이학박사학위논문

골수 및 장내에서 림프구 분화에 대한 조절 T세포의 역할에 대한 연구

Studies on the role of regulatory T cells during lymphocyte differentiation in the bone marrow and the colon

2019년 8월

서울대학교 대학원

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ABSTRACT

Studies on the role of regulatory T cells during lymphocyte differentiation in the bone marrow and the colon

Kyungsoo Park

The adaptive immune system of higher animals is a very powerful and exquisite tool to protect the host body from invasion of external pathogens. Because of its strong effect, however, improper operation of the system can cause damage to the host. Thus, in order to maintain homeostasis, cells that play a role in suppressing such inaccurate immune responses are simultaneously generated in the adaptive immune system. Regulatory T cells (Treg cells) are the specialized CD4 T lineage cells that play an indispensable role in maintaining immunological tolerance by suppressing the immune activation against self-antigens and the excessive inflammatory responses. Therefore, it is important to know how Treg cells regulate the immune responsese and act on the maintenance of immune homeostasis.

Here I verify the roles of Treg cells in B lymphopoiesis in bone marrow and pTreg cell generation in the intestine.

First, I examined the role of Treg cells in the bone marrow hematopoiesis by using Treg cell deficient mice. Development of mature blood cells is a sequential process of lineage specification, which starts from the hematopoietic stem cells (HSCs). HSCs constitute bone marrow as rare cells, and are able to self-renewal to maintain its pool. Multipotent progenitor cells (MPPs) are decreased in its self-renew potential and increased in proliferation, yet still have differentiation potential into all lineage of blood cells. Myeloid progenitors and lymphoid progenitors are diverged from MPPs. Myeloid progenitors differentiate into erythrocyte, megakaryocytes, macrophages, and granulocytes while lymphoid progenitors differentiate into early thymic progenitors, NK cells, and B cells. B lymphopoiesis first starts from pre-pro B cell step acquiring the expression of CD45R (B220) and the transcription factor EBF1 followed by B cell receptor heavy chain gene rearrangement. Pro B cells are then passed through the pre-B cell stage to differentiate into immature B cells that express the full B cell receptor. Scurfy mice that are deficient in Foxp3 gene, which is a master regulator gene of Treg cells, show systemic autoimmune responses due to the absence of Treg cells. Here, I found altered hematopoiesis in the bone marrow of scurfy mouse. Especially, B cell development was severely impaired in the bone marrow. Significant reduction of mature B cells stemmed from pre-pro B cell arrest. When I

analysed hematopoietic processes, the alteration was observed at branching point of lymphoid cells and myeloid cells. The number of lymphoid progenitor is greatly reduced but that of myeloid progenitors was increased concurrently. Furthermore, significant expansion of MPP and HSC population was observed. Analysis of bone marrow chimera of scurfy and wild type revealed that such impaired B lymphopoiesis from early hematopoietic process does not stem from intrinsic defect of HSCs. Treg cell reconstitution rescued all of these alterations of B lymphopoiesis. In vitro HSC culture in the B cell-inducing condition revealed that B cell development is inhibited by culture medium of activated effector T cell. Based on this result, the ex-vivo BM cell stimulation of scurfy mice showed the secretion of several pro-inflammatory cytokines. This secretion propensity was observed in in vivo scurfy bone marrow as well. Among the pro-inflammatory cytokines of scurfy mice, IL-6, TNF, and GM-CSF were shown to inhibit B cell development intensively. Further, neutralizing these cytokines sufficiently rescued B cell development in vitro and in vivo. These data show that Treg cells ensure B lymphopoiesis in bone marrow by suppressing pro-inflammatory cytokines.

Next, I studied the role of Treg cells in peripherally derived Treg cell (pTreg cell) generation in the intestine. Treg cells conduct its suppressive roles in the intestine where external antigens and pathogens induce continuous immune responses. Colon is the site where abundant commensal bacteria reside, and immune systems are developed in response to them. The

frequency of Treg cells that constitute the colon lamina propria is significantly higher (> 30%) than that of other tissues because it is likely to be related to the characteristics of the colon, which is constantly exposed to external antigens. A significant proportion (~ 60%) of these are peripherally derived Treg cells (pTregs) induced from naive CD4 in response to a local antigen. Moreover, these pTreg cells have a crucial role in maintaining intestinal immune homeostasis. Here, in this study, I found that the expression of CEBPβ on iTreg cells recruited into the colon have a role on pTreg cell generation to suppress intestinal inflammation. I found higher expression of C/EBPβ in colon Treg cells compared with spleen Treg cells and thereby tried to figure out the function of C/EBPβ in colon Treg cells. Co-transfer of C/EBPβ-overexpressed iTreg cells into the intestinal inflammation-induced mice resulted in significant increase of pTreg cell generation. Concurrently, C/EBPβ-overexpressed iTreg cells showed stable expression of Foxp3 as previously reported. However, substantial reduction in cell frequency was observed which was caused by increased cell death. These two different effects of C/EBPβ expression, a substantial reduction of iTreg cells and increase of pTreg cell induction, resulted in similar level of total $Foxp3^+$ Treg frequency compared with mice reconstituted with control iTreg cells in intestine. Despite the similar frequencies of $Foxp3^+$ cells in intestine, C/EBPβ-iTreg cell-transferred mice showed significantly ameliorated inflammation. However, this immune suppression of C/EBPβ-iTreg- transferred mice collapsed after selective deletion of pTreg cells. This

result indicates that the amelioration of C/EBPβ-transferred mice practically depends on the induction of pTreg cells. Finally, I found C/EBPβ expression up-regulates the pTreg cell induction-related signalling molecules in mRNA level in iTreg cells. These data collectively suggest that C/EBPβ expression in Treg cells promotes pTreg cell differentiation in a trans-manner so that it regulates intestinal inflammation.

Keywords: Regulatory T cells**,** B cell development. Hematopoiesis, CCAAT-enhancer-binding protein β, Intestinal inflammation, Peripherally derived Treg cells

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INTRODUCTION

Introduction

1-1. Hematopoietic lineage differentiation in the bone marrow

Hematopoiesis is a process of serial differentiation into blood cells from self-renewing hematopoietic stem cells (HSCs) that reside in the bone marrow as rare cells. HSCs are capable of self-renewal so that additional HSCs can be generated to maintain its pool. However, HSCs progressively lose their self-renewing potential and give rise to multipotent progenitors (MPPs) that have full potential to differentiate into every type of blood cells (Morrison et al., 1997; Reya et al., 2001). These HSCs can be classified by special surface markers. A highly dormant HSCs are $Lin^{-}c\text{-}Kit^{+}Sca1^{+}CD150^{+}CD34^{-}CD48^{-}$ (Adolfsson et al., 2005; Wilson et al., 2008), while multi-potent progenitors (MPPs) are $Lin^{-}c\text{-}Kit^{+}Sca1^{+}CD34^{+}$. Verification of the lineage differentiation of MPPs into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) (Akashi et al., 2000; Kondo et al., 1997) implicates crucial lineage decision step of HSCs cause complete separation of myelopoiesis and lymphopoiesis in the bone marrow. A compelling report demonstrated that $Flt3^{+}Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ cells lack differentiation potential to megakaryocyte and erythrocyte, but they were still retaining granulocyte, myeloid, and lymphoid potential, thereby were termed lymphoid-primed multipotent progenitors (LMPPs)

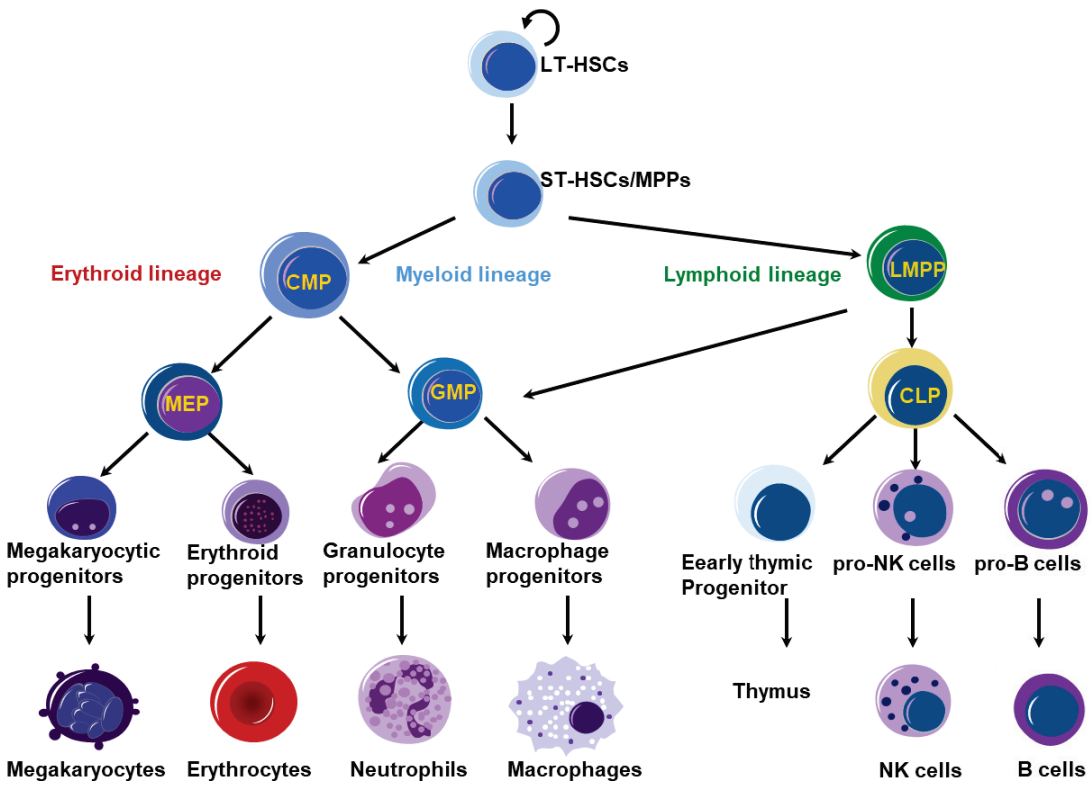


Figure 1. Overview of the hierarchical process of hematopoiesis.

later (Adolfsson et al., 2005; Iwasaki and Akashi, 2007). Early thymic progenitors (ETPs) which express recombination activating gene 1 (Rag1) was found in LMPPs, (Igarashi et al., 2002) which possess T, B, and NK cell differentiation potential. To sum up, MPPs are first divided into LMPPs ($Flt3^{+}CD34^{+}$) and CMPs ($IL\text{-}7Ra^{-}Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RII/III^{lo}$). Lineage progenitors are progressively restricted to specific mature lineage blood cells. Mature blood cells are constituted with red blood cells, megakaryocytes, myeloid cells, and lymphocytes. These mature blood cells are differentiated from lineage progenitors, that consist of megakaryocyte-erythroid progenitors (MEPs) (Akashi et al., 2000)granulocyte-myeloid progenitors (GMPs), and common lymphoid progenitor(CLP) respectively. Collectively, the current hematopoietic lineage differentiation model suggests MPPs first divide into LMPPs ($Flt3^{+}CD34^{+}$) and CMPs ($IL\text{-}7Ra^{-}Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RII/III^{lo}$) followed by differentiation of LMPPs into CLPs ($IL\text{-}7Ra^{+}Lin^{-}Sca1^{lo}c\text{-}Kit^{lo}$) and early thymic progenitors (ETPs). CMPs, a progenitor that can generate all myeloid cells, differentiate into the MEPs ($IL\text{-}7Ra^{-}Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{-}Fc\gamma RII/III^{lo}$) and GMPs ($IL\text{-}7Ra^{-}Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RII/III^{hi}$).

1-2. B cell development in the bone marrow

IL-7 signaling activates STAT5 that induces E2A and Ebf1 sequentially in CLPs contacting with CXCL12-secreting bone marrow stromal cells. These

cells start to express B220 (CD45R) and become pre-pro-B cells. In this population, EBF1 and E2A enhance the accessibility of D-JH gene locus (Lin et al., 2010). Early pro-B cells, the next step of B cell development, are appeared with D-JH recombination in virtue of recombination activating gene (Rag) activity. Early pro-B cells start to express a transcription factor Pax5, and its expression permanently blocks the expression of non-B cell lineage gene (Nutt et al., 1999). In addition, early pro-B cells begin to express Igα-Igβ heterodimer and CD19 co-receptor, which serves as a B cell marker. In early pro-B cells, Pax5 and Ebf1 further enhance the expression of each other to strengthen the lineage commitment into B cells (Nechanitzky et al., 2013). Early pro-B cells move to contact with IL-7-expressing bone marrow stromal cells. Most of the late pro-B cells have begun to rearrange the VH to DJH. Large (early) pre-B-cells emerge as successful VDJ rearrangement of immunoglobulin heavy chain (IgH) is completed. This large (early) pre-B cells express pre-B cell receptor (Pre-BCR), assembled with IgH(μ) and surrogate light chain (SLC) composed of VpreB and λ5 (Karasuyama et al., 1994). Pre-BCR signaling induces four to five rounds of clonal expansion, which is needed to generate enough numbers of B cells (Rolink et al., 2000; Winkler and Mårtensson, 2018). This expansion is resulted from the cooperation with IL-7 signaling that activates STAT5 and cyclin D3, which suppress kappa chain rearrangement (Clark et al., 2014). Pre-BCR signaling also induces the loss of TdT activity

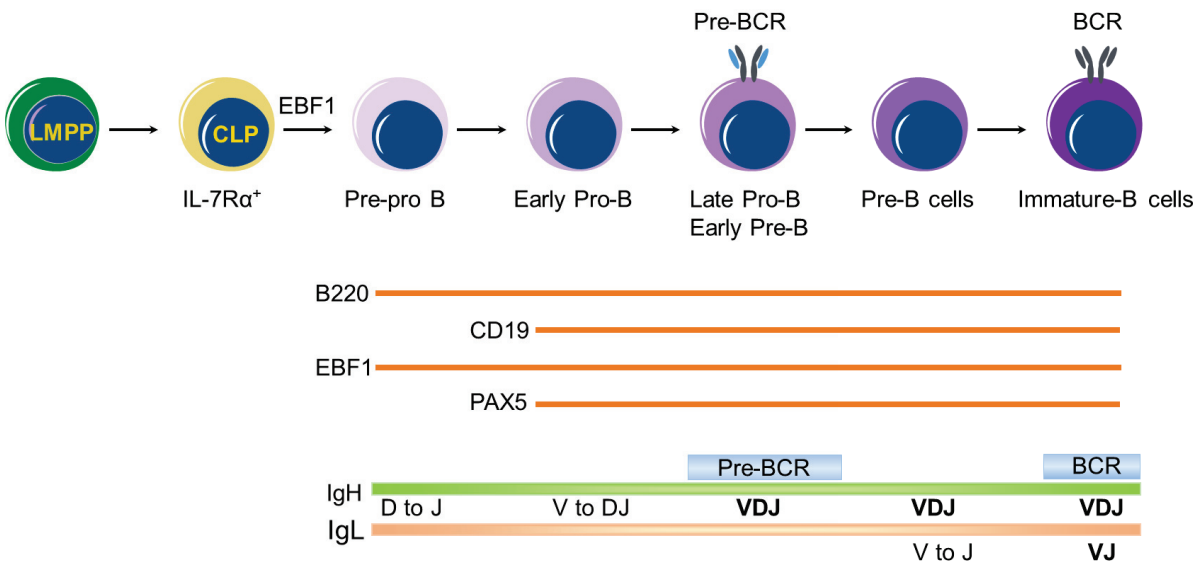


Figure 2. B cell development in the bone marrow.

and transient downregulation of Rag1 and Rag2 transcription thereby inhibits further heavy chain rearrangement. Concurrently, the serial pre-BCR signaling activates SLP65 that downregulates the expression of λ5 to lead to termination of SLC expression and downregulation of pre-BCR. At this point, the end of Pre-BCR signaling mediated proliferation, pre-BCR lost on the cell surface and become late Pre-B cells. In this resting stages, immunoglobulin light chain (IgL)(kappa, lamda) rearrangement occurs in response to re-induction of Rag1 and 2 (Clark et al., 2014). Successful completion of rearrangement of IgL leads expression of IgM on the cell surface followed by signaling entry. These cells are referred as immature B cells and are ready for export to periphery.

1-3. Regulatory T cells

Immune system is under exquisite control so that the host body can be protected against the self-antigen or excessive inflammatory responses. This immune homeostasis is maintained by central tolerance, a process that deletes the T and B cell clones that possess self-reactive receptor, and by the other process called peripheral tolerance. Regulatory T cells (Treg cells) largely take part in the peripheral tolerance that suppress the self-reactive immune cells escaped from central tolerance and inhibit the inflammation response that can cause harm to the host body.

The initial study demonstrated that autoimmune disease caused by

neonatal-thymectomize of mouse on the third day of the birth were prevented by adoptive transfer of thymocyte, suggesting the existence of suppressive cells developed from thymus (Nishizuka and Sakakura, 1969). By demonstrating that adoptive transfer of IL-2 receptor α chain $(CD25)^+$ cell-depleted CD4 T cells into athymic mouse induces systemic autoimmune disease, $CD4^+CD25^+$ T cells were proved as suppressor cells (Sakaguchi et al., 1995), termed Treg cells later. CD25, however, is not a definitive marker for Treg cells because is also expressed by activated naive T cells. Through the studies of human IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome-patient and 'scurfy' mouse mutation model, it has been reported that loss-of-function of Foxp3 gene located on X chromosome led to systemic autoimmune disease (Bennett et al., 2001). Based on this finding, the further studies demonstrated that Foxp3 is specifically expressed in $CD4^+CD25^+$ T cells, and strikingly, retroviral gene transduction of Foxp3 confers suppressive ability and regulatory phenotype to naive T cell (Hori et al., 2003). In addition, mice that carried a specific deletion of Foxp3 in CD4 T cells were not able to develop $CD4^+CD25^+$ T cells and led to lethal autoimmune disease just as scurfy mouse. However, the adoptive transfer of $CD4^+CD25^+$ T cells rescued the lymphoproliferative disease in Foxp3-deficient mice. These studies indicated that Foxp3 is one of the key transcription factors in the development of Treg cells

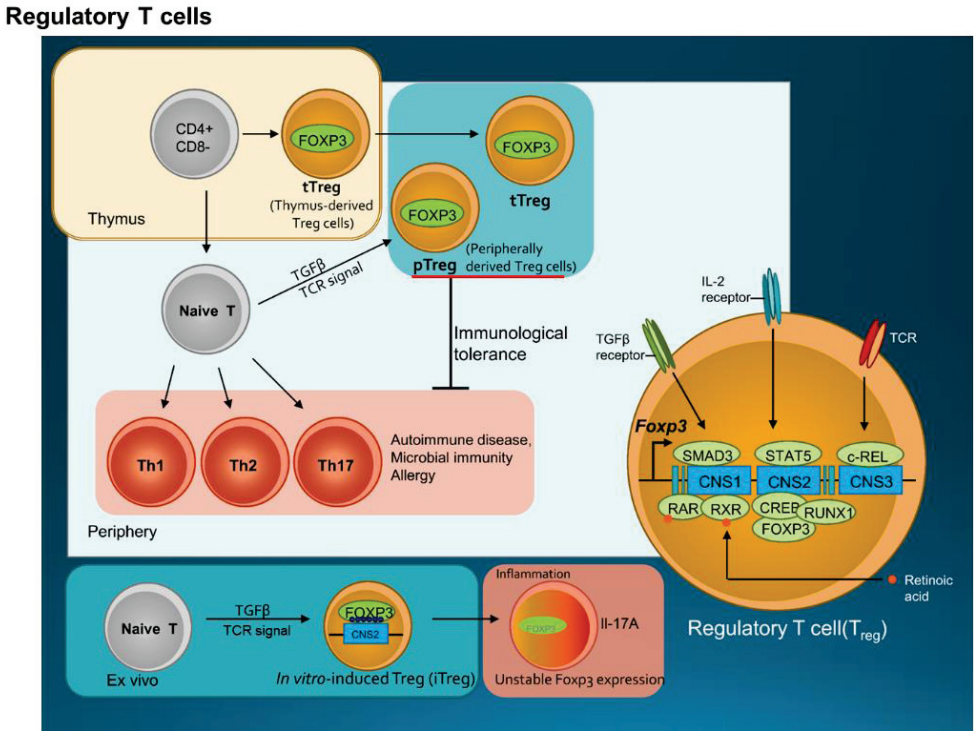


Figure 3. Overview of the regulatory T cells

(Fontenot et al., 2003; Hori et al., 2003).

Treg cells are categorized into thymus-derived Treg cells (tT_{reg} cells) and peripherally derived Treg cells (pT_{reg} cells) based on the anatomical location of their development. In the thymus, CD4 single positive thymocytes possessing TCR with affinity for self peptide-MHC ligands in the range between those that mediate positive selection and stronger signals that mediate negative selection turned to $CD25^{+}GITR^{+}Foxp3^{-}$ progenitor and followed by final differentiation into $Foxp3^{+}$ Treg cells by IL-2 signaling (Hsieh et al., 2006; Lio and Hsieh, 2008). The Treg cells generated by this process is called tT_{reg} cell. In addition to the tTreg cell, naive CD4 T cells in the peripheral lymphoid tissue are converted into Foxp3-expressing T cells under the environment where TGFβ cytokine exists and these Treg cells are called pTreg cells. Both tTreg and pTreg cells play a crucial role in suppressive function of immune systems.

1-4. Instructive role of TCR signaling in Treg cell differentiation

During Foxp3 induction, strength of TCR signaling has an instructive role for Treg cell differentiation. TCR repertoires of Treg and non-Treg cells are only partially overlapped. As TCRs of Treg cells, distinct from naive T cells, show higher self-reactivity, effector T cells transduced with TCR of Treg cells show robust expansion and induction of autoimmune disease (Hsieh et al., 2004). A study that has proved Treg-TCR of wild type mice can be

detected in activated T cells of Foxp3-deficient mouse supports the view that the Treg-TCR possess high self-affinity but lower affinity of negative selection as well (Hsieh et al., 2006).

In case of pTreg cells, when compared with the expanded Foxp3$^-$ cells, pTreg cells differentiated from Foxp3$^-$ naive T cells in the lymphopenic mouse showed a quite distinct TCR repertoire. In addition, TCR of colon Treg cells was different from that of spleen or lymph node Treg cells (Lathrop et al., 2011). Given that low dose of high affinity-antigenic peptides induced pTreg cells most efficiently in vivo (Gottschalk et al., 2010), TCR of pTreg cells are likely to have high affinity to the exogenous antigens. pTreg cells are differentiated in response to strong TCR signalling and suboptimal co-stimulation. It has been reported that induction of Foxp3 in naive CD4 T cells of periphery requires TGFβ and IL-2. TGFβ activates smad3 that binds to the conserved non-coding enhancer of Foxp3 locus (CNS1) to induce Foxp3.

1-5. Foxp3 enhancer usage in Treg cell differentiation

The enhancer elements of Foxp3 gene have functions on the differentiation and maintenance of Foxp3 expression. Upstream region of Foxp3 gene locus is composed of basal promoter and conserved non-coding sequences. CNS1 also provides the site for binding of nuclear receptor of retinoic acid. Two-retinoic acid nuclear receptor RAR/RXR binds to CNS1 and promotes

Foxp3 gene expression. Notably, deletion of CNS1 led to decrease of frequency of Treg cells in periphery, and pTreg cell development along the administration of oral antigen was impaired and Th2 type inflammation was developed. This result indicates the pivotal role of CNS1 in pTreg cell differentiation (Josefowicz et al., 2012). In case of the CNS2 (conserved non-coding enhancer element 2) in Foxp3 locus which is also known as the TSDR (Treg-specific demethylated region), it has been reported that the stability of Foxp3 expression is dependent on its CpG methylation status. The CpG of TSDR in Foxp3 locus is specifically demethylated in Treg cells, which is directly responsible for stable expression of Foxp3 (Zheng et al., 2010). In addition to the demethylation of TSDR of Foxp3, the result of methylated DNA immunoprecipitation sequencing (MeDIP-seq) and bisulfite sequencing revealed that Treg cell-dominant CpG hypomethylation is observed in Treg-associated genes such as Il2ra, Ctla4, Tnfrsf18(GITR), Ikzf4(EOS), Ikzf2(Helios)(Ohkura et al., 2012). pTreg cell differentiation can be mimicked in vitro by inducing $Foxp3^+$ Treg cells from naive $CD4^+$ T cells with TCR stimulation and TGFβ cytokine (Chen et al., 2003a; Davidson et al., 2007). Unlike nTreg cells generate in vivo, in vitro induced Treg cells by TGFβ cytokine show poor demethylation status of TSDR region, so that result in loss of Foxp3 expression along with restimulation in vitro, or cell proliferation in lymphopenic host. It has been reported that the deletion of TSDR resulted in loss of Foxp3 expression by cell division both

in vitro and in vivo. Activated STAT5 by IL-2 signal competes with STAT6 activated by IL-4 for the binding to the TSDR and maintains the stability of Foxp3 expression (Feng et al., 2014; Li et al., 2014). Recently, binding of C/EBPβ to the CRE sequence of methylated TSDR has been reported to sustain Foxp3 expression in the presence of inflammatory cytokine (Lee et al., 2018). Chromatin organizer Satb1 functions as a pioneering factor in Treg cell differentiation. Satb1 expressed on double positive (DP) and immature CD4 thymocytes, preceding the Foxp3 expression, binds to CNS0 and CNS3 to induce tTreg cell differentiation. Satb1 activates Treg cell-lineage specific super enhancer of Treg cell specific genes (Foxp3, CTLA4, IL-2ra, IKZF2) by mediating H3K27 acetylation. Collectively, these studies strongly suggest that expression of Foxp3 and other Treg cell-specific genes are under epigenetic control of cis-element during the Treg cell differentiation.

1-6. Regulatory cells in intestine

Gastro intestinal tract is the site that the exogenous antigens derived from food, commensal microbes, and pathogens are first encountered. Intestinal lamina propria is constituted by various immune cells recruited include IgA-secreting plasma cells, γδ T cells, innate lymphoid cells, and Th17 cells. Host is defended from the pathogens by the cooperation of these cells. Because such continuous exposure to the external antigens in intestine can

cause chronic immune responses, strict regulation of immune responses is required to prevent the intestinal inflammation and tissue damage. pTreg cells are primarily responsible for this regulation of excessive immune responses. Regulatory T cells constitute more than 30% of colonic lamina propria immune cells.

B cells and plasma cells in intestine express IgA, which limits the antigen access to the blood and controls the microbiota of intestine (Li and Rudensky, 2016; Pabst, 2012). The development of IgA secreting cells is greatly dependent on microbiota. In germ- free mice, the expression of IgA in plasma cells and B cells in intestine is greatly reduced, and colonization of the microbe promotes IgA production rapidly (Kawamoto et al., 2014). A subset of innate lymphoid cells (ILCs) play an inhibitory role in the intestine. ILC-reg is induced in response to inflammatory stimulation, and suppresses innate immune responses by expressing IL-10 (Wang et al., 2017). Tr-1 cells are a subset of CD4 T cells characterized by IL-10 expression and comprise main suppressive cell population in intestine (Barnes and Powrie, 2009). Previous studies reported that conditional deletion of IL-10 on CD4 T cells result in spontaneous colitis while IL-10 KO Treg cells can prevent colitis. These results indicate Tr-1 cells, not Treg cells, also contribute to immune homeostasis in intestine (Murai et al., 2009; Roers et al., 2004). However, conditional deletion of IL-10 on Treg cells also showed aged-dependent colitis (Rubtsov et al., 2008), and deficiency in Treg cells led systemic

autoimmune disease including fatal enteropathy (Lahl et al., 2007). Further, increased susceptibility to intestinal inflammation in the absence of pTreg cells (Ohnmacht et al., 2015; Sefik et al., 2015) means that the most important population in intestinal homeostasis is Treg cells.

1-7. Peripherally derived regulatory T cell (pTreg) differentiation and immune regulation in intestine

In the intestine, pTreg cells constitute up 50 to 70% of $Foxp3^+$ Treg cells. Induction of pTreg cells in intestine is largely dependent on the diet-derived antigens in small intestine and microbes-derived antigens in the colon. This antigen-dependent differentiation of Treg cells is proven by analysing the small intestine of mice that were fed antigen-free diet and the colon of mice that were bred in germ-free facility (Kim et al., 2016; Ohnmacht et al., 2015; Sefik et al., 2015).

In the colon, it has been reported that a specific species of microbiota, Clostridia, and its metabolite short chain fatty acid leads to a strong induction of $helios^-$ pTreg cells in colon (Atarashi et al., 2013; Atarashi et al., 2011; Furusawa et al., 2013). It has been long believed that Clostridia colonization increased the expression of TGFβ and indoleamine 2, 3-dioxygenase (IDO) on intestinal epithelial cells (IECs), leading induction of pTreg cells. However, the later report demonstrated that Bacteroidetes fragilis in germ-free mice showed the substantial pTreg cell induction (Sefik

et al., 2015). Furthermore, polysaccharide A (PSA) of Bacteroidetes fragilis (Round and Mazmanian, 2010) is demonstrated to be potent to induce IL-10 producing Treg cells in colon as well.

It has been recently reported that CX_3CR1^+ macrophages uptake the soluble luminal antigen and transfer it to $CD103^+$ dendritic cells (Mazzini et al., 2014). These CX3CR1 macrophages are required to induce the pTreg cell differentiation per se via secreting IL-10 in small intestine (Kim et al., 2018). $CD103^+$ dendritic cells preferentially promote the pTreg cell differentiation in the intestine as well. In the mice study, $CD103^+$ dendritic cells (DCs) express αvβ8 integrin to convert TGFβ into active form (Travis et al., 2007). In the intestine, the expression of retinal dehydrogenase (ALDH) is strictly regulated and its expression is specifically high in $CD103^+$ dendritic cells. Since ALDH metabolizes vitamin A into retinoic acid, DCs can induces the pTreg cell differentiation in TGFβ and RA-dependent manner (Coombes et al., 2007; Hall et al., 2011; Sun et al., 2007). Moreover, IDO metabolizes tryptophan into kynurenine, which has been reported to promote the pTreg cell differentiation through aryl hydrocarbon receptor (2010 JI). Cytotoxic T-lymphocyte-associated protein 4 (CTLA4), a co-inhibitory molecule, which is highly expressed on Treg cells binds to CD80/86 on the surface of DCs to increase the IDO expression.

Inhibition of AKT signaling promotes pTreg differentiation as well. When PI3K-AKT-mTOR signaling is inhibited, Treg cell differentiation is

markedly increased de novo and in vitro in response to TGFβ. Moreover, the retroviral gene transduction of constitutive active form of AKT or deletion of PTEN substantially inhibits the Treg cell differentiation. These results indicate that AKT-mTOR activation via PI3K downstream of TCR signaling pathway involved in Treg cell differentiation in the negative way (Haxhinasto et al., 2008; Sauer et al., 2008). On the basis of these studies, an experimental study of pTreg cell differentiation associated with the akt signal was reported (Francisco et al., 2009). Programmed cell death protein1 (PD-1)-programmed death-ligand 1 (PD-L1) signaling promote the pTreg cell differentiation and conduct a pivotal role in sustaining the function of pTreg cells by antagonizing AKT-mTORC pathway while activating PTEN. OX40 signaling has an independent role in peripheral differentiation of Treg cells. OX40-deficient conventional T cells are less efficiently differentiated into $Foxp3^+$ Treg cells. Similarly, the forced expression of OX40L on the antigen presenting cells abrogated Treg cell differentiation in vitro considerably (Vu et al., 2007). As a result, it appears that OX40-OX40L signaling inhibits the pTreg cell differentiation.

pTreg cells are responsible in regulation of inflammation in mucosal interfaces, especially in the intestine. Through the deletion of CNS1, a conserved enhancer of Foxp3, a study demonstrated that pTreg cells are involved in the control of th2 type gastrointestinal pathology (Josefowicz et al., 2012). pTreg cells induced by colon microbiota express rorgt, a nuclear

receptor family of transcription factors, and specific deletion of rorgt in Treg cells led to impairment in suppression of gut inflammation (Ohnmacht et al., 2015; Sefik et al., 2015). A study showed that deficiency of Treg cells in mice could be completely recovered by transferring not only nTreg cells but also naive CD4 T cells as a source of the pTreg cell differentiation. Interestingly, specific deletion of pTreg cells in this recovered mice resulted in the sharp weight loss and the inflammatory infiltration of immune cells into lung, liver and intestine (Haribhai et al., 2011). Although the gene expression profile of nTreg cells and that of pTreg cells were similar, CDR3 sequencing of TCR beta variable gene revealed that TCR repertoire of nTreg and pTreg cells were mostly unique and nonoverlapping. These results indicate that pTreg cells are essential in control of inflammation at mucosal site, especially at the intestine by means of its TCR specificity.

1-8. CCAAT enhancer-binding protein β

CCAAT enhancer binding proteins (C/EBPs) are the family of transcription factors that are composed of C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ. They bind to CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine) box motif via their leucine zipper domain in the form of homo- and hetero dimerization (Tsukada et al., 2011). C/EBPβ is broadly expressed in many cells of multiple organs. A broad expression of C/EBPβ has been reported in hepatocyte, hepatoma cell, adipoblast cell line, human peripheral

monocyte and myeloid leukemia cells. Especially, C/EBPβ is expressed in myelomonocytes and during differentiation into macrophages but not granulocytes (Scott et al., 1992). In accordance with the previous studies, C/EBPβ expression is specifically high in GMPs of mice, and analysis of C/EBPβ$^{-/-}$ mice showed its reduced granulocyte differentiation independently from C/EBPα (Hirai et al., 2006). In addition, C/EBPβ$^{-/-}$ mice showed the impaired bacteria killing and the tumor cytotoxicity by macrophages (Screpanti et al., 1995). Collectively, it seems clear that C/EBPβ is involved in myeloid lineage cell differentiation and function. C/EBPβ has functions on female fertility, and adipocytes as well. C/EBPβ deficiency results in defect of female reproduction, proliferation and differentiation of mammary gland epithelial cell, ductal morphogenesis and lobuloalveolar differentiation in the mammary gland (Robinson et al., 1998; Seagroves et al., 1998; Sterneck et al., 1997), and adipocyte genesis and differentiation (Tanaka et al., 1997; Tang et al., 2003). It is well known that C/EBP family including C/EBPβ are implicated in regulating growth arrest, thus the forced expression can inhibit cell proliferation. One report verified that C/EBPβ deficiency resulted in the reduced hepatocyte proliferation in response to hepatectomy (Greenbaum et al., 1998). On the other hand, the other study reported that C/EBPβ expression and hepatocyte proliferation are mutually exclusive during postnatal liver development, and the forced expression of C/EBPβ on human hematoma cell line arrested cell cycle at

the boundary of G1/S phase. Moreover, C/EBPβ$^{-/-}$ mice showed splenomegaly, lymphoproliferative disease (Buck et al., 1994; Screpanti et al., 1995). In keratinocyte, C/EBPβ expression was increased in growth arrest condition of primary keratinocytes and the forced expression of C/EBPβ inhibited the cell growth. The subsequent study demonstrated that C/EBPβ-deficient primary keratinocytes were resistant to the growth arrest and accompanied the epidermal hyperplasia. These results implicate that C/EBPβ functions as inhibitor of cell proliferation at least in the context of forced expression on cells.

Notably, C/EBPβ specifically interacts with SWI/SNF chromatin remodeling complex and this interaction is essential for exerting its function of activating target genes (Kowenz-Leutz and Leutz, 1999). Lastly, the recent report showed that C/EBPβ expression is increased by retinoic acid and C/EBPβ maintained the Foxp3 expression in inflammatory environments by binding to the methyl-CRE sequence in TSDR of Foxp3 locus (Lee et al., 2018). However, the regulating cell proliferation character of C/EBPβ on Treg cells remains to be elucidated.

MATERIALS AND METHODS

Materials and methods

Mice

Scurfy mice (sf), **CD45.1 SJL** mice, and **DEREG** ($Foxp^{DTR/EGFP}$) mice were purchased from the Jackson laboratory (Bar Harbor, ME). **$Rag2^{-/-}$ mice** were purchased from Taconic Farms Inc. (New York). C57BL/6 mice were purchased from Charles River Laboratories. **$Foxp3^{EGFP}$ mice** were kindly provided by Talal A. Chatila (The David Geffen School of Medicine at the University of California at Los Angeles). All mice were bred and maintained in specific pathogen-free condition facility of the Institute of Molecular Biology and Genetics at Seoul National University and experiments were performed in protocols approved by the Institutional Animal Care and Use Committees (IACUC) of Seoul National University.

Flow cytometry and cell sorting

Single cell suspensions were prepared from spleen, lymph node, bone marrow (femur and tibia), and colon lamina propria and were stained with antibodies in PBS. Stained cells were analyzed by FACSCanto II or collected by FACSAria II (BD Biosciences).

For intracellular staining, surface-stained cells were fixed and permeabilized with cytofix/permeabilization solution (BD Biosciences) or Foxp3 Fix/Perm

Kit (eBioscience) following the manufacturer's instruction. Cells were washed with 1×permeabilization buffer twice and then stained with mAbs diluted in permeabilization solution for 0.5-1hr at 4°C in the dark. Stained cells were washed twice with permeabilization solution and were resuspended in PBS and then were analyzed by a FACSCanto II. All Data were analysed with FACS Diva and Flowjo software. To confirm cytokine-producing cells, mice were injected intraperitoneally with brefeldin A (eBioscience) at 250 μg/mouse, and analyzed 6 h later by flow cytometry.

Abs against the following molecules were used in all experiments; B220 (RA3-6B2), CD11b (M1/70), CD43 (S7), Flt3 (12B6), CD24 (M1/69), BP-1 (6C3), IgM (R6-60.2), FcγRIII (93), Gr-1 (RB6-8C5), Ter119 (Ly76), Lin-, c-Kit (ACK2), Sca-1 (D7), IL-7Rα (A7R34), Foxp3 (FJK-16a), CD25 (PC61.5), CD34 (RAM34), Annexin V, CD45.1 (A20), CD3e (145-2C11), TCRβ (H57-597), IFNγ (B27), TNFα (MP6-XT22), IL-17a (TC11-18H10), IL-10 (JES5-16E3), and CD45.2 (104).

In vitro differentiation of BM cells and iTreg cells

OP-9 stromal cells received 3000 cGy γ-irradiation and were seeded in 24-well plates at 6.6×10^3 cells/well in αMEM supplemented with 20% FBS. After 1 day, the medium was changed to RPMI1640 supplemented with 10% FBS, and 1×10^4/well Lineage$^-$Sca1$^+$c-kit$^+$ (LSK) bone marrow cells purified from 5-week-old C57BL/6, scurfy, K/B or K/Bsf mice were added.

To provide B-cell differentiation conditions, cells were cultured in the presence of IL-7, SCF, and Flt3L (all at 20 ng/mL; all purchased from Peprotech) for a total of 12 days. In some experiments, Treg cells or Teff cells that had been preactivated for 3 days with 10 μg/mL anti-CD3 mAb, 1 μg/mL anti-CD28 mAb, and 20 U/mL IL-2 were added to the culture of LSK plus OP-9 cells. Transwell membranes with 0.4 μm pores were used to physically separate the T cells from LSK cells, or alternatively supernatants of cultures of activated Teff cells were added to the LSK culture, making up to 10% of the total culture volume. To assess the effects of cytokines, several cytokines such as IL-6, IL-17, IFN-γ, TNF, and GM-CSF (all at 10 ng/mL; all purchased from Peprotech) were added to the LSK culture. To neutralize cytokines in the LSK culture, anti-IL-6 mAb, anti-TNF mAb, anti-GM-CSF mAb, or a mixture of all mAbs (all at 50 μg/mL; all purchased from BioLegend) were added to the LSK culture.

For induction of regulatory T cell *in vitro*, $CD4^{+}CD25^{-}CD44^{-}Foxp3^{-}$ T cells were sorted from the spleen and mesenteric lymphnodes of $Foxp3^{eGFP}$ mice, and cultured in RPMI medium suppled with 10% FBS, 2mM glutamine, 100U/ml penicillin, 100ug/ml streptomycin, and 55uM 2-mercaptoethanol. in the presence of 5ng/ml rTGFβ (7666-MB-005/CF, R&D system), anti-IL-4 (11B11, 3ug/ml), anti-IFNγ (XMG1.2, 3ug/ml), anti-CD28 (37.51, 1ug/ml) and plate coated anti-CD3e(2c11, 3ug/ml).

Bone marrow transplantation

Bone marrow donor cells were prepared from CD45.1^{+} congenic mice and CD45.2+ scurfy mice. Donor cells were labeled with biotinylated anti-CD3ε (145-2C11; BD Bioscience), followed by negative depletion with anti-biotin MACS columns (Miltenyi Biotec). 6-week-old Rag2$^{-/-}$ recipient mice were sublethally irradiated with 5 Gy gamma-irradiation (^{137}Cs source, *NCIRF*) before the transplantation and were injected intravenously with 2 × 10^{6} donor cells from CD45.1 congenic mice, 2 × 10^{6} cells from CD45.2 scurfy mice, or a mixture of both bone marrow cell populations in a 1:1 ratio. At 6 weeks after transplantation, mice were sacrificed and the bone marrow cells were analyzed by flow cytometry.

In another experiment, 3 ×10^{6} CD4^{+}CD25^{+} Treg cells and the same number of CD4^{+}CD25^{-} T cells were sorted from CD45.1 congenic mice and mixed with whole bone marrow cells from CD45.2 scurfy mice. Mixed cells were intravenously injected into CD45.2 Rag$^{-/-}$ mice. Recipient mice were sacrificed three weeks later and bone marrow cells were analyzed flow cytometry.

Regulatory T cell reconstitution

CD4^{+}CD25^{+} regulatory T cells were sorted from the spleen and mesenteric lymph node of CD45.1 congenic mice and 3 ×10^{6} cells were injected into the ophthalmic vein of CD45.2 scurfy mice. The mice were sacrificed 3

weeks later and analyzed by flow cytometry.

$CD4^+CD45RB^{high}$ $Rag^{-/-}$ inflammatory bowel disease model.

To induce colitis model, 5 x 10^5 FACS-sorted $CD4^+CD25^-CD45RB^{high}$ cells from CD45.1 congenic mice were transferred into C57BL/6 *Rag2*$^{-/-}$ recipient by i.v with or without FACS-sorted $CD4^+$ GFP^+NGFR^+ cells (2 × 10^5) infected with control (NGFR) or C/EBPβ -encoding retrovirus and cultured for 2 days in the presence of TGF-β. Mice were monitored weekly and sacrificed at 11 weeks after transfer for analysis. For histological analysis, part of colons were fixed with 10% neutral-buffered formalin and then paraffin-embedded sections were stained with haematoxylin and eosin (H&E). For flow cytometric analysis, 1cm-size-cut colon were incubated with EDTA suppled RPMI and followed by tissue dissociation with collagenase buffer. Immune cells were collected by percoll gradient centrifugation. Prepared lamina propria cells were intracellular stained with Foxp3-APC, IL-17a-PE, IFNγ-APC, IL-10-APC, IL-4 PE antibodies. Cells were incubated at 37'C for 4hr in 10%FBS RPMI media with 10ng/ml PMA, Ionomycin 500ng/ml and brefeldin A for cytokine detection. Samples were analysed by FACS Canto II (BD)

Histological assessment of colon inflammation

For histological analysis, middle part of colon were fixed in 10% neutral-

buffered formalin solution. 6-μm paraffin-embedded sections were stained with haematoxylin and eosin (H&E). Histopathology was scored by a pathologist in a blinded fashion as a combination of intestinal inflammation (score 0–5), neutrophil infiltration (score 0–4), and gland epithelial hyperplasia (score 0–4).

Cell culture and retroviral transduction

Phoenix cells were maintained in DMEM (WelGENE) supplemented with 10% FBS (Gibco), 100U/ml streptomycin and penicillin. Naïve T cells were cultured in RPMI-1640 (WelGENE) supplemented with 10% FBS (Gibco), 100U/ml streptomycin and penicillin. Mock vector or C/EBPβ-expressing vector was transient transfected into phoenix cells by calcium phosphate transfection method following the manufacturer's instruction. After 48hr, retroviral supernatants were harvested and added 8μg/ml polybrene. Activated T cells were spin-infected with retroviral supernatant for 1hr at 2100 RPM in room temperature.

Intracellular Foxp3 staining

Before intracellular staining of Foxp3, prepared cells were stained by CD4 and CD25 flow cytometric antibodies and Stained cells washed once with PBS. For intracellular staining of Foxp3, the cells were resuspended in 1 ml of freshly prepared fixation/ permeabilization working solution and

incubated 30 minutes at 4°C in the dark. They were washed once in FACS buffer and resuspended in 2 ml of 1× permeabilization buffer. Cells were washed with 1×permeabilization buffer once more. 1 μg/tube of FITC anti-mouse Foxp3 was added to the appropriate cell groups and incubated for 1 hour at 4°C in the dark. The cells were washed twice with 2 ml 1×permeabilization buffer and were resuspended in PBS.

Diphtheria toxin depletion

Mice induced intestinal inflammation with CD4+CD45RB^{high} cells of DEREG mice were given 50 mg/kg of diphtheria toxin (SigmaAldrich) in 200ml of PBS by intra-peritoneal injection every other day for 10 days. Mice were weighed daily, before each injection when appropriate. After the last injection, mice were rested for 2 days and then examined.

Quantitative RT-PCR

RNA was extracted from the sorted populations by Trizol reagent (Invitrogen) according to the manufacturer's instruction. RNA was reverse transcribed with SuperScript III (Invitrogen) or QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was conducted using StepOnePlus (Applied biosystems) with 2X SYBR Green Master mix (Applied biosystems or Thermo Fisher). The sequences of the primers were as follows: TGFβ, forward: 5'- CTCCCGTGGCTTCTAGTGC-3' and

reverse: 5'-GCCTTAGTTTGGACAGGATCTG-3',

OX-40L, forward: 5'- AGGACCCTCCAATCCAAAGAC-3' and reverse: 5'-GAGCTGATGAATAGTTGCCCAT-3',

CTLA4, forward: 5'-TTTTGTAGCCCTGCTCACTCT-3' and reverse: 5'-CTGAAGGTTGGGTCACCTGTA-3', and

PD-L1, forward: 5'- ACAAGCGAATCACGCTGAAAG -3' and reverse: 5'-GGCCTGACATATTAGTTCATGCT-3', and

β-actin, forward: 5'-GGCTGTATTCCCCTCCATCG-3' and reverse: 5'-CCAGTTGGTAACAATGCCATGT3'. β-actin was used as an internal control for RT-PCR analysis.

Statistical analysis

Statistical analysis was performed by Prism software (GraphPad). Two-tailed Student's *t-tests* were used to calculate p values and p values <0.05 were considered statistically significant.

RESULTS

Preface

The contents of this chapter 3-1 was published **in *European Journal of Immunology*** on January 2015 as follows.

Kim, S*., **Park, K***., Choi, J., Jang, E., Paik, D.J., Seong, R.H., and Youn, J. (2015). Foxp3+ regulatory T cells ensure B lymphopoiesis by inhibiting the granulopoietic activity of effector T cells in mouse bone marrow. European Journal of Immunology *45*, 167-179.

Part.1

Foxp3$^+$ regulatory T cells ensure B lymphopoiesis by inhibiting the granulopoietic activity of effector T cells in mouse bone marrow

Impaired B cell development in Foxp3-deficient mice

Scurfy mice have a Foxp3 gene mutation and do not generate regulatory T (Treg) cells, so they have severe multi-organ inflammation. It has been reported that regulatory T cells participated in early hematopoiesis and suppress neutrophil generation. Also previous study has shown that B cell differentiation is altered in Foxp3-deficient mice. To gain insight on the role of Foxp3$^+$ Treg in general mouse bone marrow (BM) hematopoiesis, I analyzed bone marrow cell population in scurfy mice by flow cytometry. In bone marrow of scurfy mice, B220$^+$ cells and Ter-119$^+$ erythroid cells were severely reduced, while on the other hand, Gr-1$^+$Mac-1$^+$ myeloid cells were dramatically increased (Figure 4A). I further analyzed B-lineage cells by developmental stage and found that most populations of B cells including Pro B cells (B220^{lo}CD43$^+$IgM$^-$), pre B cells (B220^{lo}CD43$^-$IgM$^-$), immature B cells (B220^{lo}CD43$^-$IgM$^+$), and mature B cells (B220^{hi}CD43$^-$IgM$^+$) were reduced (Figure 4B). To check the pro B to pre B cell maturation state, CD43 expression was verified in B220$^+$IgM$^-$ cells. As a result, in scurfy mice, the percentage of CD43$^+$ pro-B cells were increased

while that of $CD43^-$ pre-B cells were lower than control mice (Figure 4C). This result indicates that developmental arrest at pro B cell stage occurred in scurfy mice. Then, pro B cell stage was further subdivided into pre-pro-B (also referred as Fr.A; $B220^+CD43^+CD24^-BP\text{-}1^-$), early-pro-B (Fr.B; $B220^+CD43^+CD24^+BP\text{-}1^-$), and late-pro-B (Fr.C; $B220^+CD43^+CD24^+BP\text{-}1^+$) cells. A significant developmental arrest was observed in pre-pro-B cell stage in scurfy mice (Figure 4D).

To determine whether B lymphopenia was due to early developmental problems of B-cell lineage, progenitor cells in bone marrow of 3-week-old scurfy mice were analyzed. In normal hematopoiesis, B lineage cells go through the path first from long-term hematopoietic stem cells (LT-HSCs) into multipotent progenitors (MPPs), followed by lymphoid-primed multipotent progenitors (LMPPs) into common lymphoid progenitors (CLPs). Flow cytometry analysis showed that LT-HSCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^-Flt3^-$) and MPPs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^+Flt3^-$) were increased, while LMPPs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^+Flt3^+$) and CLPs ($Lin^-IL\text{-}7R\alpha^+Sca\text{-}1^{lo}c\text{-}Kit^{lo}$) were dramatically reduced in bone marrow of scurfy mice (Figure 5A-5C). These results suggested that B cell development in Foxp3-deficient mice was impaired from the most primitive progenitors, LMPPs.

Because MPPs further differentiate into LMPPs or common myeloid progenitors (CMP), I analyzed scurfy bone marrow to determine whether CMP populations were changed. As a result, the cellularity of CMP

population in scurfy mice were found to be much lower than that of control mice (Figure 5D). This result indicated that developmental arrest at the ST-HSCs and MPPs in Foxp3 deficient mice also blocked the differentiation of CMP as well as LMPPs. CMPs further differentiate into granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs). Intriguingly, despite the decrease of CMPs, GMPs in Foxp3 deficient mice were much more increased than that of control mice, while MEPs were decreased (Figure 5D). Taken together, these results demonstrated that Foxp3 deficiency led to altered hematopoiesis and resulted in impairment of B cell development.

Impaired B lymphopoiesis is caused by Treg cell deficiency

Previous studies indicated that non-Treg cells, such as mesenchymal stromal cells, thymic stromal cells, and mammary cells, also express the Foxp3 gene. So, I checked whether impairment of hematopoiesis and B cell development in Foxp3-deficient mice was due to absence of Treg cells or not. $CD4^+CD25^+$ Treg cells were obtained from the spleen and lymph node of $CD45.1^+$ congenic mice, and injected into $CD45.2^+$ scurfy mice. Three weeks later, the mice were analyzed. As a result, $B220^+$ B cells were restored, arrested pre-pro B cells were released, and pre-B and pro-B cells were regenerated (Figure 6A and 6B). Moreover, CLPs were restored and developmental arrest at early hematopoietic lineage was resolved as well

(Figure 6C). Therefore, these results suggested that Treg cells are needed for proper regulation in hematopoiesis, especially in B cell development. Next, to investigate whether perturbed hematopoiesis was due to the intrinsic or extrinsic role of Treg cells, I conducted mixed bone marrow transplantation (Figure 7A). First, I used T-cell depleted whole bone marrow for transplantation to ensure that the defect of B cell development could be rescued by intact whole bone marrow cells. B cell development was failed in $Rag^{-/-}$ recipient mice that were transferred of scurfy BM cells (Figure 7B and 7C). However, in recipient mice that were transplanted with a mixture of BM cells of congenic $CD45.1^{+}$ mice and BM cells of $CD45.2^{+}$ scurfy mice in a 1:1 ratio, not only the $CD45.1^{+}$ cells but also the $CD45.2^{+}$ cells from scurfy mice could generate B lineage cells (Figure 7C and 7D). This result indicated that B lymphopenia in scurfy mice is not due to an intrinsic defect in BM progenitors but some extrinsic factors derived from BM cells. Next, to test whether rescue of B cell development is caused by Treg cells among the whole BM cells, I analyzed mixed BM chimera mice that were transferred with a mixture of $CD45.2^{+}$ scurfy BM cells and $CD45.1^{+}$ $CD4^{+}CD25^{+}$ Treg cells or $CD4^{+}CD25^{-}$ cells (Figure 8A). As a result, only the recipient mice that were transferred with the scurfy BM cells along with Treg cells showed rescued B cell development (Figure 8B). Thus, Treg cells were demonstrated to be sufficient to ensure that Foxp3-deficient BM cells

Figure 4. B cell developmental impairment in scurfy Foxp3-deficient mouse.

(A) Representative flow cytometric analysis of the bone marrow from scurfy mice and littermates. Numbers show the relative percentage of cells within the live bone marrow cell gate. Erythrocytes are defined as Ter119 positive cells and B cells are defined as B220 positive cells.

(B) Pro-B and pre-B cells are defined as $B220^{+}$ and IgM^{-}. Immature B cells are defined as $B220^{+}IgM^{+}$, and mature recirculating B cells defined as $B220^{hi}IgM^{+}$.

(C) Numbers show the relative percentage of cells within the pro-B and pre-B cell gate. Pro-B cells are defined as $B220^{+}CD43^{+}$ and pre-B cells are defined as B220+CD43-.

(D) Numbers show the relative percentage of cells within the B220+CD43+ pro-B cell gate. Pre-pro B cells (Fr. A) are defined as $CD24^{-}BP\text{-}1^{-}$, early pro-B cells are defined as $CD24^{+}BP\text{-}1^{-}$, and late pro-B cells are defined as $CD24^{+}BP\text{-}1^{+}$. Data are representative of five independent mice set.

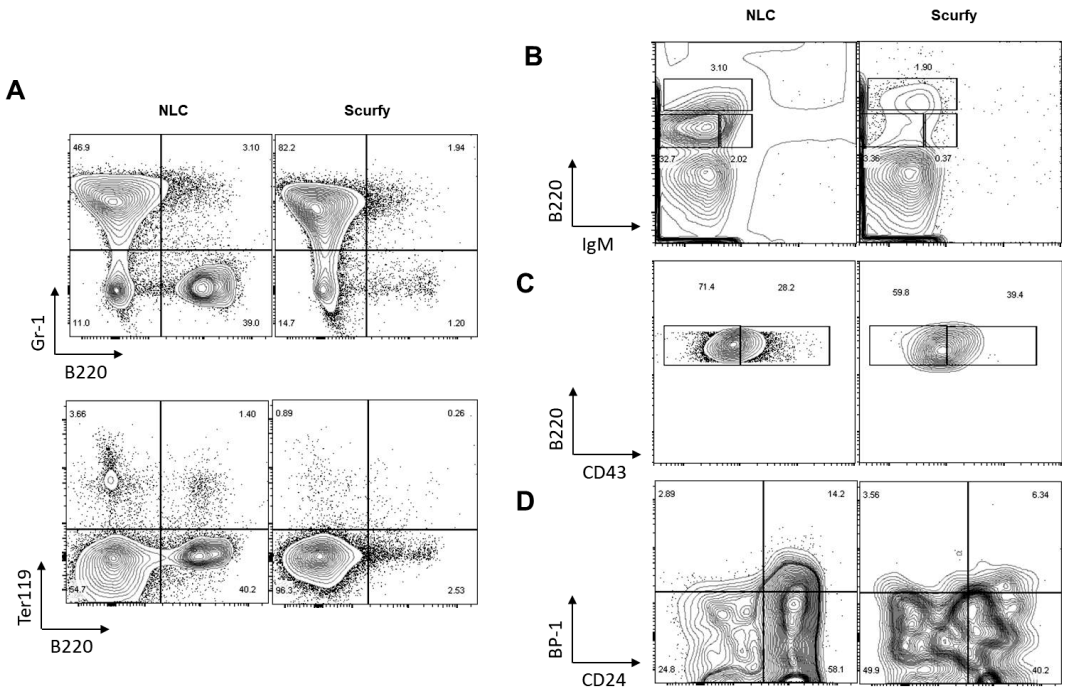

A
NLC
Scurfy
46.9
3.10
11.0
39.0
82.2
1.94
14.7
1.20
Gr-1
B220
3.66
1.40
54.7
40.2
0.89
0.26
96.3
2.53
Ter119
B220
B
NLC
Scurfy
3.10
32.7
2.02
1.90
0.36
0.37
B220
IgM
C
71.4
28.2
59.8
39.4
B220
CD43
D
2.89
14.2
24.8
58.1
3.56
6.34
49.9
40.2
BP-1
CD24

Figure 5. B cell development is blocked at the early stage of hematopoiesis in the scurfy mice.

(A) Representative flow cytometric analysis of the bone marrow from scurfy mice and normal littermates. Numbers show the relative percentage of cells within the $Lin^{-}IL\text{-}7R\alpha^{+}$ cell gate. The part shown in line represents the CLP population.

(B) Data show relative percentage of cells within the Lin- cell gate. The part shown in line represents LSK population.

(C) Numbers show the relative percentage of cells within the Lin-Sca1+c-Kit+ cell gate. Long term hematopoietic stem cells are defined as $Lin^{-}Sca1^{+}c\text{-}Kit^{+}Flt3^{-}CD34^{-}$. Multipotent progenitors defined as $Lin^{-}Sca1^{+}c\text{-}Kit^{+}Flt3^{-}CD34^{+}$. Lymphoid primed multipotent progenitors (LMPPs) defined as $Lin^{-}Sca1^{+}c\text{-}Kit^{+}Flt3^{+}CD34^{+}$.

(D) Relative percentage of cells within the $Lin^{-}Sca1^{-}c\text{-}Kit^{+}$ cell gate are shown. Common myeloid progenitor cells are defined as $Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RIII^{-}$. Granulocyte-monocyte progenitor cells are defined as $Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RIII^{+}$. Megakaryocyte-erythrocyte progenitor defined as $Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{-}Fc\gamma RIII^{-}$. Data are representative of three to five independent mice set.

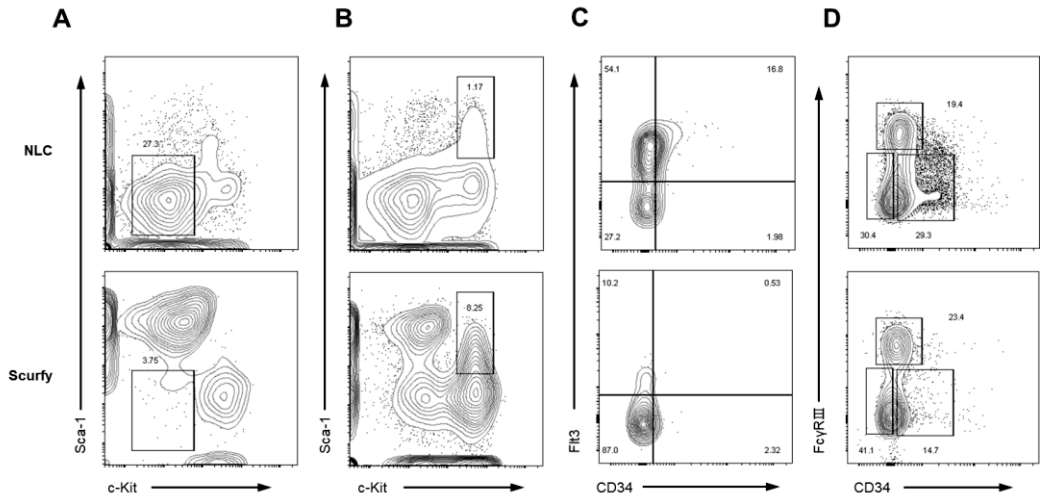

A
B
C
D
NLC
Scurfy
Sca-1
c-Kit
Flt3
CD34
FcγRIII
27.3
3.75
1.17
8.25
54.1
16.8
27.2
1.98
10.2
0.53
87.0
2.32
19.4
30.4
29.3
23.4
41.1
14.7

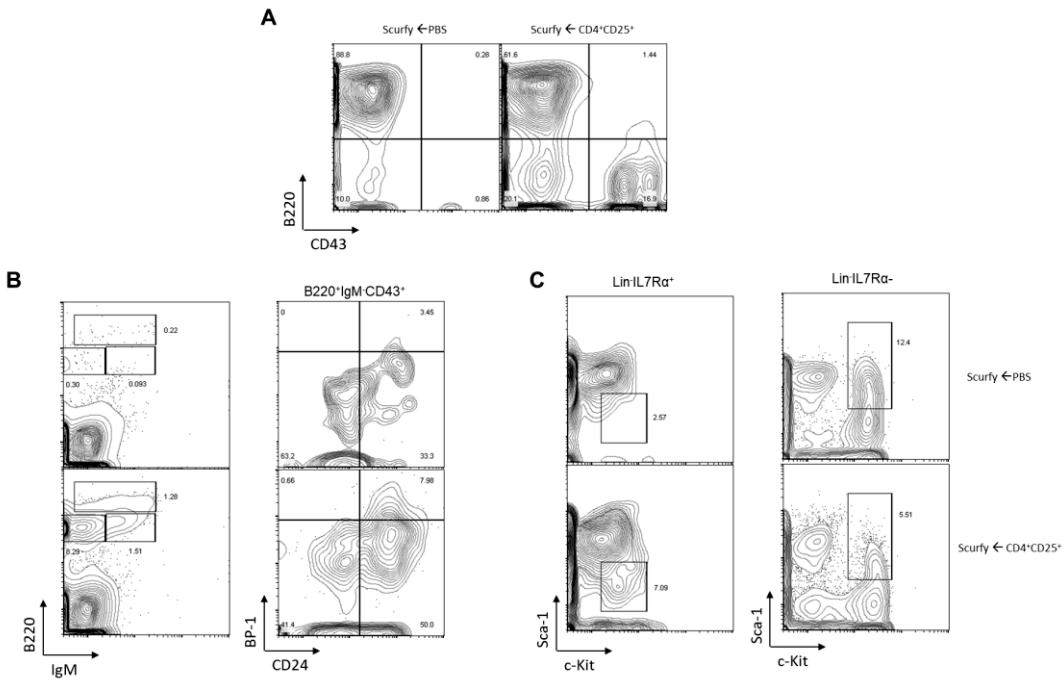
Figure 6. Regulatory T cells can restore B lymphopoiesis in scurfy bone marrow.

$CD4^+CD25^+$ Treg cells were sorted from the spleen and lymph node of CD45.1 congenic mice and injected into the ophthalmic vein of CD45.2 scurfy mice. Three weeks after injection, the mice were sacrificed and flow cytometric analysis of the bone marrow was carried out. Data are representative three independent experiments.

(A) Representative flowcytometric analysis of the bone marrow cells from recipient scurfy mice and controls. Numbers show the relative percentage of cells within the live cell gate. B cells are defined as $B220^+$, and myeloid cells are defined as $Gr\text{-}1^+$.

(B) Pro-B and pre-B cells ($B220^+IgM^-$), pre-pro B cells ($B220^+CD43^+CD24^-BP\text{-}1^-$), early pro-B cells ($B220^+CD43^+CD24^+BP\text{-}1^-$) and late pro-B cells ($B220^+CD43^+CD24^+BP\text{-}1^+$) are shown.

(C) Numbers show the percentage of cells within the Lin^-, IL-7Rα^+ cell gate on the left, and IL-7Rα^- cell gate on the right.


A
Scurfy ←PBS
Scurfy ← CD4+CD25+
88.8
0.28
10.0
0.86
61.6
1.44
20.1
16.9
B220
CD43
B
0.22
0.30
0.093
1.28
8.29
1.51
B220
IgM
B220+IgM-CD43+
0
3.45
63.2
33.3
0.66
7.98
41.4
50.0
BP-1
CD24
C
Lin-IL7Rα+
2.57
7.09
Sca-1
c-Kit
Lin-IL7Rα-
12.4
5.51
Sca-1
c-Kit
Scurfy ←PBS
Scurfy ← CD4+CD25+

generated B lineage cells normally. To sum up the data, it is appeared that impairment of B cell development in Foxp3-deficient mice is due to the absence of Treg cells and especially not by an intrinsic role of Treg but by some factors regulated from Treg cells.

B lymphopoiesis was protected by Treg cells via suppression of the Teff cells

I demonstrated that Treg cells play a critical role in B cell development by conducting Scurfy BM profile analysis and several bone marrow transplantation experiments. Next, to identify that Treg cells directly regulate B cell lineage differentiation, I used Treg cells for co-culture in in vitro B cell differentiation system. LSK cells ($Lin^{-}Sca1^{+}c\text{-}Kit^{+}$) purified from C57BL/6 mice were cultured on OP-9 bone marrow stromal cell line in the presence of IL-7, SCF, and Flt3L for 12 days with or without Treg cells. Regardless of the presence or absence of Treg cells, $B220^{+}CD19^{+}$ B cells were normally generated in LSK cells (Figure 9). This result implicated that Treg cells do not directly control the B cell lineage differentiation from the early progenitors. Also, it is well known that because Treg cells suppressed effector T cells (Teff cells) so that repress the excessive immune responses, Treg deficiency leads severe activation of effector T cells. In view of such character of Treg cells, activated

CD4+Foxp3– cells (referred Teff cells) were co-cultured with the LSK cells in B cell differentiation condition. As a result, B220+CD19+ B cells were dramatically reduced (Figure 9). Given the fact that Teff cells exert influence on immune response through direct contact or indirectly releasing the cytokine, I checked whether the suppression observed in co-culture is contact dependent or not. Despite the Teff cells were separated from the LSK cells by using trans-well systems, B cell differentiation was still blocked (Figure 9). In addition, adding the Teff cell culture medium could also suppress the generation of $B220^{+}CD19^{+}$ B cells (Figure 9). These data indicated that suppression mechanism of Teff cells on B cell development is contact independent. Strikingly, the suppressive effect of Teff cells on B cell development was fully restored by the adding the Treg cells. In conclusion, these data suggested that Treg cells protect B cell differentiation from the suppressive activity of soluble factors derived from Teff cells.

The cytokine milieu of Foxp3-deficient BM is different from that of normal BM

Based on my observations up to this point, I hypothesized that soluble factors produced by $CD4^{+}$ Teff cells are abundant in the milieu of Foxp3-deficient BM and perturb normal B lymphopoiesis. To test this idea, first I examined whether these cytokines are actually produced in higher levels in the BM of Foxp3-deficient mice than in the control mice. However, scurfy

Figure 7. Wild-type bone marrow cells induce the generation of B lymphoid lineage cells originating from scurfy mice

2×10^6 cells of CD45.1 bone marrow cells were mixed with same number of bone marrow cells obtained from CD45.2 scurfy mice. The mixture of bone marrow cells were injected into ophthalmic vein of CD45.2 $Rag^{-/-}$ mice. Six weeks later, recipient mice were sacrificed and analyzed. Data are representative of two independent experiments.

(A) Schematization of mixed bone marrow transplantation experiment.

(B) Representative flow cytometric analysis of intracellular stained bone marrow from recipient mice 6 weeks after injection. The part shown in the box on the left-hand side figure represents $Foxp3^+$ Treg cells derived from congenic mice. The part shown in the box on the right-hand side figure represents $Foxp3^+$ Treg cells derived from scurfy mice.

(C) Numbers show the relative percentage of cells within live bone marrow cells. B cells are defined as $B220^+$, and myeloid cells are defined as Mac-1^+.

(D) Representative flowcytometric analysis of the bone marrow cells from recipient mice. Immature B cells are defined as $B220^+IgM^+$ and mature recirculating B cells are defined as $B220^+IgM^+$, pro-B and pre-B cells ($B220^+IgM^-$), pre-pro B cells ($B220^+CD43^+CD24^-BP\text{-}1^-$), early pro-B cells ($B220^+CD43^+CD24^+BP\text{-}1^-$) and late pro-B cells ($B220^+CD43^+CD24^+BP\text{-}1^+$) are shown.

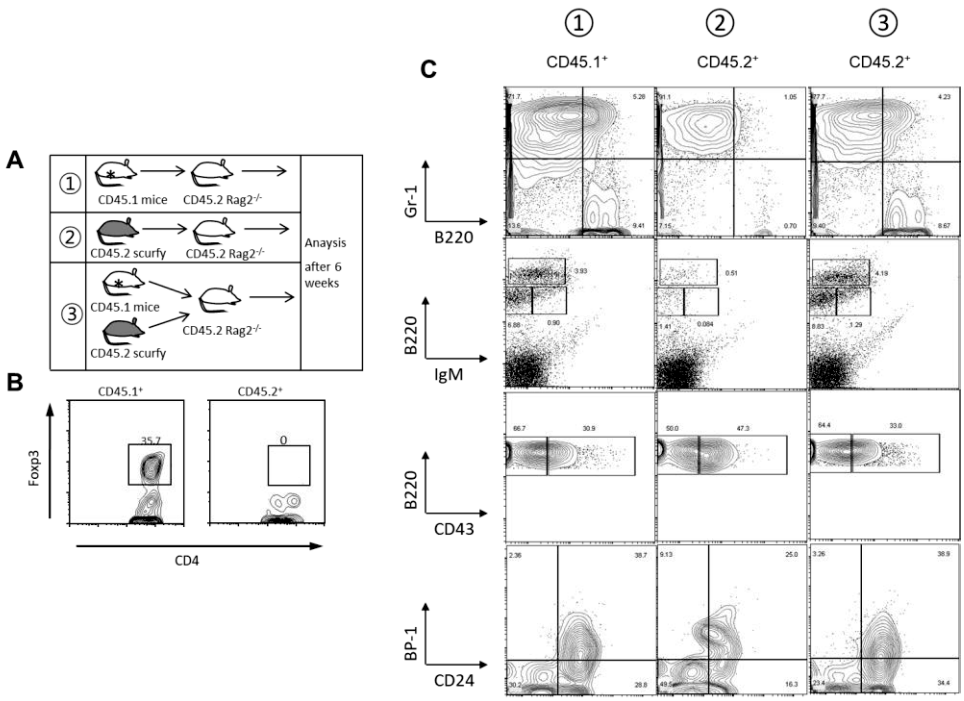
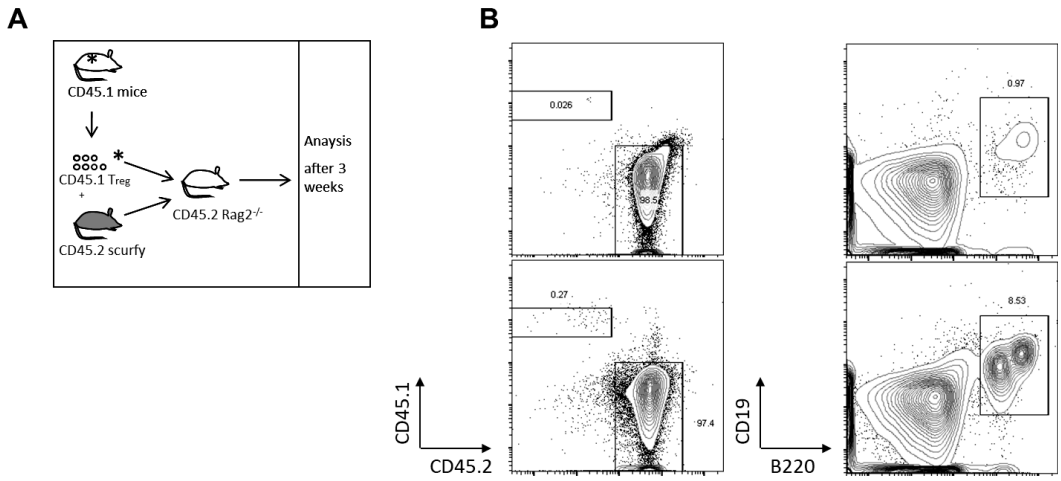

A
①
CD45.1 mice
CD45.2 Rag2-/-
②
CD45.2 scurfy
CD45.2 Rag2-/-
③
CD45.1 mice
CD45.2 Rag2-/-
CD45.2 scurfy
Anaysis after 6 weeks
B
CD45.1+
CD45.2+
35.7
0
Foxp3
CD4
C
①
②
③
CD45.1+
CD45.2+
CD45.2+
Gr-1
B220
B220
IgM
B220
CD43
BP-1
CD24

Figure 8. Regulatory T cells can restore B lymphopoiesis.

$CD4^+CD25^+$ 3 × 10^6 Treg cells and the same number of $CD4^+CD25^-$ T cells were sorted (MACS, >95% purity) from the spleen and lymph node of CD45.1 congenic mice. Sorted T cells were mixed with whole bone marrow cells of CD45.2 scurfy mice. Mixed cells were injected into the ophthalmic vein of CD45.2 $Rag^{-/-}$ mice. Three weeks after the injection, mice were sacrificed and flow cytometric analysis of the bone marrow was carried out. Data are representative of two independent experiments.

(A) Schematization of the mixed bone marrow transplantation experiment.

(B) Representative flow cytometric analysis of the bone marrow from recipient mice. The part shown in the box on the left-hand side figures represent the origin of the cell populations. $CD45.1^+$ rectangle shows mixed regulatory T cells or $CD4^+CD25^-$ T cells, respectively. $CD45.2^+$ rectangle shows bone marrow cells of scurfy mice. The part shown in the box on the right-hand side figures show regenerated $B220^+CD19^+$ B cells.


A
CD45.1 mice
CD45.1 Treg
+
CD45.2 scurfy
CD45.2 Rag2-/-
Anaysis
after 3
weeks
B
0.026
98.5
0.27
97.4
CD45.1
CD45.2
0.97
8.53
CD19
B220

mice on the C57BL/6 background (referred as to B6sf) usually succumb to extremely severe inflammatory symptoms at about 4 weeks of age, so there are practical difficulties associated with using them. To overcome these issues, I generated male KRN TCR transgenic C57BL/6 mice bearing a scurfy allele on the X chromosome (referred to as K/Bsf). As expected, K/Bsf mice exhibited much more modest symptoms of inflammation, and about 80% survived until at least 7 weeks of age by which time all our experiments were completed (data not shown). To answer the question, the cytokine production was analysed on BM cells of mice that were treated with brefeldin A were analysed by flow cytometry. Pro-inflammatory cytokine production by BM cells of K/B sf were higher than of K/B mice(Figure 10A and 10B). The TNF were produced by both of $CD4^+$ and $CD4^-$. IFNg was predominantly secreted by $CD4^+$ rather thatn $CD4^-$. On the other hands, IL-6 and GM-CSF were produced predominantly by CD4- rather than CD4+. In summary, K/B sf BM cells produced proinflammatory cytokines in higher levels than K/B BM cells.

IL-6, TNF, and GM-CSF promote in vitro granulopoiesis at the expense of B lymphopoiesis

In order to determine whether proinflammatory cytokines that expressed in higher levels in K/B sf BM than K/B BM affects B lymphopoiesis directly, each cytokines were treated into LSK cell culture of B cell-culture condition.

Pro-inflammatory cytokines blocked the $B220^{+}CD19^{-}$ and $B220^{+}CD19^{+}$ cells almost completely and induced Gr-1+ myelopoiesis at the same time even under B-cell differentiation conditions. (Figure 11). IFN-γ reduced the percentage of $B220^{+}$ $CD19^{+}$ cells but increased the percentage of $B220^{+}$ $CD19^{-}$, pointing to arrest at the pre–pro B-cell stage and this phenomenon was not accompanied by enhanced development of myeloid cells. IL-17 did not significantly alter the development of either cell line. These results suggested that the elevated concentrations of IL-6, TNF, and/or GM CSF in K/Bsf BM prevent the development of B lymphocytes and conversely enhance granulopoiesis, whereas the inhibitory effect of IFN-γ is restricted to B lineage cells.

TNF, IL-6, and GM-CSF produced by Teff cells collaborate to inhibit B-cell development

To directly assess the role of IL-6, TNF, GM-CSF on defective B cell development in the K/B sf BM, I neutralized each one cytokine or three cytokines at once with antibodies in the presence of activated Teff cells during in vitro B cell differentiation. Neutralizing single pro-inflammatory cytokines restored B cell development to B220+CD19- stage, while neutralizing three cytokines at once could make B cell development proceed to B220+CD19+ stages and inhibited the development of Gr-1+ cells.

Figure 9. Treg cells ensure B lymphopoiesis by controlling the suppressive activity of Teff cells.

LSK cells purified from CD45.1^{+} C57BL/6 mice were cultured in the presence or absence of pre-activated Treg cells or/and Teff cells purified from Foxp3-GFP reporter mice. In some experiments, Teff cells were replaced by the supernatants from cultures of activated Teff cells (Teff sup) or Teff cells were added into transwells (tw). After 12 days of culture, differentiated B cells (B220^{+} CD19^{+}) were examined by flow cytometry, gated on whole live cells. Data are shown as mean SEM percentages of B220^{+}CD19^{+} cells and are pooled from three independent experiments. ***$p < 0.001$; Student's t-test. ns: not significant.

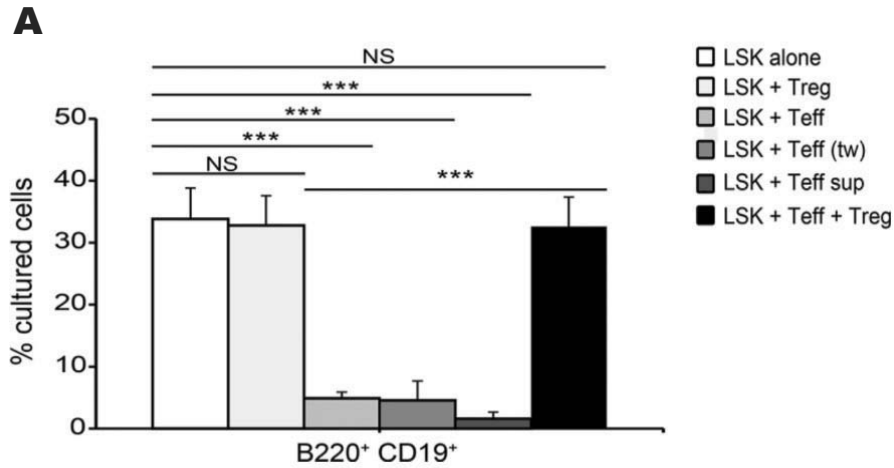

A
NS
***
***
***
NS
***
50
40
30
20
10
0
% cultured cells
B220+ CD19+
LSK alone
LSK + Treg
LSK + Teff
LSK + Teff (tw)
LSK + Teff sup
LSK + Teff + Treg

Figure 10. The cytokine milieu of the BM of K/Bsf mice is different from that of K/B mice.

K/B and K/Bsf mice were injected with brefeldin A and assayed by flow cytometry.

(A) Representative FACS profiles within a live cell gate and percentages of cytokine-producing cells are shown.

(B) The percentages of cytokine-producing cells among $CD4^+$ cells and non-$CD4^+$ cells. Data are shown as mean + SEM. *$p < 0.05$, and **$p < 0.01$; Student's t-test.

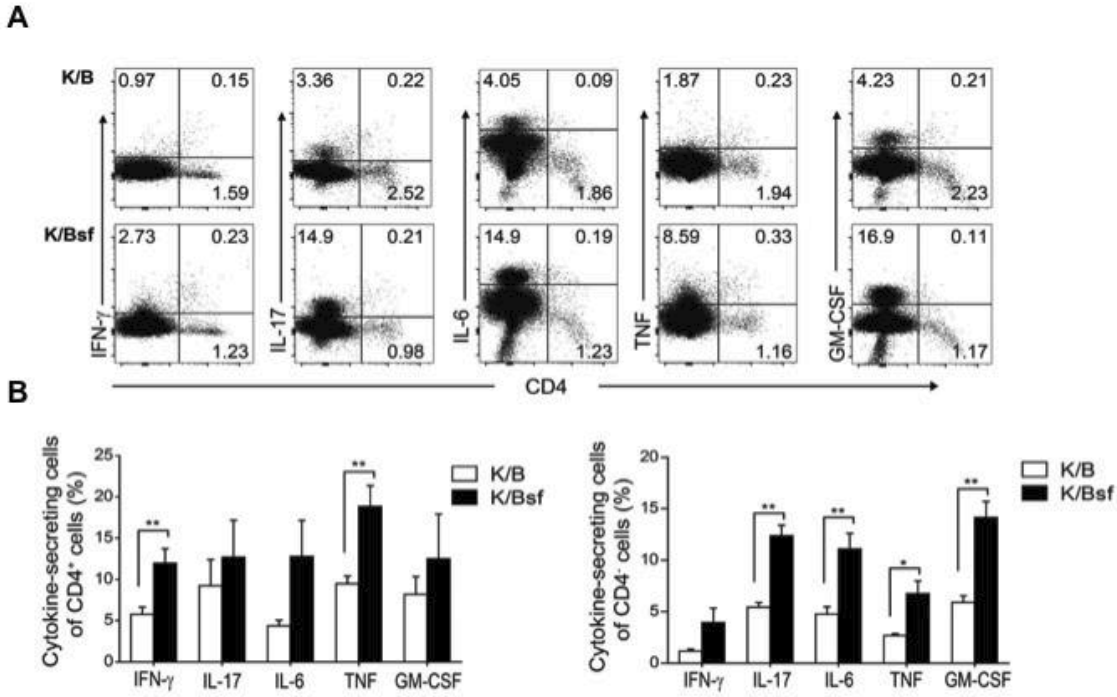
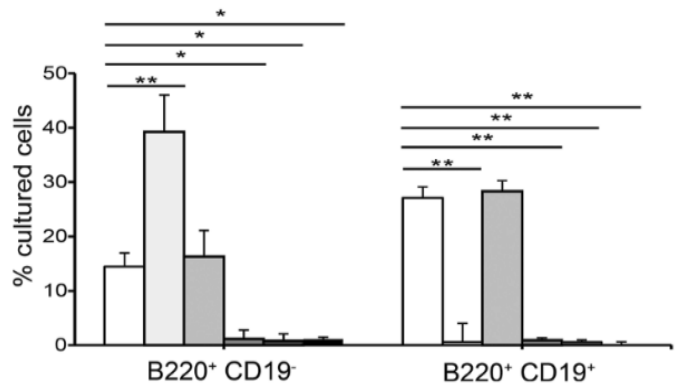

A
K/B
K/Bsf
0.97
0.15
1.59
2.73
0.23
1.23
3.36
0.22
2.52
14.9
0.21
0.98
4.05
0.09
1.86
14.9
0.19
1.23
1.87
0.23
1.94
8.59
0.33
1.16
4.23
0.21
2.23
16.9
0.11
1.17
IFN-γ
IL-17
IL-6
TNF
GM-CSF
CD4
B
Cytokine-secreting cells of CD4+ cells (%)
K/B
K/Bsf
IFN-γ
IL-17
IL-6
TNF
GM-CSF
Cytokine-secreting cells of CD4- cells (%)
K/B
K/Bsf
IFN-γ
IL-17
IL-6
TNF
GM-CSF

Figure 11. IL-6, TNF, and GM-CSF arrest B lymphopoiesis and promote granulopoiesis.

LSK cells purified from C57BL/6 mice were cultured for 12 days under B-cell differentiation conditions in the presence or absence of cytokines as indicated. Differentiated B cells and granulocytes were analyzed by flow cytometry, gated on whole live cells.

Data are shown as mean + SEM percentages of the indicated populations and are pooled from three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; Student's t-test.

A


% cultured cells
50
40
30
20
10
0
*
*
*
**
**
**
**
**
B220+ CD19-
B220+ CD19+

B

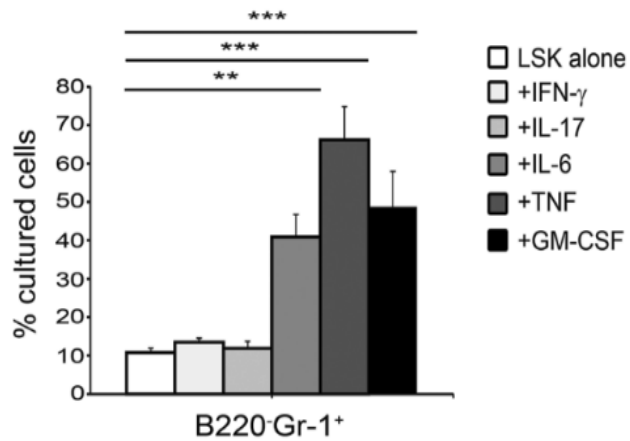
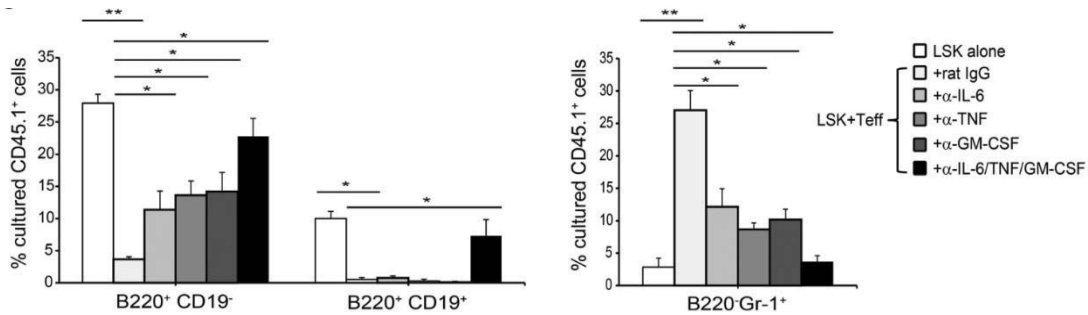

% cultured cells
80
70
60
50
40
30
20
10
0
***
***
**
B220-Gr-1+
LSK alone
+IFN-γ
+IL-17
+IL-6
+TNF
+GM-CSF

Figure 12. Cytokines secreted by activated Teff cells inhibit B lymphopoiesis.

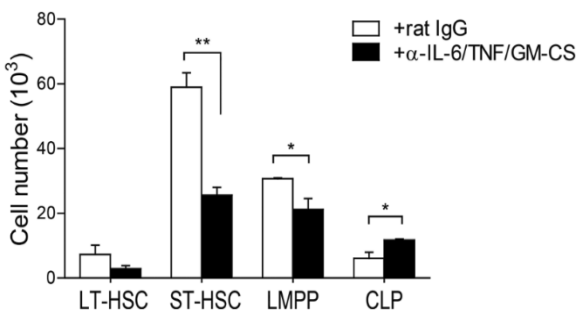
(A) LSK cells purified from CD45.1$^+$ C57BL/6 mice were cultured alone or together with activated Teff cells from CD45.2$^+$ Foxp3-GFP reporter mice under B cell differentiation conditions in the presence or absence of neutralizing mAb as indicated. After co-culture, differentiated B cells and granulocytes were assessed by FACS, gated on live CD45.1$^+$ cells. The percentages of B220$^+$CD19$^-$, B220$^+$CD19$^+$, and B220$^-$Gr-1$^+$ CD45.1$^+$ cells in the different conditions are shown.

(B) K/Bsf and K/B mice were injected with a mixture of neutralizing mAbs or rat IgG and analyzed by flow cytometry. Cell numbers of each population are displayed as mean + SEM (n = 3 per strain), and data are pooled from two independent experiments. *$p < 0.05$ and **$p < 0.01$; Student's t-test.

A


% cultured CD45.1+ cells
B220+ CD19-
B220+ CD19+
B220-Gr-1+
LSK alone
+rat IgG
+α-IL-6
+α-TNF
+α-GM-CSF
+α-IL-6/TNF/GM-CSF
LSK+Teff

B


Cell number (10^3)
+rat IgG
+α-IL-6/TNF/GM-CS
LT-HSC
ST-HSC
LMPP
CLP

These effects depended on the presence of Teff cells because the same Abs did not interfere with the developmental profiles of LSKs when cultured without Teff cells. This in vitro finding was confirmed in vivo: administration of these Abs to K/Bsf mice reversed their BM defects, such as reduced CLPs and increased ST-HSCs (Figure 12B). In the light of these in vitro and in vivo findings, I conclude that the ability of BM Teff cells to inhibit B lymphopoiesis is largely dependent on their secretion of myelopoietic cytokines such as TNF, IL-6, and GM-CSF.

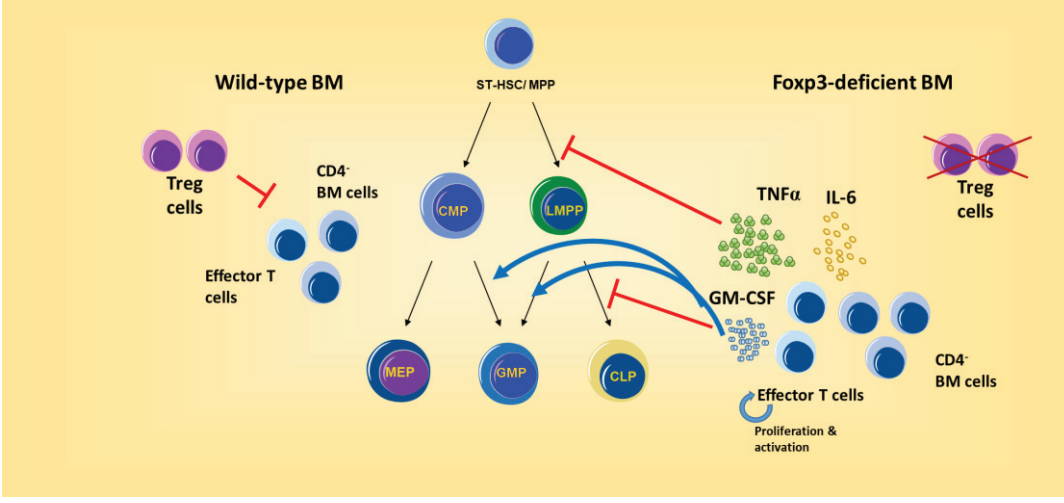


Figure 13. A model for the role of regulatory T cells to ensure normal hematopoiesis

PART 2.

CEBPβ-overexpressed iTreg cells mitigate inflammatory bowel disease by promoting pTreg differentiation

Enhanced pTreg cell differentiation in the presence of C/EBPβ-expressing iTreg cells in colon

It has been reported that C/EBPβ expression is up-regulated in in vitro-induced regulatory T cells (iTreg cell) in the presence of retinoic acid (RA) (lee et al). RA is a vitamin A metabolite and is produced by $CD103^{+}$ dendritic cells, epithelial cells, and stromal cells of intestine (Hall et al., 2011). As intestinal lamina propria environment is rich in RA (Hall et al., 2011), I speculated that the Treg cells in the intestine might have a higher C/EBPβ expression than the Treg found elsewhere. To test this idea, I first examined the expression of C/EBPβ in colon regulatory T cells. Treg cells from colon and spleen of Foxp3-GFP knock-in mice were sorted and mRNA expression of C/EBPβ were analysed by qPCR. Notably, colon Treg cells expressed C/EBPβ highly when compared with splenic Treg cells (Figure 14A).

Based on the differential expression of C/EBPβ in the intestinal Treg and splenic Treg, I investigated the possible roles of C/EBPβ on Treg cells in intestine. Intestinal tissue is exposed to continuous antigen challenge

derived from food or microorganisms, which leads to immune responses. Concurrently, suppressive cells in the intestine prevent excessive immune responses to maintain immune homeostasis**.** Previous study demonstrated that the antigen-specific peripherally derived regulatory T cells (pTreg cell) development is abrogated in mouse deficient in vitamin A (Hall et al., 2011). In addition, pTreg cells generated from naive CD4 T cells play crucial roles in regulation of intestinal inflammation (Haribhai et al., 2009; Haribhai et al., 2011; Ohnmacht et al., 2015; Sefik et al., 2015). Based on these studies, I sought to verify whether C/EBPβ expression could affect pTreg cell differentiation in intestinal inflammation. To gain insight into the role of C/EBPβ on differentiation of pTreg cells in intestinal inflammation, I used 1) iTreg cells transduced with C/EBPβ retrovirally, 2) well-established mouse model of intestinal inflammation, and 3) CD45.1 congenic naive T cells for tracing pTreg cells generated in vivo. Purified $CD4^{+}CD45RB^{high}CD45.1^{+}$naive T cells from spleen and lymph node of wild-type mice were adoptively transferred into $Rag^{-/-}$ immunodeficient recipient mice followed by sorted iTreg (45.2^{+}) cells that were retrovirally transduced with C/EBPβ-encoding (C/EBPβ-iTreg) or mock (Mock-iTreg) vector. Colonic lamina propria cells and mesenteric lymph node (mLN) cells were isolated and the $Foxp3^{+}$ cells derived from $CD45.1^{+}$ naive T cells were analysed by FACS 11 weeks after transfer. Strikingly, co-transfer of C/EBPβ-iTreg resulted in pronounced induction of pTreg cells from naive T

Figure 14. Forced expression of C/EBPβ on iT_{reg} cells induced elevated numbers of $Foxp3^+$ pT_{reg} cells derived from $CD45RB^{high}$ naive T cells in the intestine

(A) Relative expression of C/ebpβ mRNA in sorted $Foxp3^+(GFP^+)$ Treg cells of spleen or colon from wild type mice measured by quantitative RT-PCR, normalized with Gapdh. Data are shown as mean SEM (n=3); ***P <0.001

(B and E) Representative flow cytometry profiles of pTreg cells. Flow cytometric analysis of $CD3^+CD4^+$ Foxp3+ CD45.1+ pTreg cells in the colon lamina propria (B) and mesenteric lymph node (E) from the $rag^{-/-}$ recipient mice that were transferred mock-iTreg or C/EBPβ-iTreg cells. Colon lamina propria of recipient mice was analysed 11weeks after transfer of $5x10^5$ $CD4^+CD45RB^{high}$ T cells along with $2x10^5$ mock-iTreg or C/EBPβ-iTreg cells.

(C, D, F, G) Frequencies of $CD45.1^+CD4^+CD3^+Foxp3^+$ cells as described in (B) among CD45.1+ cells (C and F) and among total live cells (D and G)

Data represent the average ± SEM *P <0.05, and **P <0.01; Student's t-test.

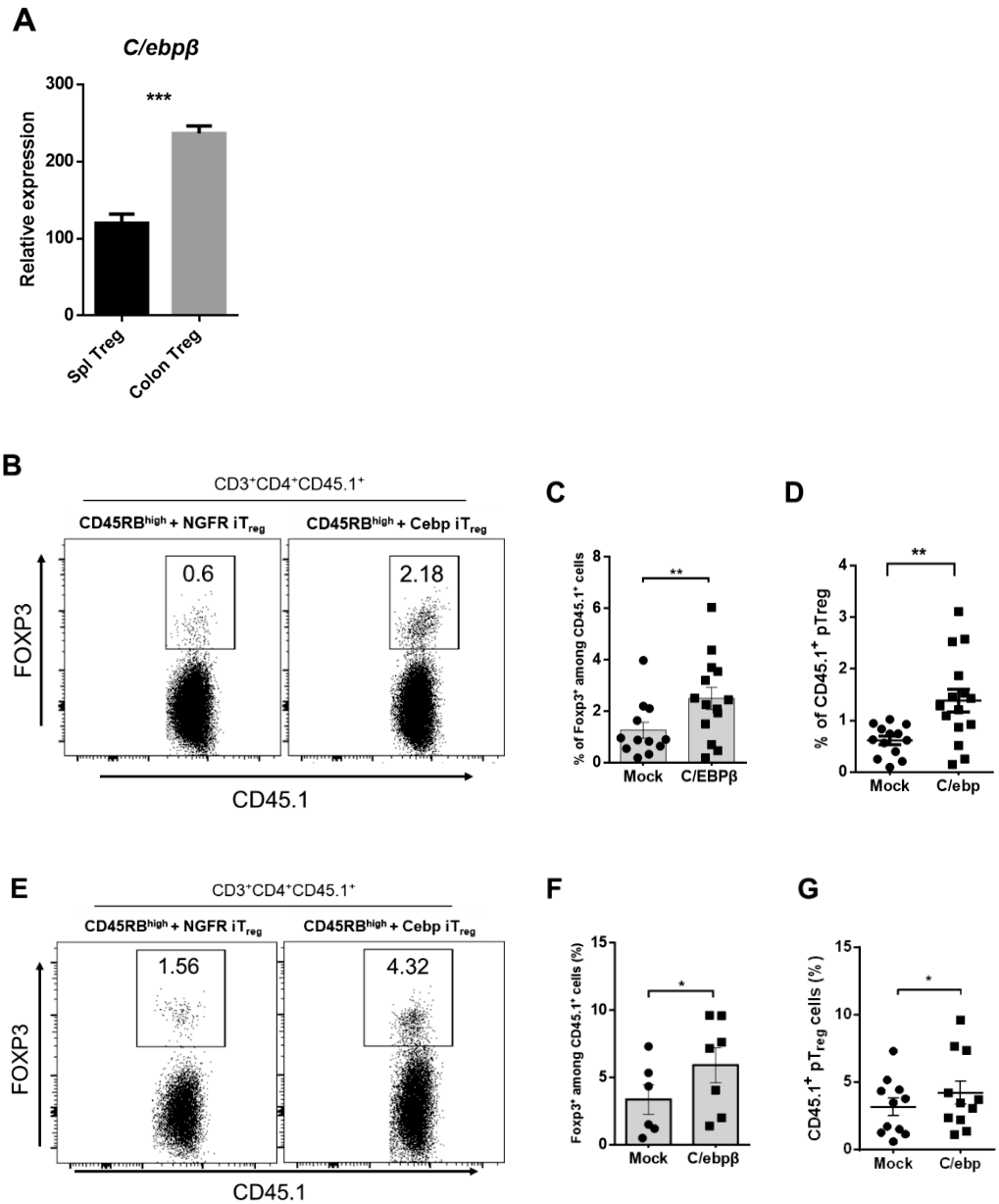

A
C/ebpβ
Relative expression
300
200
100
0
***
Spl Treg
Colon Treg
B
CD3+CD4+CD45.1+
CD45RBhigh + NGFR iTreg
CD45RBhigh + Cebp iTreg
FOXP3
0.6
2.18
CD45.1
C
% of Foxp3+ among CD45.1+ cells
**
Mock
C/EBPβ
D
% of CD45.1+ pTreg
**
Mock
C/ebp
E
CD3+CD4+CD45.1+
CD45RBhigh + NGFR iTreg
CD45RBhigh + Cebp iTreg
FOXP3
1.56
4.32
CD45.1
F
Foxp3+ among CD45.1+ cells (%)
*
Mock
C/ebpβ
G
CD45.1+ pTreg cells (%)
*
Mock
C/ebp

Figure 15. C/EBPβ-iTreg cells showed stable Foxp3 expression under the inflammation condition of the IBD mouse colon

(A) Representative flow cytometry profiles of iTreg cells. Flow cytometry analysis of $CD45.2^{+}CD4^{+}CD3^{+}Foxp3^{+}$ Treg cells in the colon lamina propria of the $rag^{-/-}$ recipient mice (A,B) that were transferred mock-iTreg or C/EBPβ-iTreg cells. Colon lamina propria of recipient mice was analysed 11weeks after transfer of $5x10^{5}$ $CD4^{+}CD45RB^{high}$ T cells along with $2x10^{5}$ mock-iTreg or C/EBPβ-iTreg cells.

(B) Frequencies of $CD45.2^{+}CD4^{+}CD3^{+}Foxp3^{+}$ cells as described in (A) among total live cells (B)

(C) Representative flow cytometry profiles of exTreg cells. Flow cytometry analysis of $CD45.2^{+}CD4^{+}CD3^{+}Foxp3^{+/-}IL\text{-}17a^{+}$ Treg cells in the colon lamina propria of the $rag^{-/-}$ recipient mice that were transferred mock-iTreg or C/EBPβ-iTreg cells. Colon lamina propria cells of recipient mice was analysed 11weeks after transfer of $5x10^{5}$ $CD4^{+}CD45RB^{high}$ T cells along with $2x10^{5}$ mock-iTreg or C/EBPβ-iTreg cells. Lamina propria cells were stimulated 10ng/ml PMA and 500ng/ml ionomycin in the presence of 1X brefeldin A for four hours before FACS analysis.

(D) Frequencies of $CD45.2^{+}CD4^{+}CD3^{+}Foxp3^{+/-}IL\text{-}17a^{+}$ Treg cells cells as described in (A) among total live cells. Data represent the average SEM $*P < 0.05$, and $**P < 0.01$; Student's t-test.

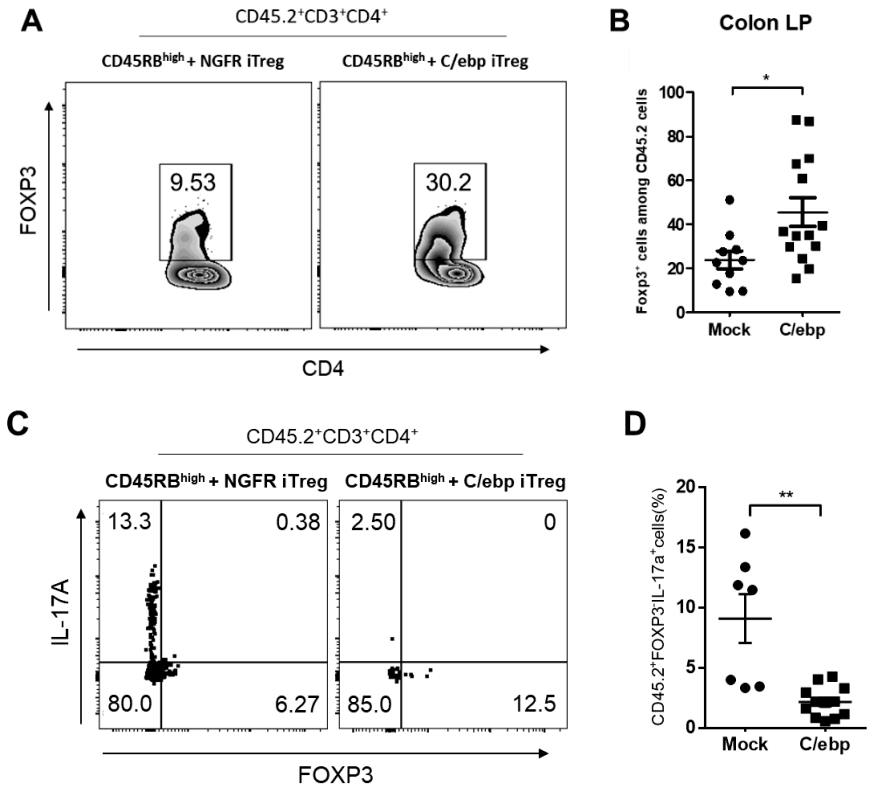
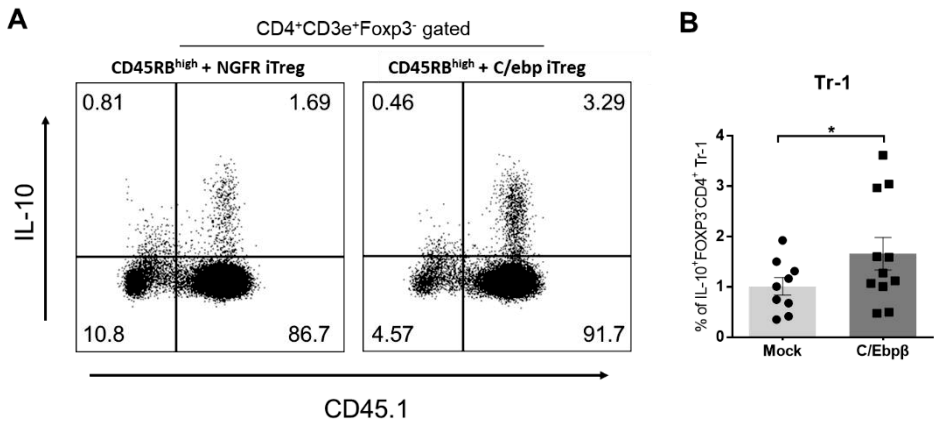

A
CD45.2+CD3+CD4+
CD45RBhigh + NGFR iTreg
CD45RBhigh + C/ebp iTreg
FOXP3
9.53
30.2
CD4
B
Colon LP
Foxp3+ cells among CD45.2 cells
*
Mock
C/ebp
C
CD45.2+CD3+CD4+
CD45RBhigh + NGFR iTreg
CD45RBhigh + C/ebp iTreg
IL-17A
13.3
0.38
80.0
6.27
2.50
0
85.0
12.5
FOXP3
D
CD45.2+FOXP3IL-17a+cells(%)
**
Mock
C/ebp

Figure 16. C/EBPβ-iT$_{reg}$ cells induced elevated numbers of IL-10^{+}Foxp3^{-} CD4^{+} Tr-1 cells

(A) Flow cytometric analysis of Tr-1 cells in the colon lamina propria from reconstituted mice with mock-iTreg or C/EBPβ iTreg cells. Representative flow cytometry profile of CD4^{+}CD3^{+}Foxp3^{-}IL-10^{+} Tr-1 cells (A). Colon lamina propria was prepared from rag-/- recipient mice 11 weeks after transferring each iTreg cells. Lamina propria cells were stimunlated with 10ng/ml PMA and 500ng/ml ionomycin in the presence of 1X brefeldin A for four hours. IL-10^{+} cells were gated from CD4^{+}CD3^{+}Foxp3^{-} cells.

(B) The frequency of gated CD4^{+}CD3^{+}Foxp3^{-}IL-10^{+} cells by flow cytometry plotted as average percentage of Tr-1 cells. Each individual dot represents one mouse; *P <0.05


A
CD4+CD3e+Foxp3- gated
CD45RBhigh + NGFR iTreg
CD45RBhigh + C/ebp iTreg
0.81
1.69
10.8
86.7
0.46
3.29
4.57
91.7
IL-10
CD45.1
B
Tr-1
% of IL-10+FOXP3-CD4+ Tr-1
Mock
C/Ebpβ
*

cells in colon and mLN compared with control (Figure 14B-14G). pTreg cells differentiated from naive T cells were found at a markedly higher proportion not only among CD4 naive T cells but also in total lamina propria or mLN cells. These results suggest that the mechanism involved in the differentiation of pTreg cells in the colon is made more efficient by C/EBPβ-expressing Treg cells.

Forced expression of C/EBPβ enhanced the stability of Foxp3 expression of iTreg cells in inflammatory environment but increased cell death concurrently

In vitro-induced Treg cells show unstable Foxp3 expression, because CpG methylation of its conserved noncoding sequence2 (CNS2) region is not fully demethylated (Baron et al., 2007; Kim and Leonard, 2007; Ohkura et al., 2012; Polansky et al., 2008). Furthermore, as Foxp3 not only functions to maintain suppressive character of Treg cells, but also suppress the expression of inflammatory cytokine genes (Gavin et al., 2007; Zheng et al., 2007), such instability frequently results in conversion of Treg cells into proinflammatory cytokine-IL17 secreting cells. A previous study has demonstrated that C/EBPβ-transduced iTreg cells showed increased stability of Foxp3 expression even in the presence of inflammatory cytokines in vitro (Lee et al., 2018). To assess whether forced expression of C/EBPβ on iTreg cells can make the expression of Foxp3 stable in inflammatory environment

in vivo, transferred iTreg cells recruited into intestinal lamina propria were analysed by FACS. Consistent with in vitro data of previous study, iTreg cells infiltrated into inflammation site of intestine retained Foxp3 expression and showed resistance to conversion into the Th17 cells in C/EBPβ expression dependent manner (Figure 15A-15D). In addition, I found elevated frequency of $CD4^{+}CD3^{+}Foxp3^{-}IL\text{-}10^{+}$ Tr-1 cells in the colon of $rag^{-/-}$ recipient mice that were transferred C/EBPβ-iTreg cells (Figure 16A and 16B).

C/EBPβ is a basic leucine-zipper transcription factor that functions in cell proliferation and differentiation (Umek et al., 1991). Previous studies showed that ectopic expression of C/EBPβ induces cell cycle arrest in hepatoma cell line, and $C/EBP\beta^{-/-}$ mice showed lymphoproliferative disorder (Buck et al., 1994; Screpanti et al., 1995). On the other hand, expression of C/EBPβ during liver cell proliferation because of hepatectomy increased while deficiency of C/EBPβ results in reduced regenerative responses including hepatocyte DNA synthesis and activation of immediate-early growth-control genes (Greenbaum et al., 1998). To examine whether the forced expression of C/EBPβ in iTreg cells affects cell proliferation arrest and subsequent cell death, iTreg cells were analysed using flow cytometry. C/EBPβ-transduced iTreg cells showed increased annexin V-positive cells compared with mock vector-transduced iTreg cells (Figure 17A). This result means that forced expression of C/EBPβ on iTreg cells

Figure 17. Induction of cell death caused by forced expression of C/EBPβ on iT_{reg} cells

(A) Flow cytometric analysis of annexin V^+ apoptotic cells of in vitro cultured iTreg cells. $CD25^-CD44^-$ naive CD4 T cells were sorted from wild-typed mice. Sorted naive CD4 T cells were activated with plate coated anti-CD3 (3ug/ml)/soluble anti-CD28 (1ug/ml) for 24hour and s subsequently followed by retroviral transduction of mock vector or C/EBPβ. 2days of incubation in the presence of 1ng/ml TGFβ with plate coated anti-CD3/ soluble anti-CD28 stimulation. Annexin V^+ cells were gated after 48 hours incubation.

(B and C) Flow cytometric analysis of annexin V^+ apoptotic cells among $CD45.2^+CD3^+CD4^+$ iTreg cells of colon lamina propria ; $*P < 0.05$

(D and E) Flow cytometric analysis of annexin V^+ apoptotic cells among $CD45.1^+CD3^+CD4^+$ transferred naive T cells. Each individual dot represents one mouse

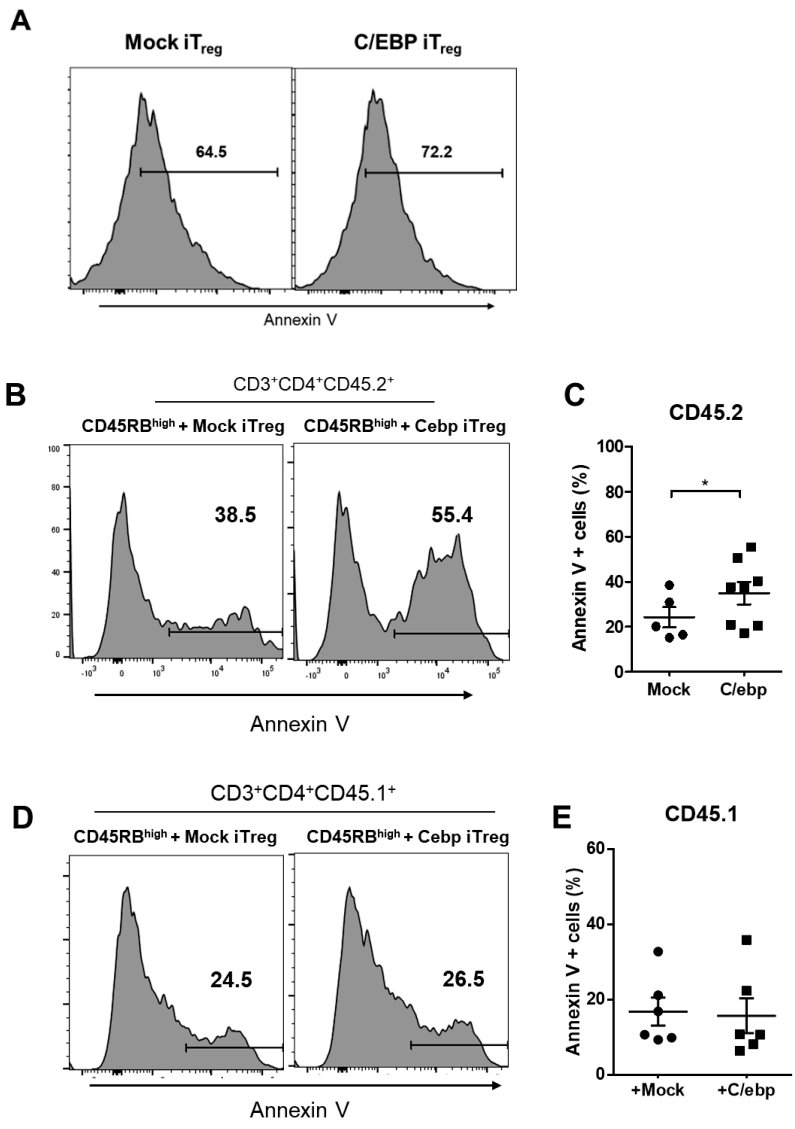

A
Mock iTreg
C/EBP iTreg
64.5
72.2
Annexin V
B
CD3+CD4+CD45.2+
CD45RBhigh + Mock iTreg
CD45RBhigh + Cebp iTreg
38.5
55.4
Annexin V
C
CD45.2
Annexin V + cells (%)
*
Mock
C/ebp
D
CD3+CD4+CD45.1+
CD45RBhigh + Mock iTreg
CD45RBhigh + Cebp iTreg
24.5
26.5
Annexin V
E
CD45.1
Annexin V + cells (%)
+Mock
+C/ebp

Figure 18. Cellularity of Foxp3^{+} C/EBPβ-iTreg cells in itself decreased despite stable foxp3 expression

(A-D) Sequential flow cytometric gating of $CD3^{+}CD4^{+}CD45.2^{+}Foxp3^{+}$ iTreg cells. Colon lamina propria cells from recipient mice that were reconstituted with $CD4^{+}CD3^{+}CD45.1^{+}CD45RB^{high}$ T cells along with mock-iTreg or C/EBPβ-iTreg cells were analysed by flow cytometry. Each individual symbol represents one mouse; Data represent the average ± SEM $^{**}P<0.01$, and $^{***}P<0.001$; Student's t-test.

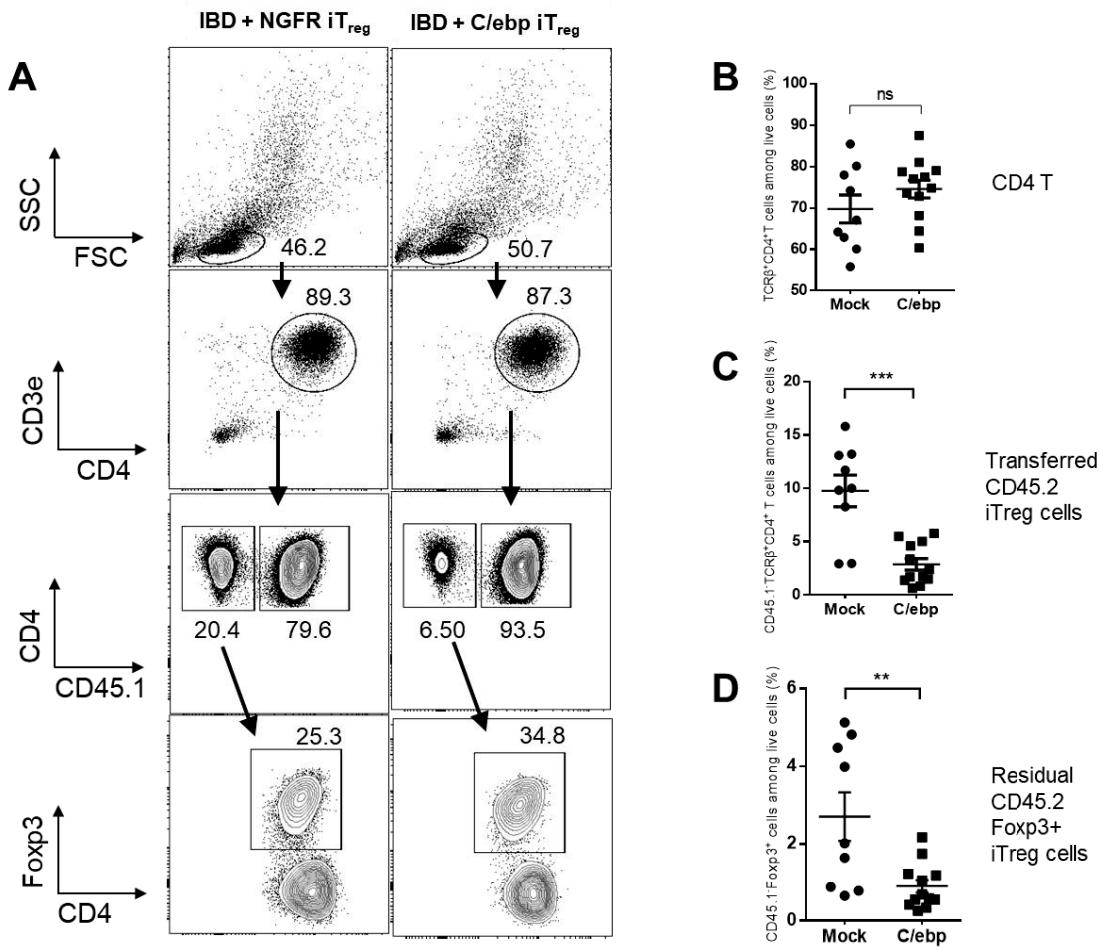
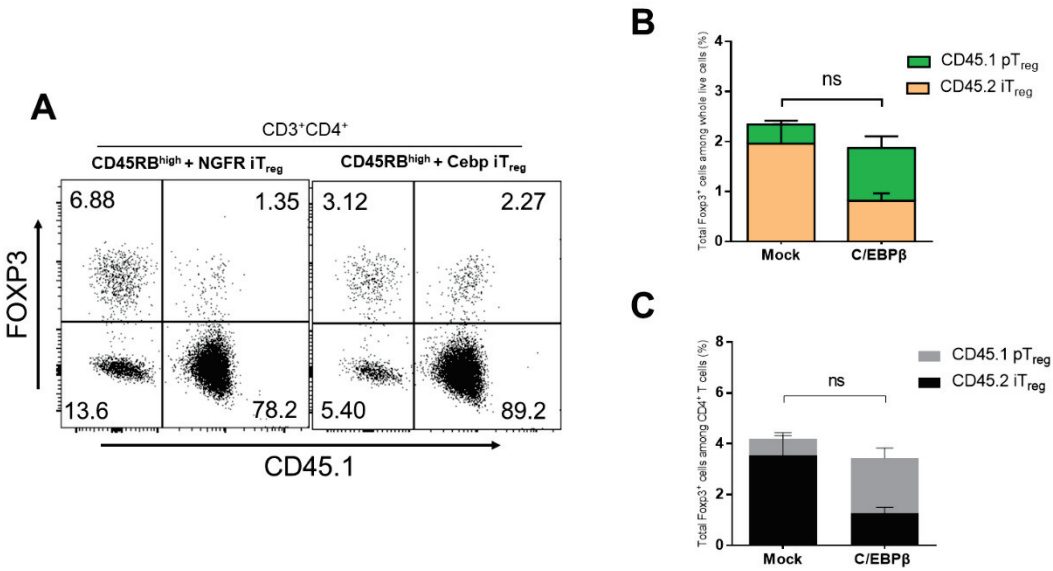

IBD + NGFR iTreg
IBD + C/ebp iTreg
A
SSC
FSC
46.2
50.7
89.3
87.3
CD3e
CD4
CD4
CD45.1
20.4
79.6
6.50
93.5
Foxp3
CD4
25.3
34.8
B
TCRβ+CD4+T cells among live cells (%)
ns
Mock
C/ebp
CD4 T
C
CD45.1+TCRβ+CD4+ T cells among live cells (%)
***
Mock
C/ebp
Transferred CD45.2 iTreg cells
D
CD45.1+Foxp3+ cells among live cells (%)
**
Mock
C/ebp
Residual CD45.2 Foxp3+ iTreg cells

Figure 19. Total Foxp3$^+$ Treg cells in the intestinal lamina propria

(A) Representative flow cytometric profiles of collective CD45.1$^+$ Foxp3$^+$ pTreg and CD45.2$^+$ Foxp3$^+$ iTreg cells in the colon of IBD induced mice.

(B) Bar graphs depicting the average sum of pTreg and iTreg cells among total live cells (B) and among CD4$^+$ T cells (C) recovered from the colon lamina propria of IBD induced mice. iTreg cells are shown in orange(B)or black(C) while pTreg cells are shown in green(B) or gray (C). Data represent the average ± SEM. ns: not significant.


A
CD3+CD4+
CD45RBhigh + NGFR iTreg
CD45RBhigh + Cebp iTreg
6.88
1.35
3.12
2.27
13.6
78.2
5.40
89.2
FOXP3
CD45.1
B
Total Foxp3+ cells among whole live cells (%)
CD45.1 pTreg
CD45.2 iTreg
ns
Mock
C/EBPβ
C
Total Foxp3+ cells among CD4+ T cells (%)
CD45.1 pTreg
CD45.2 iTreg
ns
Mock
C/EBPβ

induces cell proliferation arrest and leads subsequent cell death as well. To verify the effect of C/EBPβ expression on iTreg cells of in vivo inflammation site, based on in vitro apoptosis data, I analysed colonic lamina propria cells of $Rag^{-/-}$ mice 11 weeks after reconstitution with naive $CD4^{+}CD45RB^{high}$ cells and iTreg cells expressing C/EBPβ or mock vector by FACS. Consistent with in vitro data, iTreg cells expressing C/EBPβ showed a much higher frequencies of annexin V-positive apoptotic cells (Figure 17B and 17C). $CD4^{+}CD45RB^{high}$ cells, however, did not show any difference compared with that of control mice reconstituted with mock vector-transduced iTreg cells (Figure 17D and 17E). This result means that increased susceptibility to apoptosis is caused not by extrinsic factors such as inflammatory signals but by intrinsic effect of C/EBPβ expression.

Although stability of Foxp3 expression of C/EBPβ-iTreg cells increased (Figure 15A and 15B), because of its effect of increasing cell cycle arrest and apoptosis, total frequency of $CD45.2^{+}Foxp3^{+}$ cells of transferred iTreg cells among lamina propria cells is decreased in mice reconstituted with C/EBPβ-iTreg compared with Mock-iTreg cells (Figure 18A-18D).

Collectively, these results above mentioned led to differing composition of Treg cells of colonic lamina. Among Treg cells of lamina propria of mouse reconstituted with Mock-iTreg cells, iTreg cells are the major group that controls the immune responses while pTreg cells are the major group in the mouse reconstituted with C/EBPβ-iTreg cells. However, the total amount of

the Foxp3$^+$ Treg cells, which is the sum of iTreg and pTreg cells, showed similar sized among total lamina propria (Figure 19B) or CD4 T cells (Figure 19A and 19C) between mice reconstituted with C/EBPβ-iTreg or control iTreg.

Adoptive transfer of iTreg cells expressing C/EBPβ showed amelioration of inflammatory bowel disease

Given that inflammation is regulated by the orchestration of iTreg and pTreg cells, I verified the signs of intestinal inflammation of the recipient mice. Although mice reconstituted with Mock-iTreg or C/EBPβ-iTreg cells had similar proportion of total Foxp3$^+$ Treg cells, inflammation incidence differed significantly. Strikingly, co-transfer of C/EBPβ-iTreg cells was highly efficient to prevent inflammation compared with Mock-iTreg cells. Mice that were co-transferred of C/EBPβ-iTreg cells showed pronounced suppression in every index of intestinal inflammation (Figure 20). C/EBPβ-iTreg mice recovered from severe weight loss (Figure 20A), and colon length shortening was prevented (Figure 20B and 20C). Furthermore, histological scoring data of colitis and histological imaging of disorganized villi structure by aggressive infiltration of immune cells indicates sufficient suppression of inflammation in C/EBPβ-iTreg mice (Figure 20D).

Marked increase of IFNγ and TNFα expression has been reported in human inflammatory bowel disease patients (Imam et al., 2018; MacDonald et al.,

1990). In addition, mouse data also showed that IFN gamma is required for disease development in experimental colitis, and TNF alpha has been shown to be involved in intestinal barrier dysregulation in experimental colitis model (Goretsky et al., 2012). To gain insight into disease incidence by cytokine secretion at the cellular level, I stained colonic lamina propria cells of recipient mice 5 weeks after IBD induction and co-transfer of iTreg cells, subsequently measured by FACS. Co-transfer of C/EBPβ-iTreg cells substantially decreased the secretion of inflammatory cytokines in lamina propria cells (Figure 21A-21D). Collectively, these data indicated that transferred C/EBPβ-iTreg cells contributed to ameliorate intestinal inflammation.

Specific deletion of pTreg cells failed to maintain immune tolerance in IBD model

It has been well reported that Treg cells with antigen specific TCR repertoire exhibit much powerful suppressive activity than the Treg cells with random TCR repertoire (Levine et al., 2017; Liu et al., 2015), and pTreg cells differentiated in intestine possess local antigen specific TCR repertoire thereby play crucial role in immune homeostasis in intestine (Haribhai et al., 2009; Haribhai et al., 2011; Josefowicz et al., 2012; Ohnmacht et al., 2015; Sefik et al., 2015). Given the fact that the major Treg cell subset in the colon of mice co-transferred with C/EBPβ-iTreg cells are

pTreg cells, I speculated that amelioration of intestinal inflammation might be a result of enhanced differentiation of the pTreg cells, which has been reported to possess antigen specific TCR repertoire (Haribhai et al., 2011). To evaluate the contribution of pTreg cells on the regulation of inflammation, I utilized a mouse system for selective deletion of pTreg cells. As DEREG mice carries diphtheria toxin (DT) receptor-flanked exon1 (Lahl et al., 2007) of Foxp3 gene, injection of DT can delete $Foxp3^{+}$ Treg cells selectively (Figure 22A). By transferring naive $CD4^{+}CD45RB^{high}$ cells obtained from the DEREG mice into $Rag^{-/-}$ recipient mice, I induced intestinal inflammation, where pTreg cells generated in vivo are susceptible to DT. As with former data, co-transfer of C/EBPβ-iTreg cells was completely suppressed the intestinal inflammation compared with mock_iTreg cells. At the time point that the difference of suppression showed by weight loss, DT was injected into the mice every other day for 15 days. Surprisingly, mice reconstituted with C/EBPβ-iTreg cells showed rapid weight loss in response to DT injection that shows the deletion of pTreg cells resulted in disrupted immune regulation in the intestine (Figure 22B). This result implies that pTreg cells induced by C/EBPβ-iTreg cells have played a crucial role in suppressing intestinal inflammation. The selective deletion of pTreg cells was confirmed by flow cytometric analysis of colonic lamina propria cells using CD45.1 and Foxp3 as a pTreg marker. Collectively, dominance of pTreg among $Foxp3^{+}$ Treg cells in the intestine

Figure 20. Significant mitigation of inflammatory bowel disease by increased pTreg cells induced by C/EBP-iTreg cells

(A) Body weight trace of RAG-$2^{-/-}$ mice after transfer with $5x10^5$ $CD45.1^+CD4^+$ $CD45RB^{high}$ cells alone (circles) or with 2×10^5 FACS-sorted mock-iTreg cells (filled rectangles) or C/EBPβ-iTreg cells(empty rectangles). Each time point contains three to six mice in each group. Error bars represent average ± SEM ; **P* <0.05, ***P* <0.01

(B and C) Colon length of the mice described in (A). Each individual symbol represents one mouse; Data represent the average ± SEM; ***P* <0.01

(D) Representative histological images from haematoxylin & eosin (H&E) stained colon sections from the mice described in (A).

(E) H&E stained colon sections of depicted mice were scored in a blinded fashion for the amount of inflammation. Each individual symbol represents one mouse; Data represent the average ± SEM; ***P* <0.01

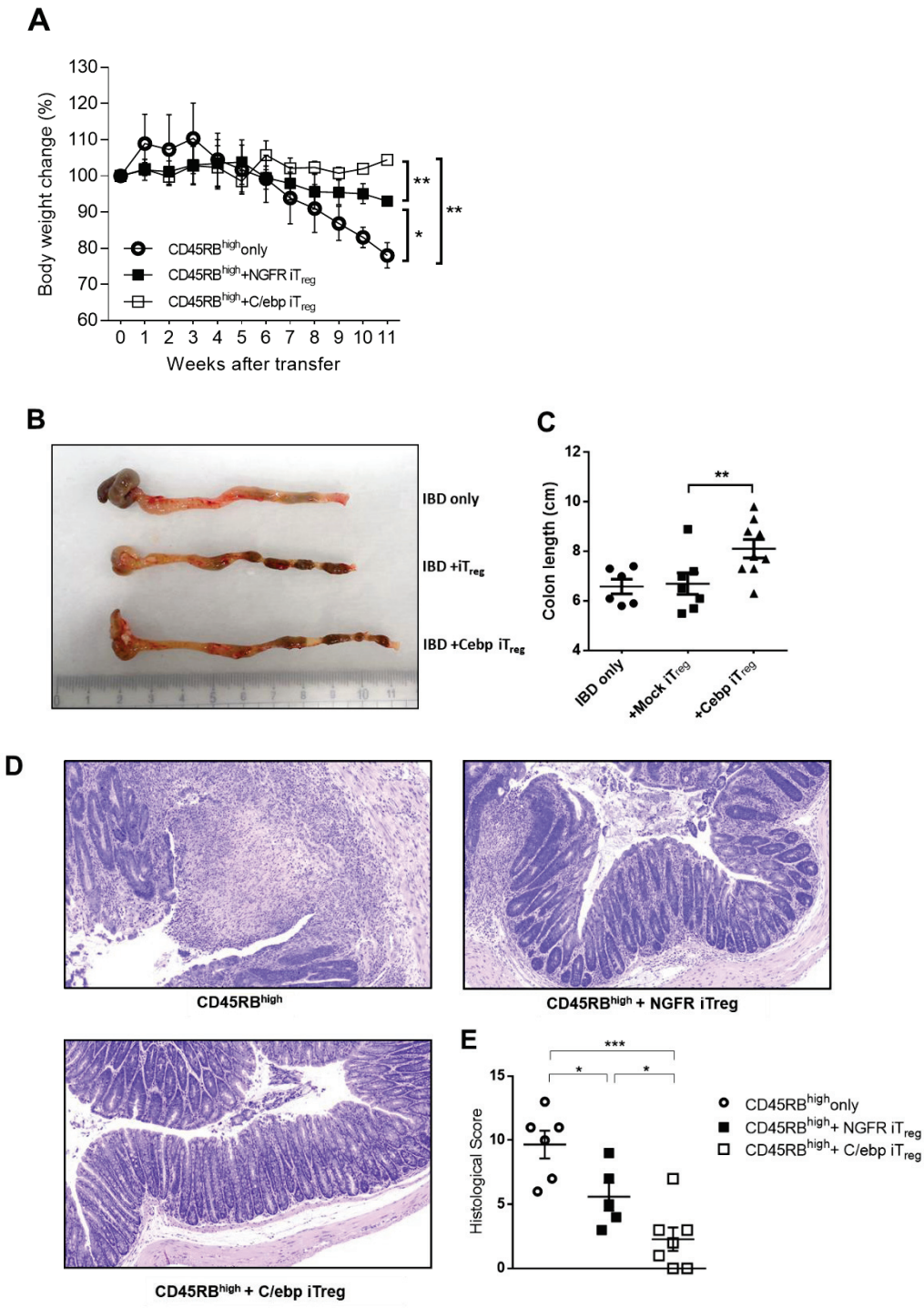
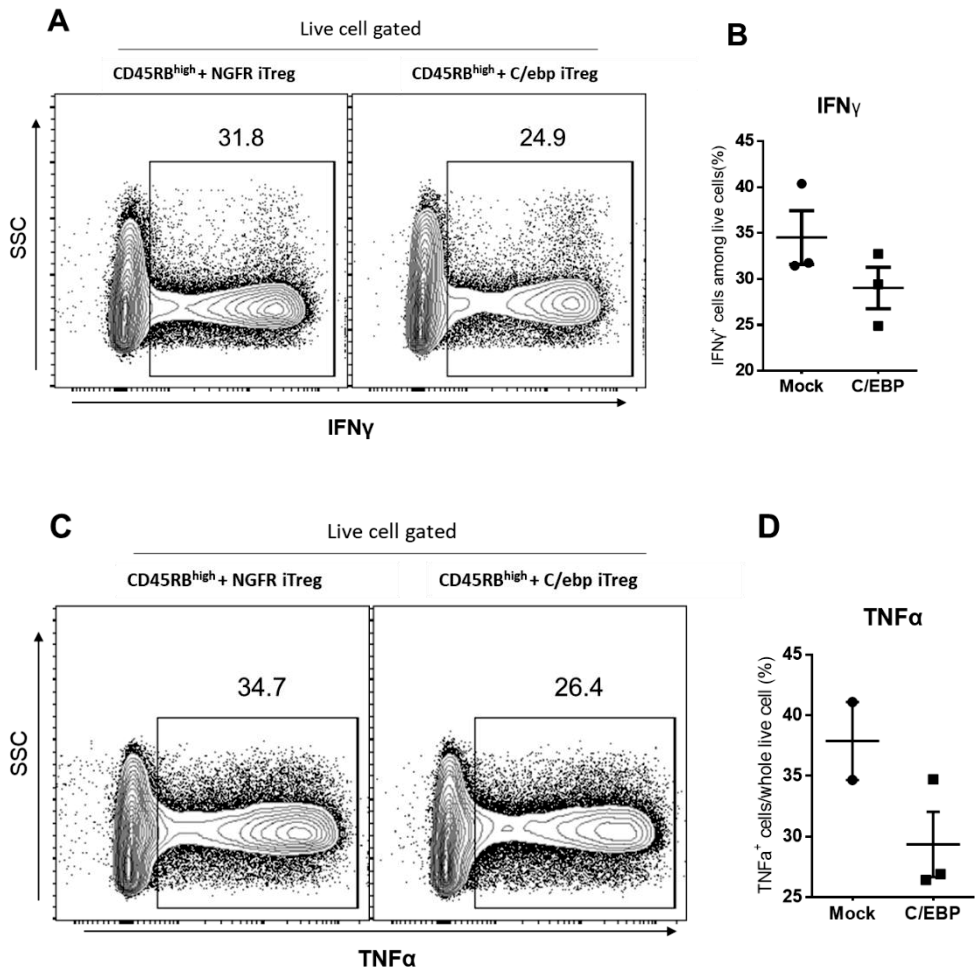

A
Body weight change (%)
130
120
110
100
90
80
70
60
0 1 2 3 4 5 6 7 8 9 10 11
Weeks after transfer
CD45RB^high only
CD45RB^high+NGFR iT_reg
CD45RB^high+C/ebp iT_reg
**
*
**
B
IBD only
IBD +iT_reg
IBD +Cebp iT_reg
C
Colon length (cm)
12
10
8
6
4
**
IBD only
+Mock iT_reg
+Cebp iT_reg
D
CD45RB^high
CD45RB^high + NGFR iTreg
CD45RB^high + C/ebp iTreg
E
Histological Score
15
10
5
0
***
*
*
CD45RB^high only
CD45RB^high+ NGFR iT_reg
CD45RB^high+ C/ebp iT_reg

Figure 21. pTreg cells induced by C/EBPβ overexpressed iTreg cells can suppress both interferon γ and TNFα expression in intestine

(A and C) Representative flow cytometry profile of inflammatory cytokine-expressing immune cells in colon from the mice described in figure 7A. Mononuclear cells of colon lamina propria were prepared and stimulated with plate coated anti-CD3/ soluble anti-CD28 stimulation in the presence of 1X brefeldin A at 37°C, 4 hour. Subsequent staining of intracellular cytokines (IFNγ and TNFα) were analysed by flow cytometry.

(B and D) Frequencies of IFNγ positive cells (B) and TNFα positive cells (D). Each individual symbol represents one mouse. Data represent the average ± SEM **P* <0.05, and ***P*<0.01; Student's t-test.


A
Live cell gated
CD45RB^high + NGFR iTreg
CD45RB^high + C/ebp iTreg
31.8
24.9
SSC
IFNγ
B
IFNγ
IFNγ+ cells among live cells(%)
45
40
35
30
25
20
Mock
C/EBP
C
Live cell gated
CD45RB^high + NGFR iTreg
CD45RB^high + C/ebp iTreg
34.7
26.4
SSC
TNFα
D
TNFα
TNFa+ cells/whole live cell (%)
45
40
35
30
25
Mock
C/EBP

of recipient mice and disruption of immune regulation caused by specific deletion of pTreg cells suggest that pTreg cells are the key population that suppress inflammation in the intestine.

C/EBPβ expression regulates the expression of signalling molecules associated with pTreg cell differentiation

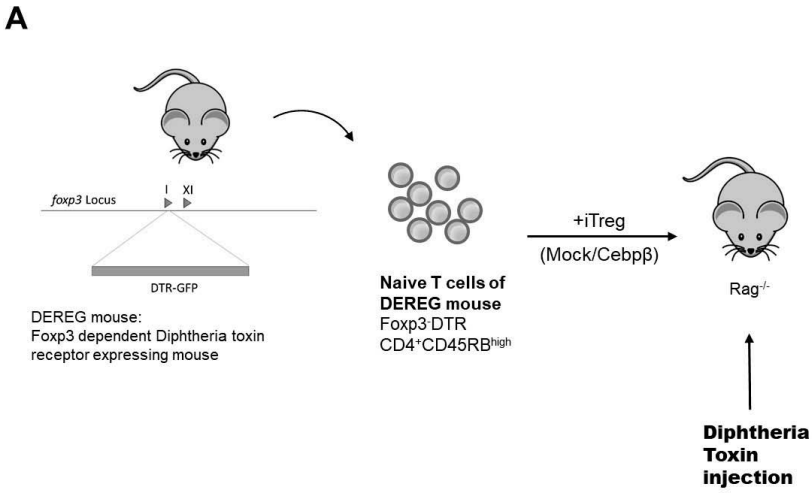
The enhanced pTreg cell differentiation from naive CD4 T cells in the presence of C/EBPβ-iTreg led me to speculate that signalling molecules expressed on C/EBPβ-iTreg cells might have a function in pTreg cell differentiation. To test this possibility, I examined the relative mRNA expression level of molecules involved in pTreg cell differentiation by qPCR. First, I analysed the expression of Foxp3, a transcription factor that confers suppressive function on Treg cells. FACS-sorted iTreg cells transduced with C/EBPβ showed increased expression of Foxp3 compared with iTreg cells transduced with mock vector (Figure 23A). Considering Foxp3 expression level is closely correlated to suppression activity (Gavin et al., 2007; Zheng et al., 2007), this result is consistent with previous study that demonstrated enhanced suppression by C/EBPβ-iTreg cells in in vitro suppression assay (Lee et al., 2018). It is well known that TGFβ signalling is required in conversion of naive CD4 T cells into $Foxp3^+$ Treg cells in periphery (Bilate and Lafaille, 2012; Chen et al., 2003b). In addition, Interleukin-10, a cytokine with anti-inflammatory character, acts on Treg

cells to maintain expression of the transcription factor FOXP3 and suppressive function and limits induction of colitis by means of limiting induction of antigen-specific Th1 responses as well (Di Giacinto et al., 2005). In addition, IL-10 produced by CX_3CR1^+ macrophage of intestine is required for induction of antigen specific Treg cells. Based on these studies, I examined relative mRNA expression level of Tgfβ1 and Il10 of iTreg cells to find the factor that affected pTreg cell induction. However, forced expression of C/EBPβ did not alter Tgfβ nor IL-10 expression on the C/EBPβ iTreg cells (Figure 22B). This result implies the existence of other factors, not canonical factors that induce pTreg cells. CTLA4, which binds to CD80 on the surface of dendritic cells, leads DC to express indolamine 2, 3-dioxygenase (IDO) that mediates tryptophan metabolism. A kynurenine and 3-hydroxyanthranilic acid (3-HAA), a first and second metabolite of tryptophan respectively, have been reported to induce Treg cells extrathymically (Mezrich et al., 2010; Yan et al., 2010). In addition, OX40L, a member of tumor necrosis factor superfamily, binds to OX40 that is highly expressed in Treg cells. The OX40-OX40L signalling has been reported inhibit the extrathymic Treg generation in vitro (So and Croft, 2007; Vu et al., 2007). Moreover, programmed cell death protein 1 and PD-Ligand 1 (PD-L1) that are highly expressed on Treg cells and activated T cells, mediate PD1-PD-L1 signalling, promotes pTreg cell differentiation by enhancing PTEN expression and inhibiting AKT-mTOR signalling

Figure 22. Ablation of pTreg cells resulted in failure to control intestinal inflammation

(A) A schematic design of experiment. FACS sorted $CD4^{+}CD45RB^{high}$ cells of $CD45.1^{+}$DEREG ($Foxp3^{DTR/EGFP}$) mice were transferred into $rag^{-/-}$ recipient mice along with mock-iTreg cells or C/EBPβ-iTreg cells. 50 days later, 50ug/kg diphtheria toxin was injected by intra-peritoneal injection every other day for 10 days. Mice were weighed daily.

(B) Weight change over time following adoptive transfer of naive T cells and iTreg cells into Rag deficient mice. Diphtheria toxin injection begins at 50 days after transfer (arrow)


A
foxp3 Locus
I XI
DTR-GFP
DEREG mouse:
Foxp3 dependent Diphtheria toxin receptor expressing mouse
Naive T cells of DEREG mouse
Foxp3·DTR
CD4+CD45RBhigh
+iTreg
(Mock/Cebpβ)
Rag-/-
Diphtheria Toxin injection

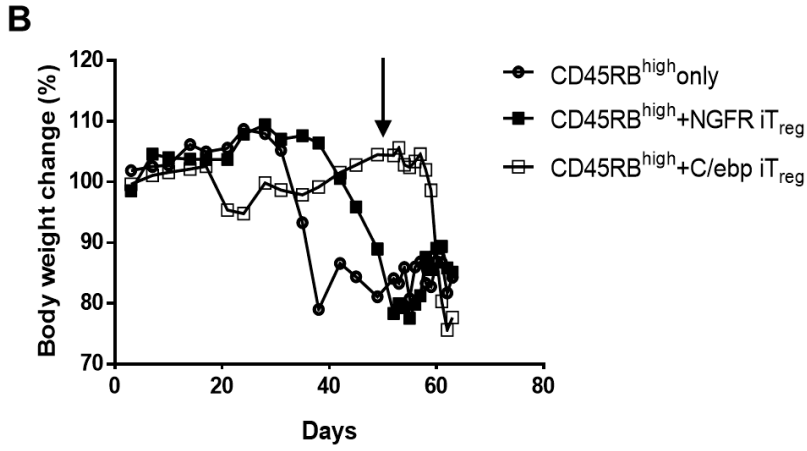
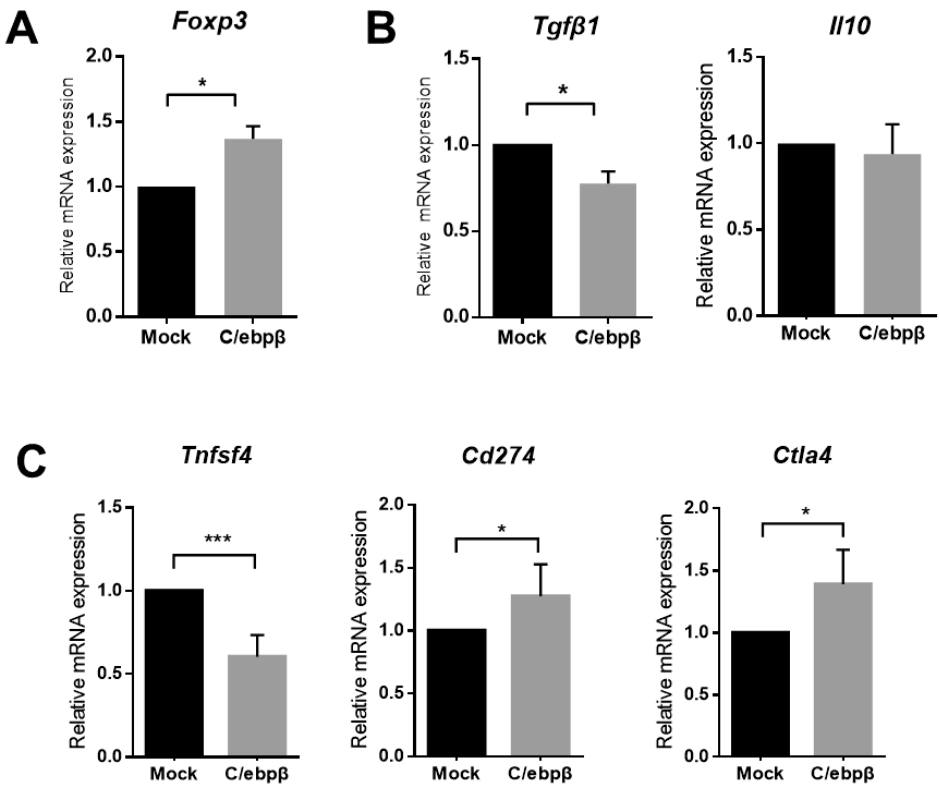

B
Body weight change (%)
120
110
100
90
80
70
0
20
40
60
80
Days
CD45RBhighonly
CD45RBhigh+NGFR iTreg
CD45RBhigh+C/ebp iTreg

Figure 23. C/EBPβ overexpression affected to the mRNA expression of the genes related to the induction of pTreg T cells

(A and B) Quantitative real-time PCR analysis of *Foxp3, Tgfβ1, Il10* in FACS sorted iTreg cells that were retrovirally transduced mock-vector (black bar), or C/EBPβ gene (gray bar). Data represent the average ± SEM of six independent experiments; **P* <0.05

(C) Quantitative real-time PCR analysis of *Tnfsf4* (OX40L)*, Cd274* (PD-L1)*, Ctla4* (CTLA4) in FACS sorted iTreg cells that were retrovirally transduced mock-vector (black bar), or C/EBPβ gene (gray bar).). Data represent the average ± SEM of six independent experiments; **P* <0.05, ****P*<0.001


A
Foxp3
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
Mock
C/ebpβ
B
Tgfβ1
Relative mRNA expression
1.5
1.0
0.5
0.0
*
Mock
C/ebpβ
Il10
Relative mRNA expression
1.5
1.0
0.5
0.0
Mock
C/ebpβ
C
Tnfsf4
Relative mRNA expression
1.5
1.0
0.5
0.0
***
Mock
C/ebpβ
Cd274
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
Mock
C/ebpβ
Ctla4
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
Mock
C/ebpβ

(Francisco et al., 2009). Based on these studies I examined the mRNA expression of those genes. Strikingly, mRNA expression of CTLA4 and PD-L1 increased in C/EBPβ-iTreg while that of OX40L decreased significantly (Figure 22C). Taken together, this result supports the idea that changes in the expression of certain cell surface signaling molecules might have affected pTreg cell differentiation by cell-cell contact dependent manner in the intestine

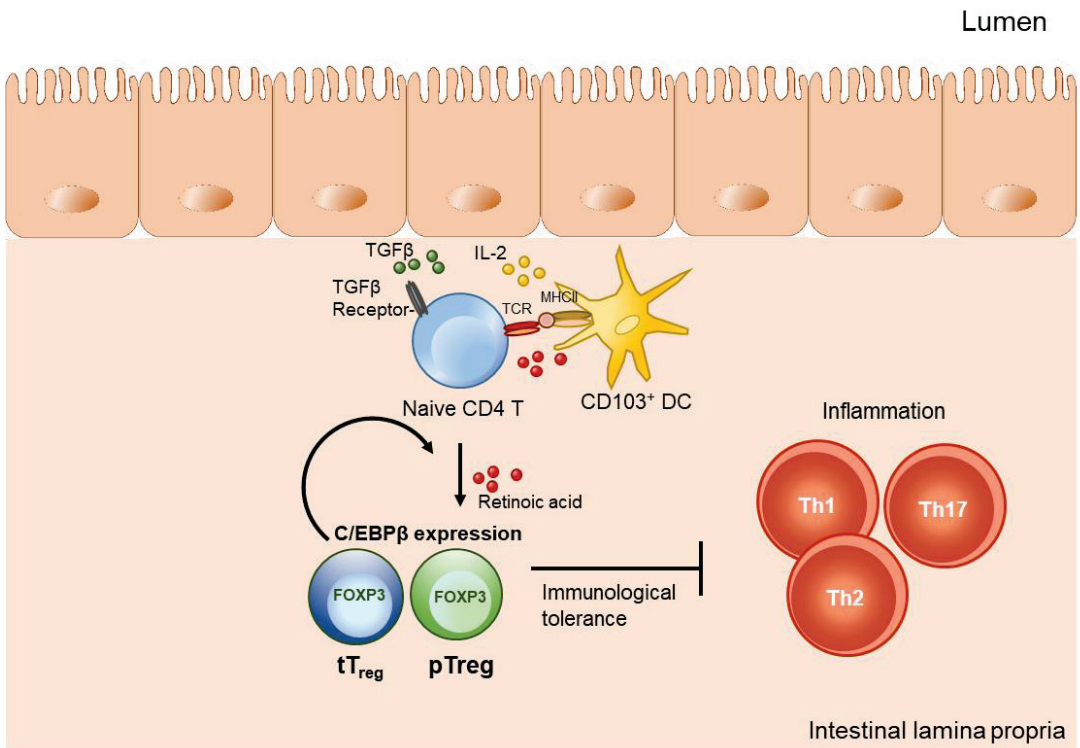


Figure 24. A model of regulatory T cell in colon promotes pTreg differentiation via C/EBPβ expression

DISCUSSION

Foxp3^{+} regulatory T cells ensure B lymphopoiesis by inhibiting the granulopoietic activity of effector T cells in mouse bone marrow

Here I show that normal B lymphopoiesis in the bone marrow (BM) requires regulatory T cells. I found that hematopoiesis in Treg deficient BM was arrested at the ST-HSC stage as well as at the pre-pro B (fraction A of pro-B) stage, and progenitors formed committed lineages biased toward myeloid cell fates over lymphoid cell fates. In addition, the ratio of GMPs to MEPs was also biased. These results suggest that the activity of Treg cells is essential at several checkpoints in the developmental pathways, including the conversion of the most primitive progenitors to LMPPs.

Despite the severe impairment of B lymphopoiesis in the BM of scurfy mice, I found that a few pro-B cells still emerged, and these were mostly at the Fr. A stage unlike those in the wild type. This phenomenon may be due to an additional developmental arrest at the Fr. A stage of pre-B cells. However, in the light of a previous report that 20–40% of B220^{lo}CD19^{-} cells are NK cell progenitors (Rolink et al., 1996), I cannot rule out the possibility that the increase in Fr. A cells (B220^{lo}IgM^{-}CD43^{+}HSA^{-}BP-1^{-}) in BM of scurfy reflects an increase in NK cell progenitors.

An interesting finding is that BM of scurfy mice contained more GMPs than BM of control mice, despite reduction CMPs—their immediate progenitors. This may indicate that the deficiency of Treg cells leads the

CMPs to give rise predominantly to GMPs at the expense of the MEP fate. It is also possible that an alternative pathway to developing GMPs was activated in the BM of scurfy mice, and I suspect LMPPs were the progenitors, since they were previously reported to give rise not only to CLPs but also to GMPs (Adolfsson et al., 2005). If that is true, it may mean that Foxp3 deficiency licenses LMPPs to preferentially differentiate to GMPs at the expense of the alternative fate: differentiation to CLPs. These data also explain how the rate of reduction of CLP numbers was much greater than that of LMPPs in the scurfy mice.

Unlike the regulation of myeloid lineages demonstrated previously (Urbieta et al., 2010), the mechanism underlying the effect of Tregs seems not to include their direct action on progenitors, because they did not directly alter the developmental fate of LSK cells cultured under B cell polarizing conditions. Rather, Treg cells exerted their effects by constraining the activity of effector T cells. Apart from their action on Tregs, effector T cells directly affect B lymphopoiesis through the cytokines they produce. Thus, the mechanism by which marrow Treg cells protect normal B lymphopoiesis seems to largely rely on suppressing cytokine production by effector T cells.

IL-6 has been reported that its levels were elevated in systemic lupus erythematosus (SLE) model mice and accompanied by losses of B cells and CLPs. This defect in B lymphopoiesis was recovered in IL-$6^{-/-}$ background.

In this SLE mice, IL-6Ra was shown to be expressed on HSCs and MPPS and signal of IL-6 induced Id1, which has been known to bind to E2A so as to inhibit B lymphopoiesis in MAP kinase dependent way (Maeda et al., 2009). This result implicates that IL-6 affects B lymphopoiesis by acting directly on HSCs. On the contrary, a notable report showed that IL-6 inhibited IL-7 induced colony forming unit(CFU) formation of whole BM cells while CFU of $B220^{+}IgM^{-}$ cell was unaffected (Fernandez et al., 2003), which indicates that IL-6 inhibits B lymphopoiesis in indirect way.

It has been reported that when GM-CSF injected into mice great reduction of bone marrow B cells and immediate progenitors was observed (Dorshkind, 1991). GM-CSF is reported to be secreted from T, B, and epithelial cells (Becher et al., 2016) and its receptor (GM-CSFR) is expressed on GMPs, and myeloid lineage cells but not in T,B, NK, and DC cells (Becher et al., 2016; Iwasaki-Arai et al., 2003). It is noteworthy that GM-CSF enhances IL-6 production in DCs and macrophage (Sonderegger et al., 2008). This is because the result indicates that GM-CSF can affect lymphopoiesis through IL-6. Although forced expression of GM-CSFR on CLP led differentiation in to myeloid cells (Iwasaki-Arai et al., 2003), still there is no physiological evidence that GM-CSF directly abrogates B lymphopoiesis. TNFα directly inhibits IL-7 induced CFU of $B220^{+}IgM^{-}$ cells (Fernandez et al., 2003).

Given that my data of LSK co-culture with OP9 bone marrow stromal cells

showed inhibitory effect of GM-CSF, IL-6, and TNFα on lymphopoiesis, although the inhibitory effect of such cytokines is evident, but whether such effect acts on progenitors or stromal cells is not clear. Interestingly, it has been reported that hematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. The study showed osteoblasts promotes the maintenance of lymphoid progenitors. While HSCs niches are perivascular mesenchymal stromal cells and endothelial cells (Morrison and Scadden, 2014).

In accordance with these findings, deletion of heterotrimeric G protein α subunit in osteoblast attenuated IL-7 expression and resulted in impaired B lymphopoiesis, showing attenuated pre-pro B cells and significant decrease of pro-B cells, pre B cells, and total $B220^{+}$ B cells. In addition, CXCR4 deficiency in HSC led substantial reduction in CLP population. Therefore, it is a possible explanation of disrupted B lymphopoiesis in scurfy mouse that elevated proinflammatory cytokines affected the expression of CXCL12, IL-7 in the bone marrow osteoblasts.

Indeed, a recent study showed that IL-7 expression on subpopulation of ICAM1 perivascular stromal cells were decreased in Treg-depleted mice, thereby leading to disruption of B lymphopoiesis (Pierini et al., 2017). In my study, treatment of GM-CSF, IL-6, and TNFα showed inhibition of $B220^{+}CD19^{+}$ B cell development in vitro co-culture. Furthermore, administration of neutralizing antibodies of those cytokines partially rescued

of common lymphoid progenitor cells and LMPPS in scurfy mouse. Given the aforementioned studies, it is possible that proinflammatory cytokines affected bone marrow stromal cells, which compose niche of lymphopoiesis, resulting in B cell defect in scurfy mouse. A study that showed that TNFα inhibits osteoblast differentiation and induces osteoclastogenesis (Gilbert et al., 2000; Zhao et al., 2012) also supports this view. However, it is still possible that IL-6 directly affect progenitor cells by inducing Id1 expression through MAPK pathway, so that lead granulopoiesis in the expense of B cell differentiation. Furthermore, TNFα affected on IL-7 dependent B lymphopoiesis directly to progenitor cells (Fernandez et al., 2003). In conclusion, impaired B cell development in scurfy mice bone marrow can be a result of direct or indirect effect of proinflammatory cytokines. Intriguingly, the action of IFN-γ, whose level was also enhanced in the K/Bsf BM, was different from the action of the cytokines mentioned above. Like them, IFN-γ interfered with the maturation of B cells, in line with previous observations indicating that it has a negative effect on B lymphopoiesis (Garvy and Riley, 1994; Grawunder et al., 1993); however, surprisingly, this was not coupled with enhanced emergence of myeloid-lineage cells.

Gene profiling studies in LSK cells including HSC and MPPs (possess full myeloid and lymphoid lineage differentiation potentials) showed that HSCs expressed megakaryocyte, erythrocyte, and granulocyte, myeloid related

genes, but not lymphoid genes (Adolfsson et al., 2005). Moreover, this result is supported by many other studies. One of such studies showed the first hematopoietic stem cells appeared during embryonic development have potential to differentiate into erythroid and macrophages but no lymphoid cells. That means that default genetic settings of hematopoietic lineage commitment is set to myeloid lineage cells. These results may show the evolutionary aspect of nature that innate immune cells precede adoptive immune cell formation during evolution. This perspective is in agreement with our data that without safeguard of regulatory T cells, granulopoiesis dominates hematopoiesis.

In conclusion, I demonstrate here that Treg cells ensure B lymphopoiesis in the BM by suppressing cytokine production by effector T cells. I suggest a novel role for bone marrow Treg cells in B-cell homeostasis (Figure 13).

CEBPβ-overexpressed iTreg cells mitigate inflammatory bowel disease by promoting pTreg differentiation

Because the intestine is the site of continuous antigen challenge, many lymphocyte populations cooperatively function in defense against external pathogens. However, uncontrolled immune responses may cause tissue damage and inflammation. Therefore, diverse suppressive cells in intestine have a role to maintain the immune homeostasis. Here, I showed that expression of C/EBPβ, which is induced in intestinal Treg cells, is involved in pTreg cell induction to regulate intestinal inflammation.

A previous study of our group demonstrated that C/EBPβ is expressed on iTreg in RA dependent manner. Further, retinoic acid, which is the metabolite of vitamin A, is essential for generation of antigen-specific pTreg cells in intestine. I checked whether expression of C/EBPβ is up-regulated in intestinal Treg cells rather than the Treg cells found elsewhere and pTreg cell induction is different with or without C/EBPβ expression. As a result, intestinal Treg cells expressed C/EBPβ highly when compared with splenic Treg cells and pTreg cells induced from naive CD4+ T cells in intestinal inflammation was increased in mice reconstituted with C/EBPβ-iTreg cells (Figure 14A).

These results are in the same context as the previous study, which reported

an abrogated antigen-specific pTreg cell generation in the intestine of mice fed without vitamin A (Hall et al., 2011). However, $CD103^+$ dendritic cells are reported to have preferential roles in pTreg cell generation by producing RA and active form of TGFβ (Coombes et al., 2007; Hall et al., 2011; Sun et al., 2007). In addition, CX_3CR1^+ macrophages support pTreg cell generation in intestine by expressing IL-10 in microbiota dependent manner (Kim et al., 2018). Thus, the previous studies revealed major pathways of the pTreg cell generation (Esterhazy et al., 2016). Nevertheless, given that a difference in pTreg cell generation between the

mice received control-iTreg cells and the mice received C/EBPβ-iTreg was apparent, it is evident that C/EBPβ-iTreg cells play a role in differentiation of pTreg cells (Figure 14 B-G).

Our group previously demonstrated that C/EBPβ binds to methyl TSDR of Foxp3 locus, and thereby enhances and sustains the Foxp3 expression in the inhibitory cytokines environment(Lee et al., 2018). However, it has been reported that expression of C/EBPβ and hepatocyte proliferation are mutually exclusive (Buck et al., 1994), and forced expression of C/EBPβ in hepatoma cells induced cell cycle arrest (Buck et al., 1994). Moreover, C/EBPβ deficient mice displayed epidermal hyperplasia (Songyun et al., 1999). The phosphorylation of serine 273 of C/EBPβ leads homodimerization, thereby promoting cell cycle arrest (Lee et al., 2010). Therfore, I tested whether forced expression of C/EBPβ on iTreg cells can

make the expression of Foxp3 stable in inflammatory environment in vivo as shown in previous in vitro experiments and whether C/EBPβ overexpression influences iTreg survival via the cell cycle arrest. I found not only in vivo-transferred C/EBPβ-iTreg cells showed stable expression of Foxp3 even in the intestinal inflammation environment as previously reported (Lee et al., 2018), but also showed increased cell death caused by cell cycle arrest (Figure 18 A-D).

This increased cell death significantly reduced the frequency of iTreg cells (Figure 18C). Interestingly, however, the reduction in the frequency of these iTreg cells did not lead to a reduction in the total Treg cell ratio. Although the increase in cell death has significantly reduced the frequency of iTreg cells, the total amount of Treg cells of mice reconstituted with C/EBPβ-iTreg cells has been found to be similar compared with mice reconstituted with control iTreg cells due to more pTreg cells generated. (Figure 19 A-C).

Although retaining similar frequency of Foxp3+ Treg cells (Figure 19 A-C), colitis-induced mice reconstituted with C/EBPβ-iTreg cells showed clear improvement of intestinal inflammation compared with the mice reconstituted with control-iTreg cells (Figure 20 A-E). Further, I confirmed the decreased inflammatory cytokine-expressing cell frequencies in intestine of the C/EBP-iTreg transferred mice (Figure 21 A-D). However, this inflammation-suppressing effect in mice reconstituted with C/EBPβ-iTreg cells was lost after specific deletion of pTreg (Figure 22 B). The intestinal

suppressive cells are composed of IgA secreting plasma cells, IL-10-secreting Tr-1 cells, ILC reg, and Treg cells. In my result, Tr-1 cells also increased in mice reconstituted with C/EBPβ-iTreg cells and these Tr-1 cells may also have contributed to ameliorate colitis (Figure 16 A and B). Nevertheless, the result that selective deletion of pTreg cells led to rapid disruption of immune suppression indicates that the major suppressor in inflammatory intestine is pTreg cells, not Tr-1 cells in experimental IBD condition (Figure 22).

Finally, qPCR experiment revealed that forced expression of C/EBPβ changed the gene expression of signaling molecules related with pTreg cell induction. My study therefore reports here that overexpressed C/EBPβ on iTreg cells promotes pTreg cell induction in trans manner thereby suppresses inflammation of the intestine sufficiently.

I reported here that forced expression of C/EBPβ resulted in increased cell death in iTreg cells that were transferred into colitis-induced mice (Figure 17A-C). On the other hand, I also showed increased CTLA4 expression in C/EBPβ-iTreg cells. It is well studied that CTLA4, an inhibitory co-stimulation molecule, restricts cell cycle transition from the G1 to S phage progression (Krummel and Allison, 1996). Therefore, it is possible that ectopic expression of C/EBPβ caused cell cycle arrest-induced cell death by up-regulating the expression of CTLA4. Thus, in sum, it is possible that increased cell death in C/EBP-iTreg is caused by C/EBPβ in a direct or

indirect manner.

Inflammatory cytokine TNF has been reported to down regulate the Foxp3 expression in Treg cells (Bilate and Lafaille, 2012; Valencia et al., 2006). In this study, I showed forced expression of C/EBPβ increased the mRNA expression of Foxp3 (Figure 23A). This result means that ectopic expression of C/EBPβ in iTreg cells can overcome the inhibition of Foxp3 expression presumably by binding methyl TSDR so that promote the expression of Foxp3 (Lee et al., 2018). Concurrently, as pTreg cell induction-promoting signals (PD-L1, CTLA4) increased by C/EBPβ (Figure 23C), more pTreg cells would be generated. A previous study reported that major cell source of TNF is Th1, Th2, macrophage, and dendritic cells in the inflammatory intestine (Neurath, 2014) and at least in my experimental settings of CD45RB transfer model, most of TNF+ cells (>80%) were CD4+T cells. Because antigen specificity physically led Treg cells close to effector cells, it is possible to explain that antigen specificity of pTreg cells presumably made it get close to TNF-secreting effector CD4 T cells so that suppressed the cells (Levine et al., 2017; Liu et al., 2015). To sum up, increased pTreg cell induction also led to great suppression of TNF expression. Furthermore, one possible explanation of increased pTreg cells is that the induction of pTreg cells is a result of decreased TNF cytokine, which suppresses Foxp3 expression. Decreased TNF expression can contribute to the induction of $Foxp3^{+}$ pTreg cells, which means the possibility of the feed forward loop of

pTreg cell induction against TNF suppression in this IBD model.

Although I found the C/EBP expression of colon Treg cells increased when compared with spleen Treg cells, physiological role of C/EBP in colon Treg cells remains to be elucidated. Here I showed that forced expression of C/EBPβ on iTreg cells have function on the induction of pTreg cells in the colon to control intestinal inflammation. Since C/EBPβ of Treg cells is increased in its expression and binds to methyl TSDR locus in RA-rich environment, it is possible that C/EBPβ binds to Foxp3 locus in colon Treg cells to express signalling molecules related to pTreg cell differentiation. To sum up, there is possibility that C/EBP expression on colon Treg cells may form feed forward loop for pTreg cell generation in steady state environment.

Based on the findings and considerations, I suggest that C/EBPβ of intestinal Treg cells has a role in pTreg differentiation so that enhances the suppression against the intestinal inflammation (Figure 24).

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국문초록

골수 및 장내에서 림프구 분화에 대한 조절 T세포의 역할에 대한 연구

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고등동물의 적응성 면역시스템은 외부 병원체의 침입으로부터 호스트의 신체를 보호하게 해주는 매우 강력하면서 정교하게 작동하는 방어수단이다. 그러나 이런 강력한 효과가 자칫 잘못 작동하면 오히려 신체에 손상을 줄 수 있다. 따라서 적응면역계에서는 동시에 이런 부정확한 면역반응을 억제하고 면역항상성을 유지할 수 있는 세포를 만들어낸다. 조절T세포는 이런 자기항원에 대한 면역반응과 과도한 염증반응을 억제함으로서 신체 내 면역관용을 유지하는 대체불가한 역할을 담당하는 특화된 CD4 T세포이다. 따라서 이런 조절 T세포가 어떻게 면역반응을 조절하고 면역항상성 유지에 어떻게 작용하는지를 연구하는 것은 매우 중요한 의미를 갖는다. 이 연구에서 본인은 조절T세포가 골수 내 B세포 분화 및 초기 조혈과정, 그리고 장에서 말초유래조절T세포 생성에서 수행하는 역할을 확인하였다.

첫째로, 조절T세포가 없는 마우스를 이용해 골수내 조혈과정에서 관여하는 조절 T세포의 역할을 확인하였다. 성숙한 혈액세포들의 발달은 최초 조혈모세포로부터 시작되는 순차적인 계열 결정과정을 거치게 된다. 조혈모세포는 골수 내 매우 적은 비율을 차지하고 있고 자가재생산을 통해 일정 수를 유지할 수 있다. 중분화능 전구체세포는 자가재생산 능력이 감소하는 대신 세포 증식이 증가되나 여전히 모든 종류의 혈액세포로의 분화능을 유지하고 있다. 골수성 전구체 세포 및 림프구성 전구체 세포는 중분화능 전구체세포로부터 분화된다. 골수성 전구체 세포는 추가로 분화과정을 거쳐 적혈구, 거핵구, 대식세포, 과립구로 분화가 되고, 림프구성 전구체 세포는 초기 흉선전구세포, 자연살해세포, B 세포로 분화된다. B 림프구 생성은 CD45R발현과 동시에 EBF1 전사인자를 발현하기 시작하는 pre-pro B 세포로부터 시작된다. 이어서 B 림프구는 Pro B세포 Pre B세포 단계를 거쳐 온전한 B세포 수용체를 발현하는 미성숙 B세포로 분화된 후 말초로 나가게 된다. Scurfy 마우스는 조절T세포의 성질과 기능을 관장하는 Foxp3 유전자에 돌연변이가 생겨 조절T세포가 없어서 전신에 걸친 자가면역반응을 보이게 된다. 본 연구에서는 scurfy 마우스의 골수내 조혈과정에 분제가 있는 것을 확인하였고, 특히 B세포 발달과정이 심각하게 손상되어 있음을 확인하였다. 상위단계인 조혈과정을 살펴보았을 때, 림프구성 세포와 골수성 세포로 갈라지는 분화과정에서 문제가 시작됨을 확인하였다. 림프구성 전구체의 수가 굉장히 감소되어 있었고, 반면 골수성 전구체는 증가되어 있었다. 또한 상위단

계인 중분화능 전구체세포와 조혈모세포는 증가되어 있었다. Scurfy 와 정상 쥐의 골수 혼합마우스를 만들어 확인한 결과 조혈 과정 초기부터 손상된 B림프구 생성은 scurfy 마우스의 조혈모세포의 내재적 문제가 아니란 것을 밝혔다. 또한 조절 T세포를 주입해주자 이런 모든 B 림프구 생성이 회복되었다. 시험관내 B 림프구 분화 환경에서 조혈모세포 배양을 이용한 실험으로 활성화된 반응기 T세포 배양액이 B 림프구 생성을 억제함을 확인하였다. 이런 결과를 바탕으로 scurfy 마우스의 골수세포를 꺼내 확인했을 때, 여러 염증성 사이토카인들이 분비되는 것을 확인하였고, 이는 실제 마우스내에서도 그러함을 확인하였다. 이런 염증성 사이토카인들 중에 특히 IL-6, TNF, GM-CSF가 B세포 분화를 강력하게 억제하는 것을 확인하였고, 이들을 시험관 내 실험 및 실제 마우스에서 중화시키자 B 세포 분화가 회복되는 것을 확인하였다. 이 데이터들은 조절 T세포가 염증성 사이토카인들을 억제함으로서 B 림프구 생성이 되도록 한다는 사실을 알 수 있게 한다.

다음으로 조절 T세포가 장에서 말초유래 조절T세포 생성에서의 역할을 연구하였다. 조절T세포는 외부 항원과 병원체로 노출되어 끊임없는 면역반응이 유도되는 장에서 면역억제 기능을 담당하고 있다. 대장은 공생박테리아가 풍부하게 존재하고 이에 반응하여 면역체계가 발달되어 있다. 장에서 조절 T세포는 다른 조직에 비해 그 비율이 상당히 높은데(30% 이상)이는 외부 항원에 지속적으로 노출되는 장의 특성일것이다. 이 중에 상당수(60%정도)가 무경험 CD4 T세포가 국지적인 항원에 반응해서

생성되는 말초유래 조절 T세포이다. 더욱이 이런 말초유래조절T세포는 장 면역항상성에 중요한 역할을 수행하고 있다. 본연구에서는 장으로 모인 유도성 조절T세포의 C/EBPβ 발현이 말초유래 조절T세포 생성에 역할을 하여 장 염증반응을 억제함을 밝혔다. 본인은 비장의 조절 T세포에 비해 장 내 조절 T세포의 C/EBPβ발현이 높은 것을 확인하고 조절 T세포에서의 C/EBPβ 발현의 기능을 확인해 보고자 하였다. C/EBPβ 를 과발현 시킨 유도성 조절 T세포를 장 염증을 유발시킨 마우스에 주입해 주면 말초유래 조절 T세포 생성이 유의미하게 증가됨을 확인하였다. 동시에 C/EBPβ 를 과발현 시킨 유도성 조절 T세포는 Foxp3발현에 있어서 안정성을 보였으나 세포사멸의 증가로 인해 전체 양을 현격한 감소를 보였다. C/EBPβ 를 과발현으로 인한 서로 다른 이 두가지 효과는 장내 $Foxp3^+$ 조절 T세포의 총량이 비슷하게 되는 결과로 이어졌다. 그러나 이런 비슷한 $Foxp3^+$ 조절 T세포의 양에도 불구하고, C/EBPβ 를 과발현 시킨 유도성 조절 T세포가 주입된 마우스에서 장염증이 확연히 완화된 것을 확인하였고, 이런 장 염증개선은 그러나, 말초유래 조절 T세포를 선택적으로 제거해 주었을 때 급격히 무너지는 것을 보였다. 이 결과는 C/EBPβ 를 과발현 시킨 유도성 조절 T세포가 들어간 마우스에서 보인 염증완화는 실제로는 말초 유래조절 T세포의 역할이었음을 의미한다. 마지막으로 본인은 C/EBPβ 를 과발현 시킨 유도성 조절 T세포에서 말초유래 조절 T세포 생성을 촉진하는 신호분자들의 발현이 달라지는 것을 mRNA 수준에서 확인하였다. 이런 결과들은조절 T세포에서 C/EBPβ

발현이 trans 한 방식으로 말초유래 조절 T세포 분화를 촉진할 수 있는 가능성을 암시한다.

핵심어: 조절 T세포, B 세포 발달, 조혈, C/EBPβ, 장염증, 말초유래 조절 T세포

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