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Bacterially activated B-cells drive T cell differentiation towards Tr1 through PD-1/PD-L1 expression

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ABSTRACT

Regulatory B cells (Bregs) play a crucial role in immunological tolerance primarily through the production of IL-10 in many diseases including autoimmune disorders, allergy, infectious diseases, and cancer. To date, various Breg subsets with overlapping phenotypes have been identified. However, the roles of Bregs in *Helicobacter* infection are largely unknown. In the present study, we investigate the phenotype and function of *Helicobacter* −stimulated B cells. Our results demonstrate that *Helicobacter felis* −stimulated IL-10- producing B cells (Hf_{stim}- IL-10$^+$ B) are composed of B10 and Transitional 2 Marginal Zone Precursor (T2-MZP) cells with expression of CD9, Tim-1, and programmed death 1 (PD-1). On the other hand, *Helicobacter felis* −stimulated IL-10- non-producing B (Hf_{stim}- IL-10$^-$ B) cells are mainly marginal zone (MZ) B cells that express PD-L1 and secrete TGF-β, IL-6, and TNF-α, and IgM and IgG2b. Furthermore, we show that both Hf_{stim}- IL-10$^+$ B cells and Hf_{stim}- IL-10$^-$ B cells induce CD49b$^+$LAG-3$^+$ Tr1 cells. Here, we describe a novel mechanism for PD-1/PD-L1- driven B cell-dependent Tr1 cell differentiation. Finally, we explore the capability of Hf_{stim}- IL-10$^-$ B cells to induce Th17 cell differentiation, which we find to be dependent on TGF-β. Taken together, the current study demonstrates that Hf_{stim}- B cells induce Tr1 cells through the PD-1/PD-L1 axis and Th17 cells by secreting TGF-β.

1. Introduction

B cells have been described as positive regulators of immune responses based on their ability to produce antigen-specific antibodies and T cell activation (Miyagaki et al., 2015) Recent research has demonstrated negative regulatory functions of B cells in murine models of inflammatory and autoimmune diseases (Rosser and Mauri, 2015). A regulatory role of B cells was indicated, for the first time, in a B cell-deficient experimental autoimmune encephalomyelitis (EAE) mouse model (Wolf et al., 1996). Later, it was shown that the inflammatory response in EAE, arthritis and colitis mouse models was repressed by IL-10-producing regulatory B cells (Bregs) (Mauri and Bosma, 2012). The phenotypes and mechanism of action of regulatory B cells have been recently studied, and diverse regulatory B cell subsets were reported in mouse models. The most characterized surface markers on Breg subsets are as follows; B10 cell subset; CD1d^{hi}, CD24$^+$, CD5$^+$, CD21$^+$, CD23$^+$, Transitional 2 Marginal Zone Precursor (T2-MZP) B cells; CD21^{hi}, CD23$^+$, CD24$^+$ and CD93$^+$, and marginal zone B cells; CD21^{hi}, CD23^{lo} (Mauri and Blair, 2010). Moreover two recent studies identified CD9, a tetraspanin-family transmembrane protein, as a key surface marker playing a role in the immunosuppressive activity of most mouse IL-10 competent B cells (Sun et al., 2015; Braza et al., 2015).

These studies also demonstrated that IL-10–producing B cells have elevated levels of the inhibitory receptor, programmed cell death receptor-1 (PD-1) (Sun et al., 2015; Braza et al., 2015). Also, PD-1 is induced on B cells, T cells, and monocytes after activation (Kanai et al., 2003). Recently, human hepatocellular carcinomas were shown to contain PD-1^{hi} Breg cells that suppress tumor- specific PD-L1 expressing T cells and promote tumor growth through IL-10 (Xiao et al., 2016). PD-1 has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), which are expressed in varying amounts in hematopoietic and epithelial cells (Fife and Pauken, 2011). Recent studies demonstrate elevated PD-L1 expression on Breg cells, which suppresses autoimmune diseases and cancer through inhibiting the activation of T cells and antibody production (Khan et al., 2015; Guan et al., 2016; Bodhankar et al., 2011). T cell immunoglobulin mucin domain-1 (Tim-1$^+$) B cells were shown constitute approximately 70% of the IL-10- producing B cell population (Yeung et al., 2015). Tim-1 deficient mice were unable to induce IL-10 production, and promoted Th1, Th17 and T regulatory cells (Xiao et al., 2015). TIM-1 signaling was shown to play a major role in Breg

Abbreviations: Hf_{stim}, *Helicobacter felis* -stimulated; Hf_{stim}- IL-10$^+$ B, *Helicobacter felis*–stimulated IL-10- producing B cells; Hf_{stim}- IL-10-B, *Helicobacter felis*–stimulated IL-10- non-producing B cells; Breg, regulatory B cells; PD-1, programmed death 1; PD-L1, programmed death ligand 1

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maintenance and induction (Yeung et al., 2015).

Bregs were shown to exhibit their inhibitory function through secreting interleukin- 10 (IL-10), interleukin- 35 (IL-35), and transforming growth factor β (TGF-β) (Rosser and Mauri, 2015). Also, Breg cells suppress immunopathology by preventing the development of pathogenic T cells and other pro-inflammatory lymphocytes (Rosser and Mauri, 2015). It was demonstrated that TGF-β- producing Bregs mediate immune tolerance through the induction of regulatory T cells (Lee et al., 2014).

To date, the role of regulatory B cells play in *Helicobacter* infection has not been thoroughly studied. *Helicobacter pylori* is a gram-negative bacteria which colonizes human gastric mucosa (Monack et al., 2004). *Helicobacter* infection leads to severe duodenal and gastric pathologies in nearly 20% of infected individuals by triggering a strong immune response (Marshall and Warren, 1984; Parsonnet, 1994). *Helicobacter felis (H. felis)* is the most frequently used *Helicobacter* species in murine studies because of its higher immunogenicity (Sayi et al., 2009). We previously reported that IL-10- producing B cells are activated through the *Helicobacter* – derived TLR-2 ligands. These B cells lead to differentiation of naïve T cells into IL-10- producing $CD4^+CD25^+$ T regulatory-1 (Tr1) cells (Sayi et al., 2011). The cooperation between these Tr1 cells and B cells may prevent the gastric pathology in a mouse model of *Helicobacter* infection. On the basis of these findings, we herein investigated, (1) the phenotype of *Helicobacter felis* sonicate-activated B cells; (2) the contribution of *Helicobacter felis* −stimulated IL-10- producing B (Hf_{stim}- $IL\text{-}10^+$ B) cells and *Helicobacter felis* −stimulated IL-10- nonproducing B (Hf_{stim}- $IL\text{-}10^-$ B) cells on Tr1 cell differentiation; (3) the impact of PD-1/PD-L1 and TGF-β produced by B cells on Tr1 cell differentiation *in vitro*.

In this study, we demonstrate that *Helicobacter* – induced B cell subsets, which were separated through their IL-10 expression display distinct phenotypes; Hf_{stim}- $IL\text{-}10^+$ B cells are composed of $CD1d^{hi}CD5^+$ B10 cells and $CD21^+CD23^+$ T2-MZP with expression of CD9, PD-1, and Tim-1 and Hf_{stim}- $IL\text{-}10^-$ B cells are composed of $CD21^+CD23^-$ MZ B cells with expression of PD-L1. Also Hf_{stim}- $IL\text{-}10^+$ B cells and Hf_{stim}- $IL\text{-}10^-$ B cells secrete different cytokines, IL10 and TGF-β, respectively. Moreover, we found that only Hf_{stim}- $IL\text{-}10^-$ B cells secrete IgM and IgG2b antibodies.

Although, we showed previously that *Helicobacter* could induce differentiation of naive $CD4^+$ T cells to Tr1 cells (Sayi et al., 2011), it was still unknown whether IL-10 has an impact on this differentiation. This study demonstrates that both Hf_{stim}- $IL\text{-}10^+$ B and Hf_{stim}- $IL\text{-}10^-$ B cells can induce CD49b-LAG-3 co-expressing Tr1 cells. Next, we examined whether PD-1/PD-L1 signaling in B cells have an effect on differentiation of naive $CD4^+$ T cells to Tr1 cells. We provide evidence for a novel link between PD-1 and PD-L1 expression on B cells and their impact on Tr1 cell differentiation, *in vitro*.

Finally, we show that Hf_{stim}- $IL\text{-}10^-$ B cells can also induce IL-17-producing Th17 cells from naïve T cells. To evaluate whether Hf_{stim}- $IL\text{-}10^-$ B cell-derived TGF-β has an effect on either Tr1 or Th17 differentiation, we blocked TGF-β using TGF-β neutralizing antibodies in B/T cell co-culture. Our data demonstrate that TGF-β blockade in B/T cell co-culture had a moderate effect on the differentiation of naïve T cells to Tr1 cells, whereas a significant effect on the differentiation of naïve T cells to Th17 cells.

2. Materials and methods

2.1. Animal experimentation

C57BL/6 mice were purchased from Vivarium, Bogazici University Center for Life Sciences and Technologies (Istanbul, Turkey). All mice were housed under specific pathogen-free conditions and maintained in individually ventilated cages. All animal experiments were conducted in accordance with the guidelines approved by the Turkish Ministry of Food, Agriculture and Livestock.

2.2. Bacterial strain and treatment

Helicobacter felis (H. felis) strain CS1 (ATCC 49179) was kindly provided by Prof. Dr. Anne Mueller from Institute of Molecular Cancer Research, University of Zurich, Switzerland. *H.felis* were grown as described (Sayi et al., 2009). Moreover, *H. felis* sonicates were prepared by sonication on a Bandelin Sonopuls (30 s pulse on; 50 s pulse off for 6 min 30 s at 50 W on ice with the MS 72 probe). Aliquots of sonicates were kept at 4 °C for at most 6 months. The protein concentrations of *H.felis* sonicate was measured by the BCA assay (Thermo Fischer Scientific, Germany). *H.felis* sonicates were mixed with purified cells at a final concentration of 5 μg/ml.

2.3. Isolation and cell culture

Spleens were dissected from sacrificed C57BL/6 mice, 6–12 weeks of age. Naïve $CD4^+$ T cells and B cells were purified from single-cell suspensions of freshly isolated and pooled spleens from 4 to 5 mice by immuno-magnetic sorting ($CD4^+$ T Cell Isolation Kit, mouse (130-104-453), MACS, Militenyi Biotech, Germany) and B Cell Isolation Kit, mouse (130-090-862, MACS, Militenyi Biotech, Germany), respectively according to the manufacturer's instructions. B cell and T cell purity (more than 97% pure) was determined by anti-CD19-FITC and anti-CD4-APC antibodies (Biolegend, San Diego, CA), respectively. Purified B cells were cultured at $2{,}5 \times 10^6$ cells/ml in 96-well round-bottom plates. The experiments were performed using five different groups. First group contained splenic B cells without any stimulation (unstim. B cells). Second group contained splenic B cells stimulated with PMA (Phorbol 12-myristate 13-acetate) (50 ng/ml, 35–76 sc- Santa Cruz, USA) and ionomycin (500 ng/ml, Calbiochem, Israel), which was introduced during the last 5 h of culture (P/I – stim. B). In the third group, there were splenic B cells stimulated with 5 μg/ml *Helicobacter felis* (Hf_{stim}- Total B cells) for 21–24 h. Fourth and fifth groups contained Hf_{stim}- $IL\text{-}10^+$ B cells and Hf_{stim}- $IL\text{-}10^-$ B cells, respectively. These two groups were immuno-magnetically sorted from Hf_{stim}- Total B cells that were exposed to PMA and ionomycin during the last 5 h of culture according to the manufacturer's instructions (Regulatory B Cell Isolation Kit, mouse (130-095-873), MACS, Militenyi Biotech, Germany). For B cell/T cell co-cultures, B cells and naïve $CD4^+$ T cells were cultured at a 1:1 ratio in 96-well round-bottom plates in the presence of 1 μg/ml anti-CD3 (17A2, MACS, Militenyi Biotech, Germany) and 10 ng/ml recombinant IL-2 (MACS, Militenyi Biotech, Germany). Cell viability was evaluated by Trypan blue staining (Lonza, USA) and 7AAD staining (4204023, Biolegend, San Diego).

2.4. Staining and flow cytometry

The following antibodies were used for flow cytometry: CD4- APC (clone RM-4-5), CD19- FITC (clone, 6D5), CD1d (CD1.1, Ly-38)-FITC (clone 1B1) and CD5-Alexa Fluor 647(clone 53-7.3), CD21/CD35 (CR2/CR1)-APC (clone 7E9) and CD23-PE-Cy7 (clone B3B4), CD279 (PD-1)-FITC (clone RMP1-30), CD9-Biotin (clone MZ3), CD49b-FITC (clone DX5), LAG-3-PerCpCy5.5 (clone C9B7W), CD274 (B7-1,PDL-1)-Biotin (clone, 10F.9G2), CD273 (B7-DC,PD-L2)-Biotin (clone, TY25), CD365 (Tim-1) − Biotin (RMT1-4), APC Streptavidin. All antibodies were purchased from Biolegend, San Diego, USA. Cells were stained with indicated antibodies in PBS containing 2% FBS for 60 min. Flow acquisition was performed with a BD Accuri C6 Flow Cytometry instrument (Accuri C6, Ann Arbor, MI, USA) and analyzed using FlowJo Software.

2.5. Intracellular staining

To determine intracellular IL-10 and IL-17 production levels using flow cytometry, IL-10 and IL-17 cytokine secretion was blocked by monensin (3 μg/ml, Calbiochem, San Diego, USA) 5 h before harvesting