence of IgM with anti-phosphatidylcholine reactivity has been described in patients with autoimmune hemolytic anemia [86]. These antibodies might contribute to the disease because anti-phosphatidylcholine antibodies caused Coombs' positive haemolytic anaemia in NZB mice [90, 91]. In fact, some patient with Coombs's positive haemolytic anaemia have high titres of serum IgM reacting against phosphatidylcholine, which provoke the haemolysis of bromelain-treated erythrocytes and aged human red blood cells in the presence of complement *in vitro* [86]. Such pathological role of anti-IgM might also be relevant in SLE: a higher frequency of SLE patients with haemolytic anaemia has anti-phosphatidylcholine IgM than patients without this haematological manifestation [89]. Intriguingly, some anti-dsDNA IgG cross-reacted with phosphatidylcholine in SLE patients, suggesting that the B cells producing anti-phosphatidylcholine IgM might give rise to pathogenic anti-dsDNA IgG clones. The antibodies produced by IL-10-producing B cells might thus cause immunopathology in some cases, so that their functional contribution in pathological conditions is an important issue to address.

In conclusion, our understanding of the regulatory functions of B cells, particularly those mediated through the production of IL-10, has greatly increased with regards to the phenotype of the B cells involved, and the molecular mechanisms associated with their formation as well as function. However, important gaps remain in our knowledge, in particular concerning the antigen-specificity of these cells, and the functions of the antibodies they produce. Clarifying these aspects will be key to evaluate the relevance of these cells in human diseases, as protective cells as well as potentially pathogenic actors in specific contexts, as outlined above.

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Figure legends