Chapter 5

Characterization and Immunoregulatory Properties of Innate Pro-B-Cell Progenitors

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Abstract

Control of T-cell responses can be achieved by several subsets of B cells with immunoregulatory functions, mostly acting by provision of the anti-inflammatory cytokine IL-10 or exhibiting killing properties through Fas ligand (Fas-L) or granzyme B-induced cell death. We herein describe the characterization as well as the cellular and molecular mechanisms mediating the suppressive properties of bone marrow immature innate pro-B cell progenitors that emerge upon transient activation of Toll-like receptor 9. They are licensed by activated T-cell-derived IFN-γ to become suppressive by up-regulating their Fas-L expression and inducing effector $CD4^+$ T-cell apoptosis. They also up-regulate their own IFN-γ production which dramatically reduces T-cell production of a major pathogenic cytokine, IL-21. A single adoptive transfer of as little as 60,000 of them efficiently prevents the onset of spontaneous type 1 diabetes in recipient nonobese diabetes (NOD) mice, highlighting the remarkable regulatory potency of these so-called CpG-proB cell progenitors compared to regulatory cells of diverse lineages so far described. The CpG-proB cell activity is prolonged in vivo by their differentiation after migration in the pancreas and the spleen into B-cell progeny with high Fas-L expression that can keep up inducing apoptosis of effector T cells in the long term.

Key words Regulatory B cells, B-cell progenitors, Toll-like receptors, IFN-γ, Killer B lymphocytes, Fas-L, IL-21, Type 1 diabetes, Cell therapy, Tolerance

1 Introduction

The participation of B cells in various immunopathologic settings, particularly in autoimmune diseases, has been amply documented. Today, it is well known that their function is not limited exclusively to the secretion of antibodies since they have been shown to display influence on T-cell-driven diseases either acting as antigen-presenting cells or through their ability to produce various cytokines. These properties have led to the implementation of various B-cell-targeted depletion strategies as a mean for potential treatment. Paradoxically, in autoimmune encephalomyelitis, an experimental model of multiple sclerosis, an autoimmune disease targeting the myelin components and leading to demyelination and subsequent paralysis, B-cell depletion could trigger different

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effects depending on the time of depletion. Matsushita et al. showed that when B-cell depletion, using antibodies targeted against CD20, was accomplished at the peak of the disease or afterwards at the recovery phase, it was associated with reduced disease scores, whereas worsening of disease scores were reported when depletion took place at the immunization phase, thereby suggesting that B cells at the initiation phase of the disease exhibited regulatory properties in vivo. Additionally, the same group provided evidence also for the protective B-cell type identifying a new rare subset expressing the $B220^{+}CD5^{+}CD1d^{hi}$ phenotype, exerting immune regulation through the production of the anti-inflammatory cytokine IL-10 (thereafter named B10 cells) [1, 2].

Different subsets of B cells have since been reported to display immunoregulatory properties (see for review [3–9]), including the above cited $CD19^{+}CD5^{+}CD1d^{hi}$ cells [2], transitional type 2 marginal zone precursor B cells (T2-MZP-Bs) in mice with arthritis [10], follicular B cells and marginal zone B cells and, more recently, $CD138^{+}$ plasma cells [11]. These various B-cell subpopulations at diverse maturation stages can acquire regulatory properties provided they receive several activation signals including both innate and adaptive signals. These signals include either stimulation through Toll-like receptor (TLR) associated with B-cell receptor signaling, cognate interactions with T cells through CD40, as well as provision of several cytokines [11–16]. Even though these diverse signals are effectively provided, only a small percentage of B cells gain regulatory properties, for instance the capacity to secrete the anti-inflammatory cytokine IL-10, questioning the efficiency of this process. Although today it is believed that regulatory B cells do not form a separate lineage, at variance with regulatory T cells, whether a particular stage of differentiation in the B-cell lineage is more prone to acquire regulatory properties and whether immature B-cell progenitors can exhibit tolerogenic properties and differentiate into mature regulatory B cells has not been investigated so far.

Interestingly, it has been shown that TLRs are expressed even on highly immature cells such as hematopoietic stem cells and progenitors that upon stimulation by innate or danger signals do not remain confined within the bone marrow. They can traffic throughout the body, even within tissues, towards infectious and inflammatory signals [17, 18]. It was demonstrated that common lymphoid progenitors (CLP) stimulated via their TLR receptors are diverged from the B-cell lineage and instead differentiate into myeloid dendritic cells that actively take part in the anti-infectious defense [19]. Based on previous demonstrations that mobilized [20, 21] or activated bone marrow progenitors, in tumoral and inflammatory settings [22] as well as in parasite infection [23] can acquire tolerogenic properties, we hypothesized that TLR signals could instead promote the emergence of hematopoietic progenitor

cell populations with tolerogenic properties that could be valuable for cell therapy for the control of unwanted T-cell responses and particularly autoimmune diseases.

2 Characterization of TLR-Activated B-Cell Progenitors with Tolerogenic Properties

To test this hypothesis, we performed total bone marrow cell culture with TLR agonists and investigated whether new c-kit$^+$ progenitor populations with immunoregulatory properties could emerge. Indeed, we showed that within CpG-activated bone marrow cells emerged a new population of tolerating progenitor cells able to provide protection against nonobese diabetic (NOD) mice, an experimental model for type 1 diabetes upon their adoptive transfer.

The following conditions permitted the emergence and isolation of this protective population: incubation of total bone marrow cells for 18 h at 37 °C in 5 % CO_2 in RPMI 1640 medium supplemented with 10 % fetal calf serum and antibiotics, in the presence of 10 μM CpG-B (CpG 1668). We then performed a positive selection of c-kit$^+$ cells using automated magnetic selection (Robosep, StemCell Technologies) and thereof electronically cell-sorted c-kit$^+$ Sca-1$^+$ B220$^+$ IgM$^-$ cells of small size with a FACS Aria cell sorter (Becton Dickinson). As the phenotype of these progenitors—CD127$^+$ CD24$^+$ but also CD43$^+$, CD1d$^+$ and Sca-1$^+$—appeared close to that of a pro-B-cell stage of differentiation although with some differences likely to result from CpG-stimulation, and based on preliminary evidence of their differentiation into the B-cell lineage, we named them CpG-proB cells as a population which emerges upon activation with CpG [24].

An adoptive transfer of only about 60,000 cells by i.v. injection at 6 weeks of age in NOD mice was able to provide significant and dose-dependent protection against T1D whereas transfer of only 12,500 of the same population of cells had no similar protective effect. Furthermore, these cells still protected 50 % of mice from T1D onset even if injected in 16-week-old recipients, just before disease onset. Therefore, these CpG-proB cell progenitors are far more active at a per cell basis than any other previously reported immunoregulatory cell subset and their regulatory potential thus appears inversely correlated to their frequency. In the NOD mice, in our hands, approximately 50,000 CpG-proB cells can be obtained from a single donor mouse when using bone marrow recovered from tibiae and femurs. Interestingly, injection of non-stimulated control pro-B cells had no protective effect against T1D suggesting that TLR-9 signaling may confer tolerating inprinting.

To investigate whether cell populations with the phenotype of CpG-proBs can also transiently emerge after stimulation with other

known TLR-agonists, we performed the same bone marrow cell cultures in the presence of a large array of TLR ligands. Only MyD88-dependent TLR agonists efficiently promoted the emergence of a CpG-proB like cell subset and additionally, CpG-B was unable to trigger CpG-proB cell accumulation in the bone marrow derived from MyD88-deficient NOD mice. CpG likewise prompted the emergence of the same B-cell progenitors within the bone marrow 18 h post-injection (i.p.) in NOD mice, suggesting that such highly potent regulatory population might play a role in vivo as well.

3 Migration and Differentiation Properties

In order to assess the life-span, migration and differentiation properties of the adoptively transferred CpG-proBs in NOD mice that develop spontaneous type 1 autoimmune diabetes, we used CD45.2 congenic donor mice and traced the adoptively transferred progenitors into $CD45.1^+$recipient NOD mice. We observed that the injected progenitor cells migrated within the first 5 days to the pancreas, which is the target tissue of the autoimmune response in NOD mice. A more detailed follow-up showed that they can be recovered also within the pancreatic draining lymph nodes at approximately 3 times less cell counts than in the pancreas, staying in these organs for about 15 days. Later on and only 20 days after injection, they finally accumulated in the spleen where they persisted at least 1 month after injection. The total number of recovered $CD45.2^+$ cells found in the recipient mice corresponded approximately to the number of injected cells, an observation which, taking into account the inevitable cell loss, suggested that some proliferation had occurred.

Along with their migration, differentiation of the progenitors occurred, exclusively into more mature cells of the B-cell lineage but not into any other hematopoietic lineage. While the progeny that initially migrated to the pancreas still expressed c-kit, as did the injected progenitors, when reaching the pancreatic lymph nodes they gradually lost c-kit expression and in the spleen developed into different stages of B-cell maturation including transitional type 2 marginal zone precursor B cells as well as follicular and marginal zone B cells.

4 Cellular and Molecular Mechanisms of Action: CpG-proBs Trigger Effector T-Cell Apoptosis and Dramatically Reduce Their Production of a Major Pathogenic Cytokine in T1D, IL-21

In order to clarify the protective mechanism of CpG-proBs, we investigated whether these cells had a direct effect on either the pathogenic $CD4^+$ T cells or on regulatory T cells. Isolated

CpG-proBs had no effect on the proliferation of anti-CD3 + anti-CD28-activated $CD4^+CD25^+$ (all $Foxp3^+$) regulatory T cells in a cell culture in vitro. Conversely, CpG-proBs were efficient in inhibiting the proliferation of activated $CD4^+CD25^-$ cells at a 1:1 and 1:2 T-cell:CpG-proB ratio. This inhibition of proliferation was correlated with enhanced apoptosis of co-cultured T cells. Although CpG-proB cells expressed a number of death-inducing molecules including TRAIL, PDL1, and PDL2 as well as FasL, their capacity to trigger apoptosis of the co-cultured $CD4^+$ T cells was Fas-FasL dependent, being only inhibited by co-incubation with a neutralizing anti-FasL antibody but none of the other corresponding antibodies.

Moreover, qPCR array analysis of T cells that had escaped apoptosis in the co-culture assay with CpG-proBs demonstrated that they had up-regulated by 55-fold their expression of Fas-ligand, in keeping with their propensity to apoptosis, and additionally had considerably enhanced their pro-Th1 and cytotoxic profile at the expense of a Th2-profile with increased expression of IFN-γ, t-Bet and decreased levels of IL-13 and Gata3. They also concomitantly up-regulated eomesodermin and downregulated ICOS levels, together resulting in the dramatic 77-fold reduction of IL-21 transcript levels.

These molecular pathways were likewise modulated in vivo in T cells from CpG-proB NOD recipients. The $CD4^+$ T-cell pancreatic infiltrates showed a dramatic reduction of $CD44^{hi}CD62L^-CD4^+$ effector memory T cells correlated with reduced IL-21 production capacity contrasting with enhanced IFN-γ intracytoplasmic production. Pancreatic homogenates of CpG-proB recipients likewise displayed significantly reduced IL-21 protein levels relative to control mice with T1D. Therefore, CpG-proBs target pathogenic cellular and molecular mechanisms in T1D, by killing effector T cells and controlling their production of IL-21, a major pathogenic cytokine in T1D [25–27] with *IL-21* representing a susceptibility gene for type 1 diabetes in both mice and humans [28, 29].

5 An IFN-γ Driven Interplay but No Role for IL-10 in the Suppressive Effect of CpG-proBs on Their Target T Cells

One of the most common functional properties of so far described B regulatory cells is their capacity to secrete the anti-inflammatory cytokine IL-10. However, CpG-proBs did not produce IL-10 and neither $CD4^+$ T nor $CD19^+$B cells in CpG-proB recipient mice were displaying enhanced production of IL-10.

Instead, CpG-proBs, constitutively after their isolation, massively produced IFN-γ, proposing a crucial role for this cytokine in their protective mechanism of action. The important role of IFN-γ produced either by T cells or the CpG-proBs was shown with the use of mice deficient for IFN-γ. None of the previously

described effects, induction of apoptosis or reduction of the pathogenic cytokine IL-21 occurred in the absence of IFN-γ in either T cells or CpG-proB cells. When co-cultured with $CD4^{+}CD25^{-}$ T cells isolated from IFN-γ-deficient donors, INF-γ-competent CpG-proB cells (isolated from wild-type (WT) NOD donors) were unable to inhibit the T-cell proliferation or to trigger T-cell apoptosis and to up-regulate their own expression of Fas-L, suggesting that the provision of IFN-γ by activated T cells was the signal responsible for conferring suppressive properties onto CpG-proBs. Moreover, during the co-culture with WT T cells, CpG-proBs up-regulated their own IFN-γ production. In turn, IFN-γ deficient CpG-proB cells did not enhance IFN-γ production by T cells and did not reduce their IL-21 production. This mechanism of action points out the regulatory role of IFN-γ—either direct or indirect through IFN-γ dependent gene-encoded molecules, yet to be identified—since its enhanced production by both T and CpG-proB cells was necessary for achieving a complete suppressive effect *in vitro*. In addition, its regulatory role in vivo as well was demonstrated by the incapacity of CpG-proB cells isolated from the bone marrow of IFN-γ-deficient donor NOD mice to prevent T1D onset in vivo after their adoptive transfer in NOD mice (Fig. 1).

The production of IFN-γ by B cells has been described in various settings. Constitutive production of low levels IFN-γ by immature bone marrow B cells [30] and IFN-γ production by TLR-activated follicular and marginal zone B cells have been previously reported [31, 32], the latter with functional outcomes such as regulation of the Th1 response to *Salmonella enterica* infection [33]. Mature follicular B cells from *Toxoplasma gondii*-infected mice were shown to produce IFN-γ upon ex vivo restimulation with pathogen extracts [34]. Bao et al. [35] recently reported that innate B cells with a $CD11a^{hi}$ $CD16/32^{hi}$ phenotype emerged from follicular B cells 3 days post-infection with bacterial and viral agents, even in IFN-γR deficient mice. Upon ex vivo restimulation with anti-CD40, these innate B cells produced as much IFN-γ as NK cells. Their provision of IFN-γ was required for protection against *L. monocytogenes* infection and depended on activation of the Bruton's tyrosine kinase (*btk*)—but not the*t-Bet*-dependent pathway-, as *btk*-deficient mice lacked these $CD11a^{hi}$ $CD16/32^{hi}$B cells and did not clear *L. monocytogenes* infection. Therefore, IFN-γ production by CpG-proBs as well as other described B cells has functional role and may link innate and adaptive immune responses.

6 CpG-proB Cell Progenitors Mature into FasL-Expressing Immunoregulatory Progeny

The limited life-span of hematopoietic progenitors contrasted with the long-lasting protection against spontaneous T1D provided by a single injection of only 60,000 CpG-proBs and suggested that

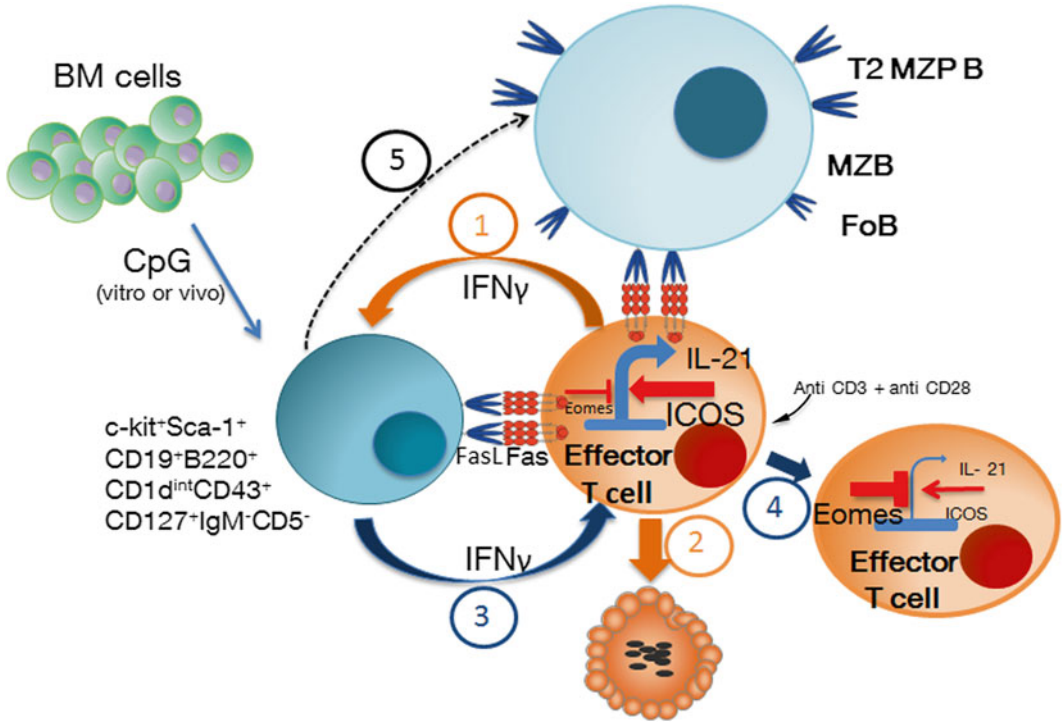

Fig. 1 Cellular and molecular mechanisms underlying the protective effects of adoptively transferred CpG-proB cells against type 1 diabetes in NOD mice. (1) IFN-γ derived from TCR-activated T cells promotes expression of Fas-L on CpG-proB cells. (2) FasL-expressing CpG-proB cells trigger $CD4^+$ T-cell apoptosis. (3) IFN-γ derived from activated T cells also enhances IFN-γ levels produced by CpG-proB cells, which (4) enhances within spared $CD4^+$ T-cells eomesodermin and ICOS levels, resulting in the dramatic reduction within T-cells of the production of IL-21, a cytokine playing a major role in T1D pathogenesis. (5) CpG-proB cells migrate to the pancreas, the pancreatic lymph nodes and the spleen sequentially where they differentiate into various B-cell mature subsets (T2-MZP-Bs, MZ, and FO B cells) all expressing higher FasL levels than the recipient's B cells in the corresponding tissues and able to trigger apoptosis of T cells in the long term

the influence of the B-cell progenitors was somehow prolonged over time. Indeed, we showed that their more mature B-cell progeny could contribute to the protection. These mature B cells, recovered either from the pancreas or the spleen of $CD45.1^+$ CpG-proB recipients, maintained a remarkably high FasL expression—higher than that of the host $CD45.2^+$B cells in the same compartments—and remained able to trigger the apoptosis of $CD4^+$ T cells, even if recovered as far as 1 month after the adoptive transfer of CpG-proBs. Therefore, a long-term control of effector diabetogenic T cells which was initially operated by the acquired FasL expression in CpG-proB cells in contact with activated T cells is further maintained in vivo through the differentiation of CpG-proBs into $FasL^{hi}$ mature B-cell progeny in both pancreas and spleen (Fig. 1). CpG-proB cells and their mature B-cell progeny thereby share the cytotoxic properties originally described in PMA + ionomycin-activated spleen B cells [30], in LPS-treated NOD mice based on FasL expression of mature B cells [36] and in

Shistosoma infection by splenic CD5⁺ B1a cells expressing high levels of FasL that triggered CD4⁺ T-cell apoptosis [37, 38]. In humans, regulatory B cells with cytotoxic activity have been reported. Leukemic, myeloma cells and EBV- and HIV-infected cells were shown to express FasL allowing them to escape immune surveillance [39, 40]. B cells producing instead granzyme B, under the influence of IL-21 production by T cells, were likewise shown to inhibit tumor defense [41].

The capacity of TLR-activated B-cell progenitors to migrate into the autoreactive inflammatory target site, develop into an immunoregulatory mature B-cell progeny that ensures long-term targeting of effector T cells represent remarkable properties than might be harnessed in cell therapy for controlling unwanted T-cell immune responses and establishing long-lasting immune tolerance.

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