博士論文

Studies on immunosuppressive mechanisms in the intestine

（腸管における免疫抑制機構に関する研究）

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Contents

Abbreviations

APCs	antigen presenting cells
BEC	blood endothelial cells
BSA	bovine serum albumin
BrdU	5-Bromo-2-deoxyuridine
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CFSE	5, 6-carboxyfluorescein-diacetate succinimidyl ester
DC	dendritic cell
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FDC	follicular dendritic cells
FITC	fluorescein isothyocyanate
Flt3L	fms-related tyrosine kinase 3
Foxp3	forkhead box protein P3
FRC	fibroblastic reticular cells
GALT	gut-associated lymphoid tissue
GF	germ free
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
HEV	high endothelial vennule
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	interferon-γ
IFR	interfollicular region
Ig	immunoglobulin
IL	interleukin
LEC	lymphatic endothelial cells
LN	lymph node
LP	lamina propria

LPS	lipopolysaccharide
MACS	magnetic cell sorting
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MLN	mesenteric lymph node
OVA	ovalbumin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-L	programmed death-1 ligand
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PFA	paraformaldehyde
PGE2	prostaglandin E2
PLN	peripheral lymph nodes
PP	payer's patch
RA	retinoic acid
RAG-2	recombination-activating gene 2
ROR	retinoic acid–related orphan receptor
SED	subepithelial dome
SLO	secondary lymphoid organs
SPF	specific pathogen free
SPL	spleen
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Tg	transgenic
Th	T-helper
TLR	Toll-like receptor
Treg	regulatory T cell
Tr1	T regulatory type 1

General introduction

The intestine is exposed continuously to vast amounts of foreign antigens, such as food proteins or pathogens. Therefore, the intestinal immune system has to discriminate between generating protective immunity against harmful antigens and tolerance against harmless materials. There are several distinctive features of the gut immune system that participate in the tolerogenic environment (1). The inductive sites for immune responses in the gut are Peyer's patches, which are macroscopic lymphoid aggregates in the submucosa along the length of the small intestine and mesenteric lymph nodes (MLNs), which are the largest lymph nodes in the body. MLNs develop distinct from Peyer's patches and peripheral lymphoid nodes and serve as a crossroads between the peripheral and mucosal recirculation pathways. A single layer of epithelial cells separates the gut microflora from the main elements of the gut immune system. Many immune cells are present in the lamina propria (LP) beneath the epitherial layer. These organs and tissues of the intestinal immune system are refered to as the gut-associated lymphoid tissues (GALT) (Fig. 0).

To induce a mucosal immune response, antigen must gain access to antigen-presenting cells by penetrating the mucus layer and then the intestinal epithelial cell barrier. Uptake of antigen occurs through a variety of mechanisms including M cells associated with Peyer's patches and uptake by columnar epithelial cells. In addition, it has been shown that dendritic cells (DCs), which are one of the most important antigen presenting cells, sample luminal contents by extending their processes through the epithelium without disruption of tight junctions (2). Major factors that condition the gut to be a tolerogenic environment are interleukin-10 (IL-10), retinoic acid (RA), and transforming growth factor-β (TGF-β).

Oral tolerance refers to physiologic induction of tolerance that occurs in the GALT. The phenomenon of oral tolerance has been known for hyporesponsiveness to a fed antigen on subsequent challenge with that antigen. Oral tolerance presumably evolved to prevent hypersensitivity reactions to food proteins. It is now recognized that there are multiple mechanisms of oral tolerance, induction of regulatory T cells (Treg), anergy, or deletion. Forkhead box protein 3 (Foxp3) is a key transcription factor for regulatory T cells, and TGF-β is now recognized as a key cytokine in the induction of $Foxp3^+$ Tregs. It has also become clear that the GALT is a rich and complex immune network that has evolved to

induce Tregs and immunological tolerance.

In addition to T cells, several cell types have been demonstrated to be involved in the induction of oral tolerance. It has been known that expanding DCs *in vivo* enhances the induction of oral tolerance (3). Moreover, LN transplantations revealed that *in vivo* the non-hematopoietic stroma network provides an indispensable scaffold shaping and directing immune responses (4)(5). However, their functional relationship and dynamics in inducing oral tolerance remain unknown. In this study, we aimed to reveal the immunosuppressive mechanisms in inducing oral tolerance focusing on relationships of distinct cell populations, and microenvironment of intestinal immune system.

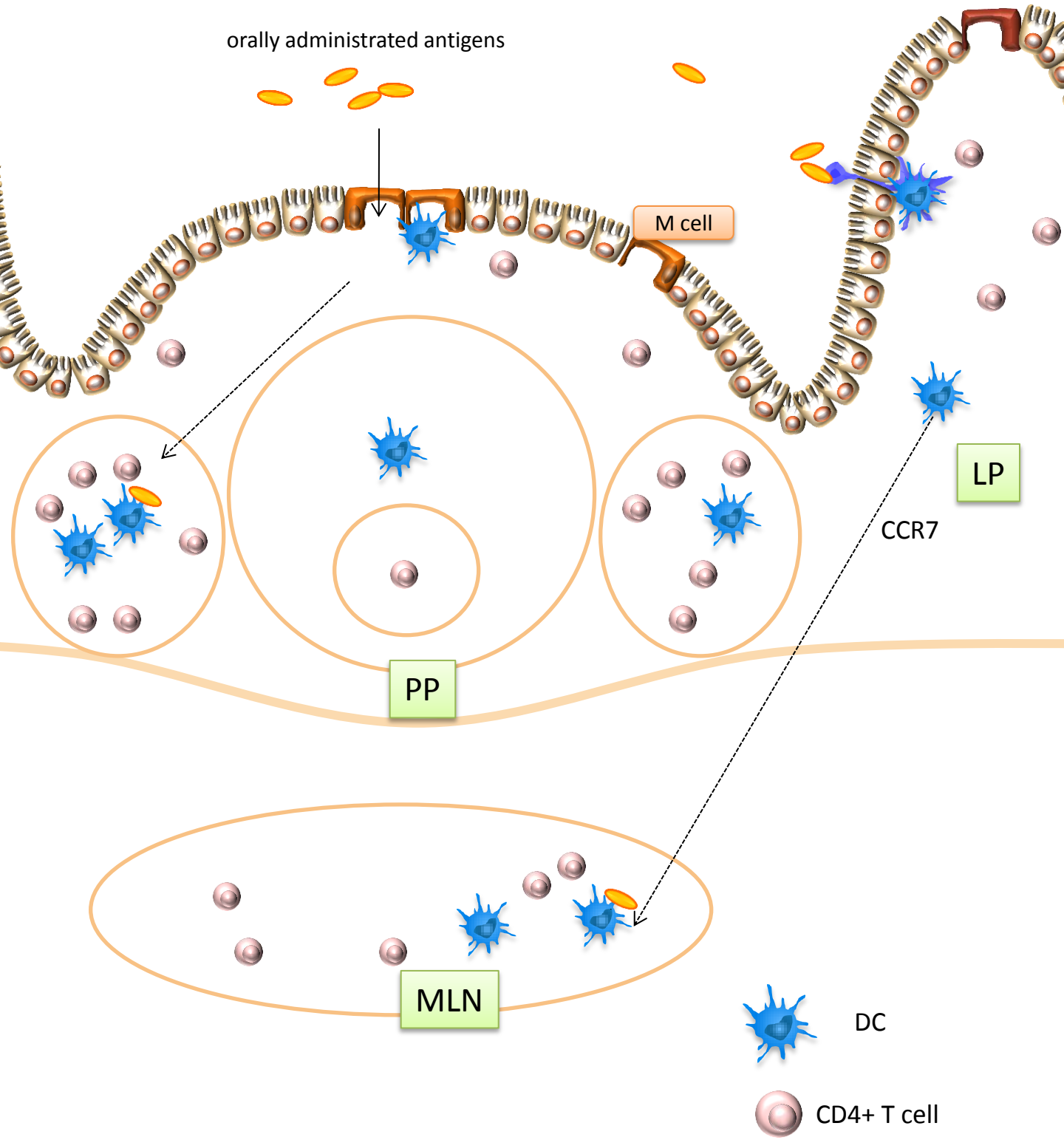

Figure 0. structure of GALT and Ag uptake

Chapter 1

Three different CD103$^+$ dendritic cell subsets in mesenteric lymph nodes have distinct function in intestinal immune regulation

1.1. Introduction

The intestine is continually exposed to antigens (Ag) from food proteins and from commensal or pathogenic bacteria. Strict regulation is thus required in the intestine, and oral tolerance represents a unique aspect of the intestinal immune system. Oral tolerance has been defined as the specific inhibition of cellular or humoral immune responses to an Ag by prior administration of Ag via the oral route, and likely evolved to prevent hypersensitivity reactions to food proteins. Oral tolerance results in Ag-specific T cell deletion, anergy, or induction of regulatory T cells (Tregs) (6), and especially Foxp3$^+$ regulatory T cells (Treg) are critical for oral tolerance induction (7). These T cell modulations are mostly dependent on the multiple signals between Ag-presenting cells (APC) and Ag-specific T cells. Dendritic cells (DCs) are essential APC that initiate primary immune response, and likely play a pivotal role in inducing oral tolerance (3). Moreover, there are several reports of DC subsets involved in inducing oral tolerance (8)(9)(10)(11)(12)(13).

Although orally administered Ag can be presented to T cells by DC in Peyer's patches (PP) and mesenteric lymph nodes (MLNs), it has been proposed that oral tolerance requires MLN (14)(15). Therefore Ag presenting DC in MLN are thought to be critical for modulating T cell response. It has been reported that Ag-captured LP DC migrate to the MLN in a CCR7-dependent manner (16)(17), however, the principal DC subsets to present oral administrated Ag in MLN remain to be fully elucidated.

CD11b, CD103, and CD8α are often used surface markers to identify DC populations (18)(19). We and others previously reported that CD11b$^+$ DC are important for oral tolerance induction in PP (9)(20). In addition, it has been shown that intestinal CD103$^+$ DC play a crucial role in oral tolerance (21). Intestinal CD103$^+$ DC highly expressed retinal dehydrogenase isoform 2 (Raldh2), which catalyzes the conversion of retinal to retinoic acid

(RA) (22). $CD103^+$ DC isolated both from the lamina propria (LP) and the MLN have been shown to drive preferentially $CD4^+Foxp3^+$ T regulatory cells via a transforming growth factor β (TGF-β) and RA dependent mechanism (23)(24)(25). In addition to $CD103^+$ DC, programmed death-1 ligand-1 (PD-L1) expressing MLN DCs are also essential for establishing oral tolerance through the induction of Ag-specific $CD4^+Foxp3^+$ Tregs (10)(26). However, the relationship of $CD103^+$ DC and $PD\text{-}L1^+$ DC remains unclear.

The aim of this study is to clarify the phenotype and role of these DC subsets for intestinal immune regulation, especially for oral tolerance induction. In this study, we demonstrated that $CD103^+$ DC in MLN are divided into phenotypically distinct three populations by CD11b and PD-L1 expression.

1.2. Materials and Methods

Mice

Balb/c mice were purchased from CLEA Japan (Tokyo, Japan). Balb/c mice and DO11.10 TCR transgenic mice (27) were used at 7–20 weeks old. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of The University of Tokyo. The protocol was approved by the Animal Use Committee of the Faculty of Agriculture at The University of Tokyo (approval number: P11-533).

Culture medium, antibodies, and reagents

Cells were cultured in RPMI 1640 medium supplemented with 5 % heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-mercaptoethanol. The following antibodies were used: Allophycocyanin (APC)-labeled anti-CD11c (N418, BioLegend), FITC-labeled anti-CD11b (M1/70, BioLegend), PE-labeled anti-PD-L1 (10F.9G2, BioLegend), biotinylated anti-CD103 (2E7, BioLegend), streptavidin-PE-Cy5 (BD Pharmingen), anti-CD3ε (145-2C11), anti-CD28 (37.51, BD Pharmingen), anti-IL-12 (C17.8)

Preparation of DCs

DC were prepared from spleen (SPL) and MLN. For isolation os SPL and MLN cells, SPL and MLN were incubated for 60 min at 37 ºC with collagenase (0.5 mg/ml; Wako, 032-10534) in RPMI containing 5 % FCS, and single cell suspensions were prepared. For isolation of $CD11c^+$ cells, cells were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech) and selected on magnetic activated cell sorting (MACS) separation columns (Miltenyi Biotech). For isolation of DC subpopulations, cells selected on the basis of CD11c expression by MACS were stained with CD11b-FITC, PD-L1-PE, and CD103-bio-Cy5. $CD103^+$ $PD\text{-}L1^+CD11b^+$ DC, $CD103^+PD\text{-}L1^+CD11b^-$ DC, $CD103^+PD\text{-}L1^-CD11b^-$ DC, and $CD103^-PD\text{-}L1^+CD11b^+$ DC were sorted by FACS using a FACSVantage (BD Bioscience).

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from MACS-purified SPL CD11c$^+$ cells and FACS-sorted MLN DC subpopulations using QIAshredder and RNeasy Mini Kits (both from Qiagen). Single-stranded cDNA was synthesized using Superscript II reverse transcriptase and Oligo dT primers (both from Invitrogen). Quantitative PCR reactions were performed using Quantitect Primer Assays with SYBR green PCR mastermix (Qiagen) with the following primers : Raldh2 forward, 5'-GACTTGTAGCAGCTGTCTTCACT-3'; reverse, 5'-TCACCCATTTCTCTCCCATTTCC-3': IL-27p28 forward: 5'-GGCCAGGTGACAGGAGACC-3', reverse: 5'-CAGCTTGTACCAGAAGCAAGGG-3': IL-6 forward, 5'-TGGAGTCACAGAAGGAGTGGCTAA-3'; reverse, 5'-TCTGACCACAGTGAGGAATGTCAA-3': IL-23p19 forward, 5'-AATAATGTGCCCCGTATCCA-3'; reverse, 5'-CTGGAGGAGTTGGCTGAGTC-3': IDO1 forward: 5'-TCCAGTGCAGTAGAGCGTTCA-3', reverse: 5'-GAAAAACGTGTCTGGGTCCA-3': IL-10 forward, 5'-CCCAGAAATCAAGGAGCATTTG-3'; reverse, 5'-CATGTATGCTTCTATGCAGTTG-3': IL-12p35 forward, 5'-TGGCTACTAGAGAGACTTCTTCCACAA-3' ; reverse, 5'-GCACAGGGTCATCAAAGAC-3': Foxp3 forward, 5'-GAGTTCTTCCACAACATGGA-3', reverse: 5'-TTTCATTGAGTGTCCTCTGC-3': CCR7 forward, 5'-GTGTGCTTCTGCCAAGAT GA-3'; reverse, 5'-CCACGAAGCAGATGACAGAA-3': CCR9 forward, 5'-TGCAGGCTGTTGACGCTTATG-3'; reverse, 5'-CATCCCAGGTTCTTCAGGGTC-3': RORγt forward, 5'-CCGCTGAGAGGGCTTCAC-3', reverse: 5'-TGCAGGAGTAGGCCACATTACA-3': HPRT forward, 5'-GAAGAGACTGGGGATCACTC-3'; reverse, 5'-CATGCCATCTTCCATATTGT-3': with the LightCycler (Roche). Gene expression levels for each individual sample were normalized to hypoxanthine phosphoribosyltransferase (HPRT).

DC-T cell cocultures

SPL CD4$^+$ T cells (2×10^5 cells) from DO11.10 mice were cultured with sorted MLN DC

subsets (2×10^4 cells) or SPL $CD11c^+$ DC (2×10^4 cells) and OVA peptide (0.3 μM) in the absence or presence of TGF-β (2 ng/ml) in 96 well plates for 72 h or 4d. LE540 (1 μM) or anti-IL-12 (5 μg/ml) was added to culture wells in some cases. Supernatants were collected, and IFN-γ, IL-4, and IL-10 levels were measured by ELISA. Cultured cells were harvested and T cells were expanded for an additional 48 h by plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml). Expanded T cells were measured for Foxp3, CCR9, and RORγt mRNA expression by quantitative RT-PCR.

Measurement of cytokine secretion

Cytokine levels in the culture supernatants were assayed by specific sandwich ELISA. IL-4 and IFN-γ were measured as previously described (28). An OPTEIA ELISA set (BD Biosciences) was used for IL-10 measurement.

Oral antigen administration and T cell proliferation assay

Balb/c mice were fed with OVA (Wako; Albumin, from Eggs, 012-09885) via drinking water (100 mg/ml) for 5 days. The daily intake of OVA was estimated to be around 100 mg/mouse. The endotoxin level of this material was tested by *Limulus* amoebocyte lysate assay using an ET-5000 Toxinometer (Wako) and was confirmed to be below approximately 1 endotoxin unit/mg. SPL $CD4^+$ T cells (2×10^5 cells) from DO11.10 mice were cultured with sorted untreated-MLN DC subsets (2×10^4 cells) and OVA peptide (0.3 μM) or sorted DC subsets (2×10^4 cells) from OVA-fed Balb/c mice without additional OVA peptide. After 48 h, 37 kBq of ^{3}[H] thymidine was added per well, and the plates were cultured for an additional 24 h. Subsequently, cells were harvested using a March III harvester (Tomtec, Hamden, CT), and incorporated ^{3}[H] thymidine was counted using a Trilux1450 Microbeta counter (Wallac, Gaithersburg, MD) and Microbeta 270.004 software (Wallac).

Adoptive cell transfer and LP cell preparation

For DC transfer, two MLN DC subsets (5×10^5 cells) from OVA-fed DO11.10 mice were

injected intravenously into untreated DO11.10 mice. 7 days after, LP cells were prepared, and Foxp3 expression in LP $CD4^+$ T cells were measured. For isolation of LP cells, PP were excised, and the small intestine was opened longitudinally and cut into 3–4 cm pieces. Epithelial cells were removed by incubating the tissue for 3×15 min at 37 ºC with 2.5 mM EDTA in HBSS supplemented with 5 % FCS. After each incubation step, tubes were shaken for 10 sec and media containing epithelial cells and debris were discarded. The remaining tissue was incubated for 30-40 min at 37 ºC with collagenase in RPMI containing 5 % FCS. Leukocytes were further enriched by density gradient centrifugation with 44–70% Percoll (GE Healthcare).

Statistical analysis

All experimental data were expressed as the mean ± standard deviation (SD). Statistical differences for all experimental data were analyzed by Student's *t*-tests.

Results

MLN DC subsets were divided into at least four subsets by CD103, PD-L1, and CD11b expression pattern

To clarify the relationship between $CD103^+$ DC, $PD\text{-}L1^+$ DC, and $CD11b^+$ DC, we analyzed expression pattern of these surface molecules. Flow cytometric analysis indicated that many MLN DC are $CD103^+$ and/or $PD\text{-}L1^+$ cells (Fig. 1A). Further analysis showed that $CD103^+PD\text{-}L1^-$ DC did not express CD11b, $CD103^+PD\text{-}L1^+$ DC contained $CD11b^+$ and $CD11b^-$ cells, and $CD103^-PD\text{-}L1^+$ cells expressed CD11b (Fig. 1B). These findings suggest that MLN DC are clearly divided into at least four subpopulations; that is $CD103^+CD11b^+$ $PD\text{-}L1^+$ DC, $CD103^+CD11b^-PD\text{-}L1^+$ DC, $CD103^-CD11b^+$ $PD\text{-}L1^+$ DC, and $CD103^+$ $CD11b^-PD\text{-}L1^-$ DC.

Three $CD103^+$ DC subsets have different character

We next investigated the mRNA expression in above shown MLN DC subsets and SPL DC as a control. In addition to RA, indoleamine-pyrrole 2,3-dioxygenase 1 (IDO1), which is an enzyme involved in tryptophan catabolism, is involved in the ability of $CD103^+$ MLN DCs to drive $Foxp3^+$ Treg cell development, and IDO1 activity is required for the establishment of oral tolerance (13) (29). Therefore we checked the expression patterns of Raldh2 and IDO1 in these three $CD103^+$ DC subsets. Raldh2 expression was prominent in $CD103^+CD11b^-PD\text{-}L1^+$ DC, whereas IDO1 expression was prominent in $CD103^+CD11b^+PD\text{-}L1^+$ DC. $CD103^-PD\text{-}L1^+CD11b^+$ DC and $CD103^+PD\text{-}L1^-CD11b^-$ DC slightly expressed Raldh2 or IDO1 (Fig. 2). IL-10 and IL-27 is important for IL-10 producing Tr1 induction. We previously showed PP $CD11b^+$ DC prominently express IL-10 and IL-27p28 (20). MLN $CD11b^+$ DC also highly express IL-10, however in MLN, IL-27p28 expressing population was not $CD11b^+$ DC but $CD103^+PD\text{-}L1^+CD11b^-$ DC (Fig. 2). We also confirmed that $CD103^+CD11b^+PD\text{-}L1^+$ DC express IL-6 and IL-23p19 (Fig. 2), which are involved in Th17 development (30). These results suggested the three $CD103^+$ DC subsets are distinct phenotypes.

$CD103^+PD\text{-}L1^+CD11b^-$ DC induce $Foxp3^+$ Treg via Raldh2

To investigate which DC subsets were involved in T cell function especially in induction of $Foxp3^+$ Treg, T cells were cocultured with each MLN DC subset and SPL DC as a control. RA has been reported to induce Foxp3 and gut-homing receptors such as CCR9 (31). Correlating with Raldh2 expression, Foxp3 and CCR9 expression was most induced in cocultures with $CD103^+PD\text{-}L1^+CD11b^-$ DC (Fig. 3A), and this function was suppressed by RAR antagonist LE540 (Fig. 3B). $CD103^+CD11b^+PD\text{-}L1^+$ DC also expressed Raldh2, nevertheless this DC subset did not induce Foxp3, but induced RORγt (Fig. 3A). To test whether $CD103^+CD11b^-PD\text{-}L1^+$ DC could induce gut-homing $Foxp3^+$ Treg *in vivo*, $CD103^+CD11b^-PD\text{-}L1^+$ DC was adoptive transferred, and $CD103^+CD11b^+PD\text{-}L1^+$ DC was also transferred as a control. Foxp3 expression in LP $CD4^+$ T cells from mice transferred with $CD103^+CD11b^-PD\text{-}L1^+$ DC was increased (Fig. 3C). These results suggested that $CD103^+CD11b^-PD\text{-}L1^+$ DC are the best $Foxp3^+$ Treg inducer cells of the three DC subsets.

$CD103^+PD\text{-}L1^+CD11b^+$ DC strongly present orally administrated Ag

LP $CD103^+$ DC migrate to MLN (18)(32). Oral tolerance induction is a key feature of intestinal immunity, initiated by Ag recognition of Ag-specific T cells. Therefore the phenotype of Ag presenting DC is important for subsequent response. To investigate which MLN DC subset present Ag in MLN, MLN DC subsets were sorted from OVA-fed mice, and cocultured with naïve $CD4^+$ T cells. $CD103^+PD\text{-}L1^+CD11b^+$ DC strongly induced proliferation of $CD4^+$ T cells, and $CD103^+PD\text{-}L1^+CD11b$- DC also induced T cell proliferation, but $CD103^+PD\text{-}L1^-CD11b^-$ DC did not (Fig. 4A). To test whether there was difference in inherent Ag-presenting function between MLN DC subsets, MLN DC were sorted from untreated mice and cocultured with naïve $CD4^+$ T cells with OVA peptide. All DC subsets equally had ability to induce T cell proliferation (Fig. 4B). CCR7 is required for DC to migrate from LP to MLN (17), and oral tolerance cannot be induced in CCR7-deficient mice that display impaired migration of DC from the intestine to the MLN (16). Correlating with presentation of oral administrated Ag, CCR7 expression was prominent in

$CD103^+CD11b^+PD\text{-}L1^+$ DC, and also highly expressed in $CD103^+CD11b^-PD\text{-}L1^+$ DC, but was not expressed in $CD103^+CD11b^-PD\text{-}L1^-$ DC (Fig. 4C). This result suggested that $CD103^+PD\text{-}L1^+$ DC subsets may catch Ag in LP and migrate to MLN. On the other hand $CD103^+PD\text{-}L1^-CD11b^-$ DC is probably MLN resident DC subset.

$CD103^+CD11b\text{-}PD\text{-}L1^-$ DC induce IFN-γ production in $CD4^+$ T cells by IL-12 independent mechanism

Finally we analyzed whether MLN DC subsets induce cytokine secretion in T cells. IFN-γ production was promptly induced when T cells were cocultured with $CD103^+CD11b^-PD\text{-}L1^-$ DC (Fig. 5A). IL-12 is a key cytokine to induce IFN-γ, but the expression level of IL-12p35 mRNA in $CD103^+CD11b^-PD\text{-}L1^-$ DC was not so high (Fig. 5B). Moreover anti-IL-12 did not abrogate IFN-γ production (Fig. 5C). These results suggested that $CD103^+CD11b^-PD\text{-}L1^-$ DC promptly induce Th1 response via IL-12 independent mechanism.

1.4. Discussion

In the experiments of this study, at least three MLN CD103$^+$ DC subsets with different phenotypes were identified. In general, CD103$^+$ DC are assumed to be a single subset of DC. The present study, together with others demonstrated that CD103$^+$ DC are more heterogeneous with different phenotype than previously assumed. It was reported that CD103$^+$CD11b$^+$ DC and CD103$^+$CD11b$^-$ DC are different populations, however, our study revealed that even CD103$^+$CD11b$^-$ DC could be divided into at least two distinct phenotypic subets by PD-L1 expression. First, CD103$^+$CD11b$^-$PD-L1$^+$ DC have an ability to induce Foxp3$^+$ Treg by producing RA. Second, CD103$^+$CD11b$^+$PD-L1$^+$ DC have an ability to present orally administrated Ag. Third, CD103$^+$CD11b$^-$PD-L1$^-$ DC have an ability to induce IFN-γ production.

CD103$^+$ DC are known to be a key DC subset for intestinal immune regulation, especially in oral tolerance (21)(33). In oral tolerance induction, gut-homing Foxp3$^+$ Treg are required (7)(34), and Foxp3 and gut-homing molecules are generated by CD103$^+$ DC via RA (25)(24)(35)(31)(36). Overall, the ability of RA production is a key feature for oral tolerance inducing DC subset. Therefore, and together with reasons below mentioned, it was assumed that CD103$^+$CD11b$^-$PD-L1$^+$ DC subset is the most critical CD103$^+$ DC subset for oral tolerance induction.

In addition to Raldh2 expression, prominent IL-27 expression is the other characteristic of CD103$^+$CD11b$^-$PD-L1$^+$ DC. IL-27 is known to induce IL-10 producing type 1 regulatory T (Tr1) cells, and suppresses the development of Th17 cells (37)(38). In our experimental condition, CD103$^+$CD11b$^-$PD-L1$^+$ DC did not induce IL-10 production. Since IL-27 worked together with TGF-β to further enhance Tr1 differentiation (37), it is possible that TGF-β producing DC subset was different DC subset and Tr1 inducing function could not detected *in vitro*. As for TGF-β producing DC, it is reported that CD103$^+$ DC but not CD8α$^+$ DC express αvβ8 and activate TGF-β (39). CD103$^+$CD11b$^+$PD-L1$^+$ DC and CD103$^+$CD11b$^-$PD-L1$^+$ DC have no or low expression of CD8α. In vitro, CD103$^+$CD11b$^-$PD-L1$^+$ DC was not so efficient in inducing Foxp3$^+$ Treg without additional TGF-β, but previous study showed CD103$^+$ DC could induce Foxp3$^+$ Treg without additional TGF-β compared to CD103$^-$ DC (23). It is possible that CD103$^+$CD11b$^-$PD-L1$^+$ DC are not

TGF-β producing DC, but $CD103^+CD11b^+PD\text{-}L1^+$ DC are TGF-β producing DC. *In vivo*, since several DC subsets will cooperate with each other, $CD103^+CD11b^-PD\text{-}L1^+$ DC subsets could be inducer of two regulatory T cell populations; $Foxp3^+$ Treg and IL-10 producing Tr1.

Next we showed that $CD103^+CD11b^+PD\text{-}L1^+$ DC efficiently present orally administrated Ag. For inducing oral tolerance, CCR7 dependent DC migration from LP to MLN is also required (16)(17). Furthermore, CCR7 dependent migration DC was mostly $CD103^+$ DC, especially $CD103^+CD11b^+$ DC (40)(18). It was shown that LP goblet cells delivered Ag to $CD103^+$ DC (41), so it is assumed that $CD103^+$ DC captured Ag from goblet cells in LP, then migrate to MLN in CCR7 dependent manner. We showed $CD103^+PD\text{-}L1^+CD11b^+$ DC actually express CCR7. Therefore $CD103^+PD\text{-}L1^+CD11b^+$ MLN DC are assumed that they capture Ag in LP then migrated from LP to MLN in CCR7 dependent manner, and worked as potent Ag presenting cell in MLN. In line with a previous report that LP derived MLN DC express IDO (29), $CD103^+CD11b^+PD\text{-}L1^+$ DC express prominently IDO1. $IDO1^+CD103^+$ DC also critical for $Foxp3^+$ Treg generation and oral tolerance induction (13), but our results demonstrated that $IDO^+CD103^+CD11b^+PD\text{-}L1^+$ DC did not induce $Foxp3^+$ Treg but induced RORγt *in vitro*. Because $CD103^+CD11b^+PD\text{-}L1^+$ DC highly expressed IL-6 and IL-23, the potency of Th17 induction may have predominated over Foxp3 induction. A possible reason for this discrepancy was that the potency of $Foxp3^+$ Treg induction by $IDO1^+$ DC may require other DC subsets. It is possible that other $CD103^+$ DC subsets, especially $IL\text{-}27^+CD103^+CD11b^-PD\text{-}L1^+$ DC could help to suppress Th17 developing condition, because IL-27 prevents Th17 development as mentioned above.

Finally, We showed $CD103^+CD11b^-PD\text{-}L1^-$ DC could not present orally administrated Ag, and induced IFN-γ production when cocultured with $CD4^+$ T cells. The mechanism to induce strong IFN-γ production by this DC subset remains unclear. However PD-L1 can inhibit IFN-γ production (42), so this might be reason for relatively higher IFN-γ induction by $CD103^+CD11b^-PD\text{-}L1^-$ DC. Recently, It was reported that TLR3,7,9 stimulated $CD103^+CD11b^-CD8\alpha^+$ LP DC induce IFN-γ. $CD103^+CD11b^-PD\text{-}L1^-$ MLN DC are also $CD8\alpha^+$, so $CD103^+CD11b^-PD\text{-}L1^-$ MLN DC resemble $CD103^+CD11b^-CD8\alpha^+$ LP DC. Nevertheless results from the oral Ag presentation assay suggested this MLN DC population may be MLN resident DC subset, rather than migrated from LP. Moreover, TLRs mRNA expressions in MLN DC were significantly lower than LP DC (data not shown). Therefore we assumed that

$CD103^+CD11b^-PD\text{-}L1^-$ MLN DC are not the same as $CD103^+CD8\alpha^+$ LP DC.

Food allergies presumably result from either a failure to establish oral tolerance, or a breakdown in existing tolerance (43). Allergy results in an excessive Th2-type immune response, so to prevent food allergy, suppressing Th2 response was critical. It is reported that polarizing Th1 response was useful to prevent food allergy (44). Our study showed that $CD103^+CD11b^-PD\text{-}L1^-$ DC could not present oral administrated Ag, but induced Th1 response. *In vivo*, $CD103^+CD11b^-PD\text{-}L1^-$ DC may work functionally to prevent Th2 polarization in oral tolerance induction.

Most studies of MLN $CD103^+$ DC are focused on two subsets; $CD103^+CD11b^+$ DC and $CD103^+CD11b^-$ DC. This study showed $CD103^+CD11b^-$ DC were heterogenic and were clearly divided according to PD-L1 expression. Although the function of PD-L1 is unclear, $PD\text{-}L1^{-/-}$ DC cannot induce $Foxp3^+$ Treg (10)(45). PD-L1 bind to PD-1 on T cells, and inhibitory signaling through PD-1 is well known (46). In addition to PD-1, CD80 also binds to PD-L1. CD80 is known to be expressed on APC but T cells also express CD80, and PD-L1/CD80 signaling results in T cell anergy (26). Overall, PD-L1 could be a functional cell surface marker on MLN DC, modulating T cell function.

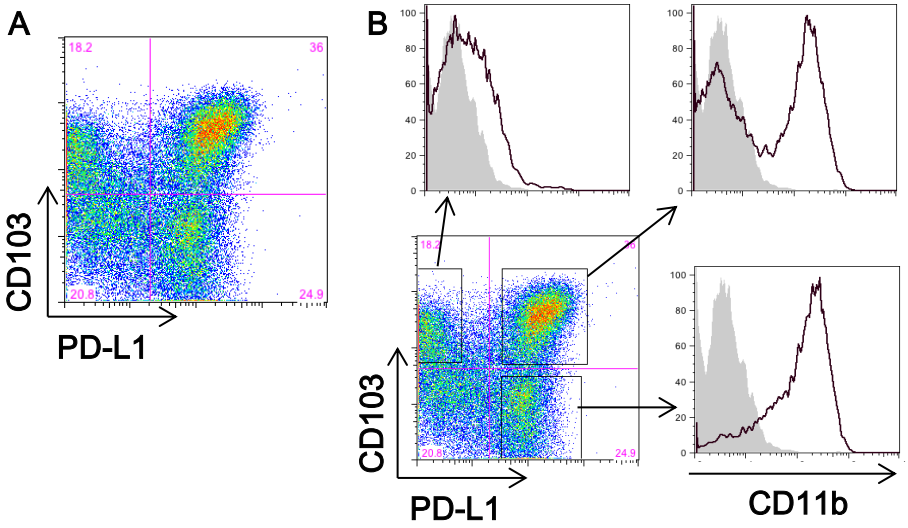

Figure 1. MLN DC subsets are divided by CD103, CD11b, and PD-L1 expression pattern.
(**A**) MLN DCs were stained for CD11c, CD103, PD-L1, and CD11b. CD11c$^+$ cells were analyzed for the expression of CD103 and PD-L1. Numbers in quadrants indicate percentage of each DC subset among CD11c$^+$ cells. (**B**) Surface expression of CD11b on CD103$^-$PD-L1$^+$ DC, CD103$^+$PD-L1$^+$ DC, and CD103$^-$PD-L1$^+$ DC were analyzed. ■not stained, □stained cells. Data are representative of three independent experiments.

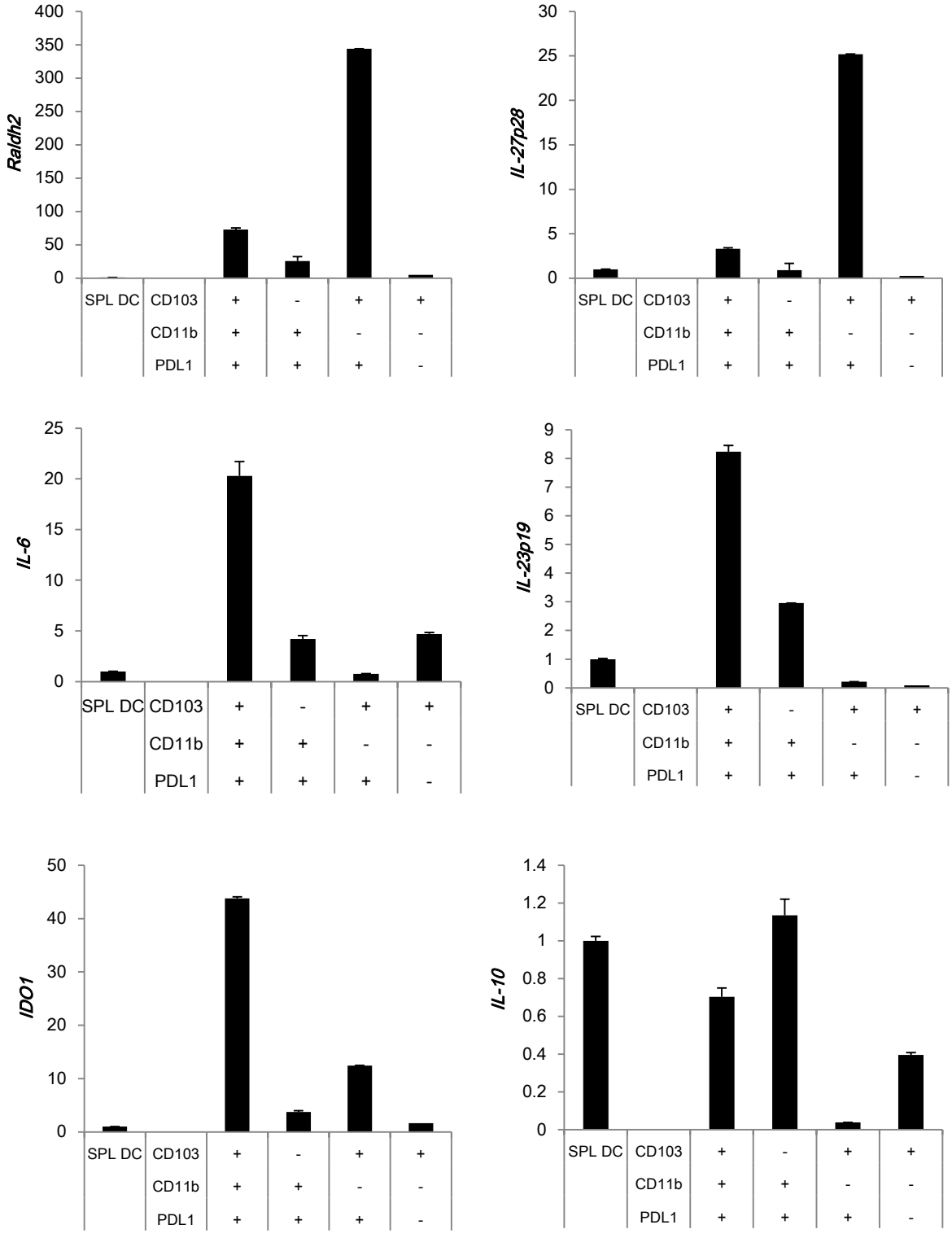

Figure 2. Three CD103⁺ DC subsets express different mRNA.
Quantitative RT-PCR analysis of Raldh2, IL-27p28, IL-6, IL-23p19, IDO1, and IL-10 mRNA expression in sorted MLN DC subsets or SPL CD11c⁺ DC. The mRNA expression level of each sample was displayed as ratio to that of SPL DC. Data are representative of four independent experiments.

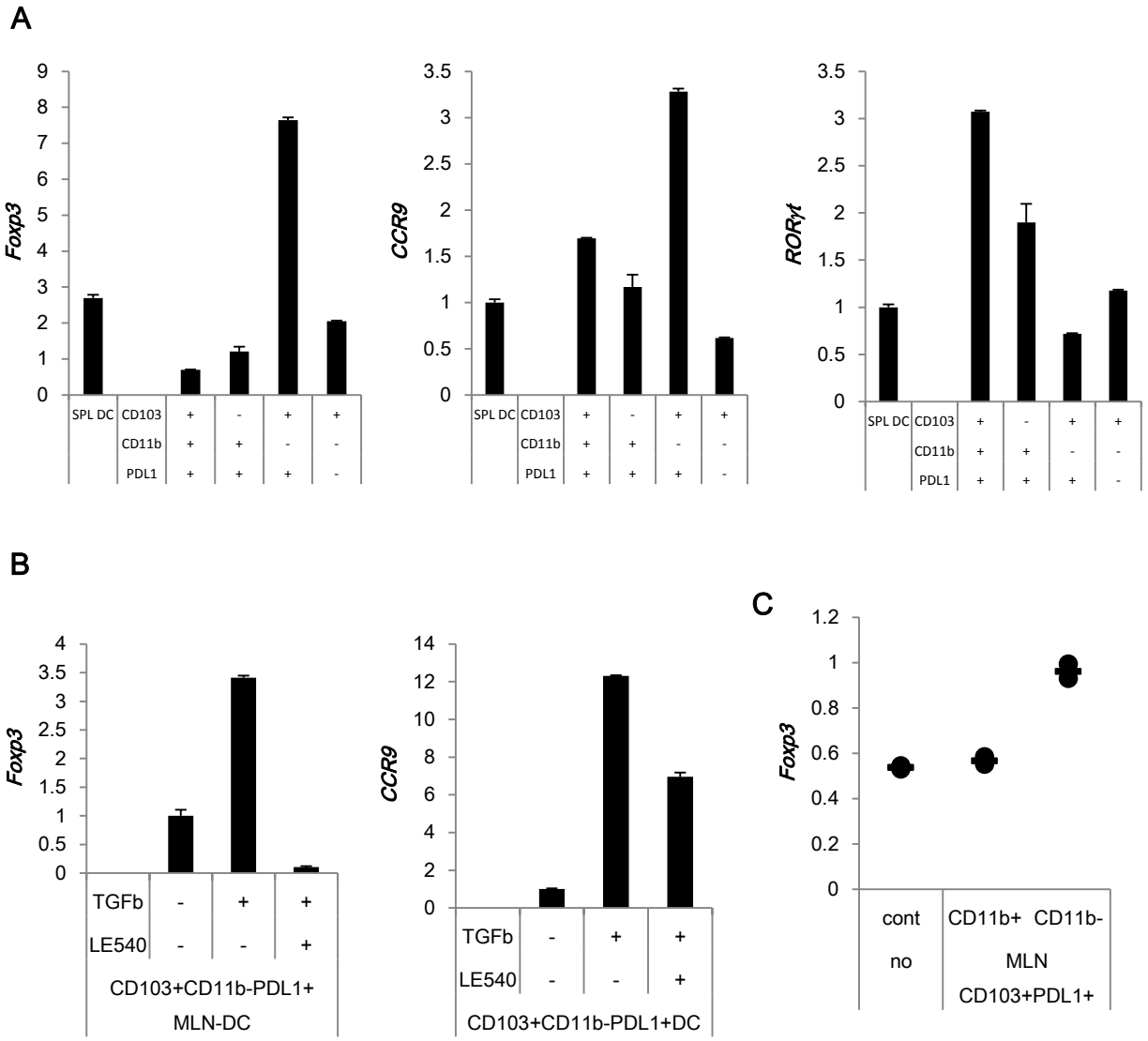

Figure 3. CD103^{+}CD11b^{-}PD-L1^{+} DC induce gut-homing Foxp3^{+} Treg via retinoic acid production. (**A**) SPL CD4^{+} T cells (2 × 10^{5} cells) from DO11.10 mice were cultured with sorted DC subsets (2 × 10^{4} cells) and OVA peptide (0.3 mM) in the presence of TGF-b (2 ng/ml) in 96 well plates for 4 d. Cultured cells were harvested and restimulated with plate bound anti-CD3 and CD28 for an additional 48h. Cells were collected and Foxp3, CCR9, and RORgt mRNA was measured. The mRNA expression level of each sample was displayed as ratio to that of cultured with SPL DC. (**B**) CD103^{+}CD11b^{+}PD-L1^{+} DC or CD103^{+}CD11^{-}PD-L1^{+} DC were cultured as described for Figure 3A in the absence or presence of LE540 (1 mM) for 4 d. Cultured cells were harvested and restimulated with plate bound anti-CD3 and CD28 for an additional 48 h, and Foxp3 and CCR9 mRNA was measured. The mRNA expression level of each sample was displayed as ratio to that of T cells cultured without TGF-b nor LE540. (**C**) DO11.10 mice were adoptive transferred (i.v.) with CD103^{+}CD11b^{+}PD-L1^{+} DC or CD103^{+}CD11b^{-}PD-L1^{+} DC (5 × 10^{5} cells) from OVA-fed mice. 7 d after, Foxp3 mRNA expression in CD4+ T cells from LP were measured. Data are representative of four independent experiments.

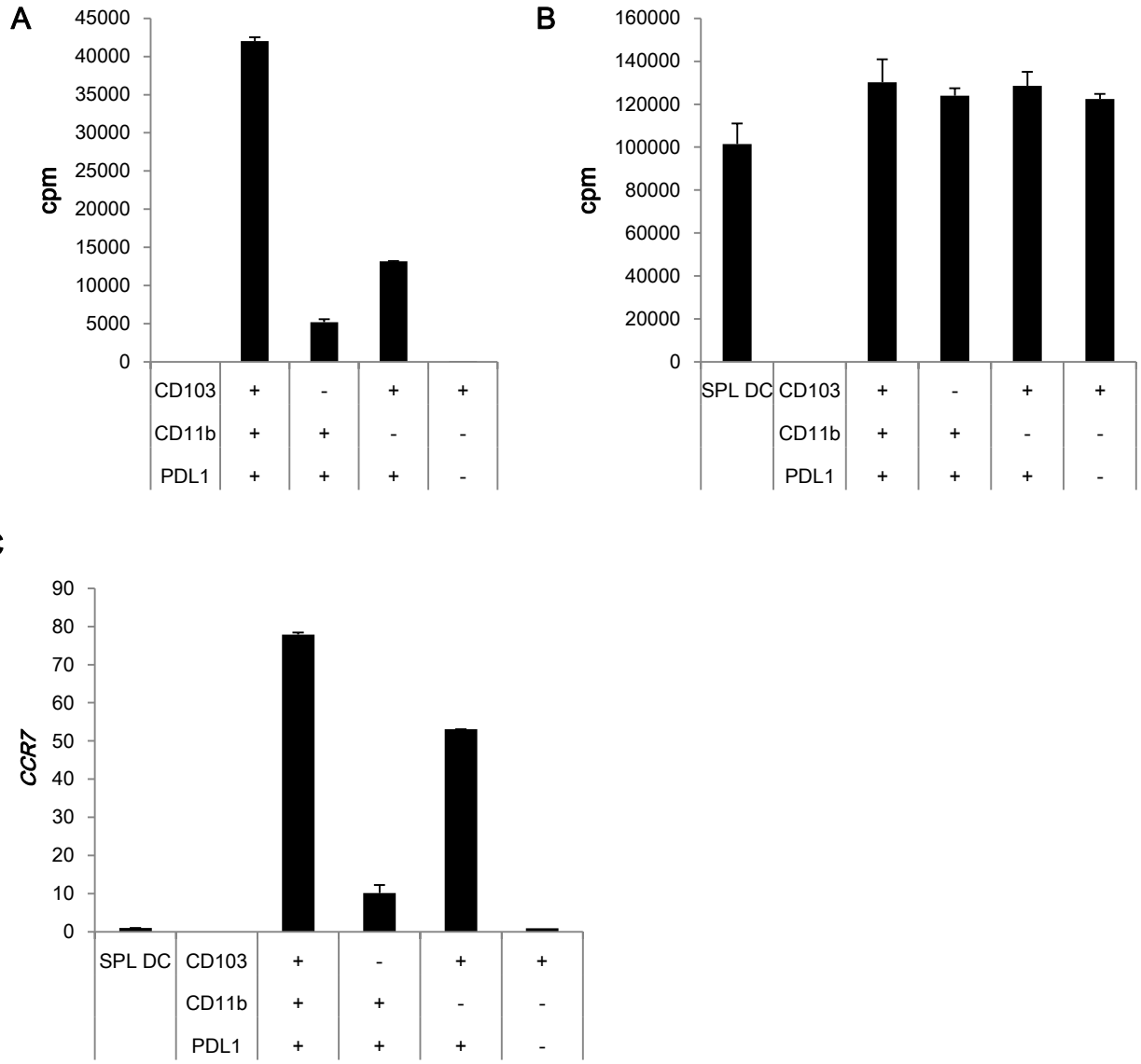

Figure 4. CD103⁺CD11b⁺PD-L1⁺ DC efficiently present orally administrated Ag.
(**A**) Balb/c mice were given OVA in drinking water 5 d. SPL CD4⁺ T cells (1×10^5 cells) from DO11.10 mice were cultured with sorted MLN DC subsets (2×10^4 cells) from OVA-fed Balb/c mice for 72 h. ^{3}[H] thymidine was pulsed for the last 24 h, and proliferative response was determined by ^{3}[H] thymidine uptake. (**B**) SPL CD4⁺ T cells (2×10^5 cells) from DO11.10 mice were cultured with sorted DC subsets (2×10^4 cells) from untreated Balb/c mice and OVA peptide (0.3 mM) for 72 h. proliferative response was determined as described for Figure 4A. (**C**) Quantitative RT-PCR analysis of CCR7 mRNA expression in sorted MLN DC subsets or SPL CD11c⁺ DC. The mRNA expression level of each sample was displayed as ratio to that of SPL DC. Data are representative of three independent experiments.

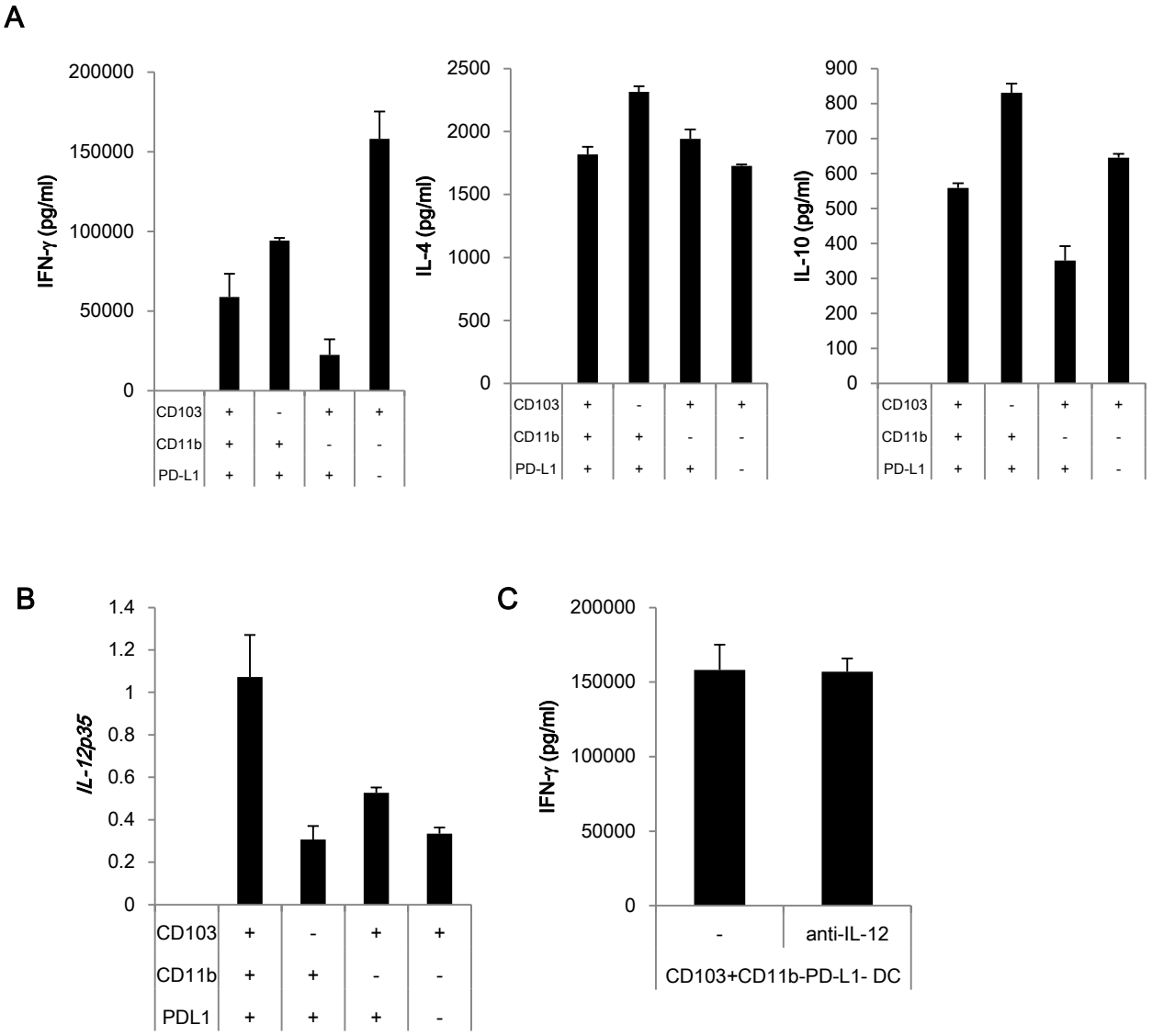

Figure 5. CD103⁺CD11b⁻PD-L1⁻ DC induce IFN-g by IL-12 independent mechanism.
(**A**) SPL CD4⁺ T cells (2×10^5 cells) from DO11.10 mice were cultured with sorted DC subsets (2×10^4 cells) from Balb/c mice and OVA peptide (0.3 mM) for 72 h. IFN-g, IL-4, and IL-10 in the supernatants were measured by ELISA. Bars represent mean±S.D. of triplicate cultures. (**B**) Quantitative RT-PCR analysis of IL-12p35 mRNA expression in sorted MLN DC subsets. The mRNA expression level of each sample was displayed as ratio to that of CD103⁺CD11b⁺PD-L1⁺ DC. (**C**) SPL CD4⁺ T cells (2×10^5 cells) from DO11.10 mice were cultured with CD103⁺CD11b⁻PD-L1⁻ DC (2×10^4 cells) and OVA peptide (0.3 mM) in the absent or presence of anti-IL-12 (5 mg/ml) for 72 h. IFN-g in the supernatants was measured by ELISA. Bars represent mean±S.D. of triplicate cultures. Data are representative of three independent experiments.

Chapter 2

IL-10 and IL-27-producing dendritic cells capable of enhancing IL-10 production of T cells are induced in oral tolerance.

2.1. Introduction

Oral tolerance is the specific suppression of cellular or humoral immune responses to an antigen (Ag) by means of prior administration of Ag through the oral route. There are several effector mechanisms for inducing oral tolerance; the primary ones being the induction of regulatory T cells (Treg) that mediate active suppression and the induction of clonal anergy or deletion. Inhibitory cytokines, such as IL-10 and transforming growth factor-β (TGF-β) are one of the important mechanisms for Treg mediated suppression. Evidence has been reported that these forms of tolerance are not mutually exclusive, and may overlap (6).

As mentioned in the previous chapter, after Ag-captured lamina propria (LP) dendritic cells (DCs) migrate to the mesenteric lymph nodes (MLN) in a CCR7-dependent manner, $Foxp3^+$ Treg induction by retinoic acid(RA)-producing $CD103^+$ DCs in the MLN is suggested to be critical for the induction of oral tolerance (16)(17). These studies investigated the LP and MLN, but not PP. The results of these studies suggested that LP and MLN DC were indispensable for establishing oral tolerance by Foxp3 induction in the MLN, followed by maintenance/expansion in the LP. However, the role of PP DC remains unclear. Some previous studies have suggested that PP is not necessary for inducing oral tolerance (47)(14), while others indicated that PP played an important role in this process (48)(49)(50). Therefore, we aimed to elucidate the characteristics of PP DC in the induction of oral tolerance.

In this study the induction of IL-10 secretion was examined in particular. IL-10 has an important role in limiting inflammation or autoimmunity (51)(52)(53)(54). Antigen specific T cell suppression by IL-10 contributes to peripheral tolerance to allergens, autoantigens,

transplantation antigens, and tumor antigens. Several reports have shown that a particular subset of DC can induce IL-10 producing regulatory T cells (55)(56). Furthermore, IL-10-secreting regulatory T cells have been shown to be induced in the PP of orally tolerized mice (50).

Here, it is demonstrated that after oral antigen administration, IL-10- producing DC increased especially in PP through interaction with antigen specific T cells. These DC can induce IL-10 producing T cells. It is also shown that DC from orally tolerized mice produce IL-27, recently revealed to be an important inducer of IL-10 in T cells (37)(57)(58). Furthermore, it was found that $CD11b^+$ DC, which are capable of producing these cytokines, increased during oral tolerance induction.

2.2. Materials and methods

Mice

BALB/c mice were purchased from CLEA Japan (Tokyo, JAPAN). Rag2$^{-/-}$ DO11.10 TCR Tg mice were kindly provided by Dr. Y. Wakatsuki (Kyoto University). These mice and DO11.10 TCR Tg mice (27) were used 7-10 weeks of age. All work was performed in accordance with guidelines for animal use and care of the University of Tokyo.

Oral tolerance induction

Oral tolerance was induced as in chapter 1. In brief, DO11.10 mice were fed ovalbumin (OVA) (Wako) via drinking water (100 mg/ml) for 7d.

Culture medium

Cells were cultured in RPMI 1640 medium supplemented with 5% heat inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-ME.

Antibodies

The following antibodies were used. FITC-labeled anti-CD4 (H129.19, BD Pharmingen), PE-labeled anti-Foxp3 (FJK-16s, eBioscience), FITC-labeled anti-CD11c (N418), PE-labeled anti-CD11b (M1/70, eBioscience), FITC-labeled anti-CD103 (2E7, eBioscience), PE-labeled anti-CD86 (PO.3, eBioscience), APC-labeled anti-CD11c (HL3, BD Pharmingen), Biotinylated anti-CD3 (145-2C11, eBioscience), Biotinylated anti-IgM (eB121-15F9, eBioscience), streptavidin-APC (BD Pharmingen), PE-labeled anti-IL-10 (JES5-16E3, eBiscience).

Measurement of cytokine secretion

Cytokine levels in the culture supernatants were assayed by specific sandwich ELISA. IL-2, IL-4, IFN-γ was measured as previously described(28). OPTEIA ELISA set (BD Biosciences) was used for IL-10 measurement. For IL-27p28 measurement, IL-27p28 ELISA kit (R&D Systems) was used in accordance with the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was isolated from MACS purified $CD4^+$ T cells and $CD11c^+$ DC by QIA shredder and RNeasy Mini Kit (both from QIAGEN). Single-stranded cDNA was synthesized using Superscript II reverse transcriptase and Oligo dT primers (both from Invitrogen). Quantitative PCR reactions were performed either using quantitect Primer Assays with SYBR green PCR mastermix (QIAGEN) with the following primers : IL-10 forward, 5'-CCCAGAAATCAAGGAGCATTTG-3'; reverse, 5'-CATGTATGCTTCTATGCAGTTG-3': IL-27p28 forward, 5'-GGCCAGGTGACAGGAGACC-3'; reverse, 5'-CAGCTTGTACCAGAAGCAAGGG-3': HPRT forward, 5'-GAAGAGACTGGGGATCACTC-3' reverse 5'-CATGCCATCTTCCATATTGT-3' with the LightCycler (Roche). Gene expression levels for each individual sample were normalized to HPRT.

Preparation of $CD4^+$ T cells and analysis of freshly isolated T cells

$CD4^+$ T cells were prepared from spleen (SPL) and PP of untreated or OVA-fed DO11.10 mice in parallel. SPL was mashed and PP was digested with collagenase type I 1mg/ml; Sigma-Aldrich), and single cell suspension was prepared. The cells were incubated with anti mouse CD4-coated magnetic beads (Miltenyi Biotech) and selected on MACS separation columns (Miltenyi Biotech). These $CD4^+$ T cells were subjected to cultures to examine cytokine response and regulatory activity. In addition, expression of IL-10 mRNA and Foxp3 protein of freshly isolated $CD4^+$ T cells was examined by RT-PCR and flowcytometry, respectively. For culture with DC, SPL $CD11c^-CD4^+$ cells from DO11.10 mice or $Rag2^{-/-}$ DO11.10 mice were prepared by CD11c-negative selection and CD4-positive selection using the MACS systems.

Measurement of T cell cytokine response and regulatory activity

For antigen presenting cells, splenocyte from BALB/c mice were treated with mytomicin C for 30 min at 37 ºC MACS purified $CD4^+$ T cells ($5x10^4$) were cultured with antigen presenting cells ($2x10^5$) and OVA peptide (1 μM: ISQAVHAAHAEINEGR; Biologica) in 96 well plates for 48 h. To examine regulatory activity, $CD4^+$ T cells ($5x10^4$) were cultured with SPL $CD4^+$ T cells from untreated mice ($5x10^4$), antigen presenting cells ($2x10^5$), and OVA (1 μM) in 96 well plates for 48 h. Supernatants were collected, and IL-2 and IL-10 levels were measured by ELISA.

Preparation of DC and analysis of freshly isolated DC

DC were prepared from spleen (SPL), mesenteric lymph node (MLN) and PP in parallel. SPL, MLN and PP were digested with collagenase type I and $CD11c^+$ cells were prepared using MACS. These DC were subjected to DC-T cell cultures. In addition, freshly isolated DC were measured for IL-10 and IL-27p28 mRNA expression by quantitative RT-PCR, and analyzed for cell surface molecule expression by flow cytometry. For flow cytometric analysis, cells were stained as follows; CD11c-FITC/CD11b-PE, CD11c-FITC/CD86-PE, and CD11c-APC/CD103-FITC. Percentages of each surface marker expressing cells in $CD11c^+$ cells were calculated. For time-dependent CD11b expression analysis, whole PP cells were stained with CD3-biotin, IgM-biotin, streptavidin-APC, CD11c-FITC, and CD11b-PE. $CD3^-IgM^-$ fraction was counted, and percentage of $CD11b^+$ cells in $CD3^-IgM^-CD11c^+$ cells was calculated. For isolation of DC subpopulations, cells selected on the basis of CD11c expression by MACS were stained with CD11c-FITC and CD11b-PE, and $CD11b^+CD11c^+$ DC and $CD11b^-CD11c^+$ DC were sorted by using FACSVantage (BD Bioscience). Sorted DC were measured for IL-10 and IL-27p28 mRNA expression by quantitative RT-PCR.

DC-T cell co-cultures

SPL $CD11c^-CD4^+$ T cells ($1x10^5$) from $Rag2^{-/-}$ DO11.10 mice (for Foxp3 induction analysis) or

DO11.10 mice (for cytokine secretion analysis) were cultured with $CD11c^+$ DC ($1x10^4$) and OVA peptide (0.3 μM) in 96 well U-bottom plates for 72 h. Supernatants were collected, and IL-10, IL-4, and IFN-γ levels were measured by ELISA. Cultured cells were harvested and stained for intracellular Foxp3.

Stimulation of DC in vitro

$CD11c^+$ DC ($2x10^5$) were incubated with anti-CD40 (5 μM; 1C10, R&D Systems) on 96 well U-bottom plates for 72 h. Supernatants were collected, and IL-10 and IL-27p28 levels were measured by ELISA.

Intracellular staining for Foxp3 and IL-10

For Foxp3 staining, cells were washed in FACS buffer (1% FCS, 0.1% NaN3 in PBS) and incubated with mAb 2.4G2 to block Fcγ receptors in 15 min. Surface CD4 were stained with CD4-FITC for 20 min at 4ºC. Intracellular Foxp3 staining was performed with Foxp3-PE and Foxp3 staining buffer set (eBioscence) in accordance with manufacturer's instructions. Data were acquired on a BD LSR (BD Bioscience) and analyzed using Cell Quest software. Percentage of $Foxp3^+$ cells in $CD4^+$ cells was calculated.

For IL-10 staining, $CD11c^-CD4^+$ Tcells ($1x10^5$) were cocultured with $CD11c^+$ DC ($1x10^4$) and OVA peptide (10 μM) on 96 well U-bottom plates for 48 h. For the last 6 h, Goldistop (BD Pharmingen) was added to the cultures. Cultured cells were harvested and stained with CD4-FITC. Then cells were fixed with 4% Para formaldehyde in PBS for 15 min at room temperature and permeabilized with saponin buffer (0.5% saponin; SIGMA, 0.5% BSA, 0.2% NaN3 in PBS) for 10 min at room temperature. After blocking Fcγ receptor for 20 min, cells were stained with IL-10-PE for 30 min. These steps were performed with saponin buffer at room temperature. Data were acquired on a BD LSR.

2.3. Results

IL-10 producing T cells and regulatory T cells are induced by oral tolerance

OVA were fed to DO11.10 OVA specific TCR transgenic mice in their drinking water for 7 days. $CD4^+$ T cells derived from SPL and PP from OVA-fed or untreated control mice were examined. As shown in previous studies (59), IL-2 secretion (Fig. 1A) and proliferative response (data not shown) decreased in T cells from OVA-fed mice (OVA-fed T cells) compared with that of T cells from untreated mice (untreated T cells). One of the major effector mechanisms for oral tolerance is immune regulation by regulatory T cells. Untreated splenic T cells were cocultured with or without untreated T cells or OVA-fed T cells. IL-2 production of this culture revealed that OVA-fed T cells suppressed response of untreated T cells (Fig. 1B). It has been shown that IL-10-secreting regulatory T cells are induced in the PP of orally tolerized mice. Examination of IL-10 production revealed that the secretion and mRNA expression of IL-10 increased in OVA-fed T cells than untreated T cells (Fig. 1C, D). Another indicator of regulatory T cells is Foxp3 expression. Expression of this molecule was increased in SPL and PP (Fig. 1E). These results showed that oral tolerance was induced in this system, accompanying induction of regulatory T cells.

DC from OVA fed mice induce IL-10 production in DC-T cell culture

The role of T cells in oral tolerance has been well examined, but the mechanism of inducing these T cells has yet to be clearly defined. To examine whether DC is involved in induction of IL-10 production or Foxp3 expression in T cells, $CD11c^+$ cells derived from SPL, MLN and PP of OVA-fed (OVA-fed DC) or untreated (untreated DC) DO11.10 mice were cocultured with splenic $CD11c^-CD4^+$ T cells derived from $Rag2^{-/-}$DO11.10 TCR transgenic mice. When cocultured with OVA-fed DC, IL-10 production of supernatant increased especially in the case of PP DC (Fig. 2A), but $Foxp3^+$ T cells were not induced (Fig. 2B). IL-4 and IFN-γ production was not increased when T cells were cocultured with OVA-fed DC (Fig. 2C, D). These results suggest OVA-fed DC were capable of inducing IL-10 producing T cells without polarizing Th2 cells or inducing $Foxp3^+$ Tregs.

IL-10 and IL-27 producing DC are induced in the induction of oral tolerance

We next examined further the characteristics of DC induced in the orally tolerant animals. IL-10 producing Type 1 regulatory T cells (Tr1) are induced by repeated stimulation in the presence of IL-10 or with immature DC *in vitro*. We firstly investigated IL-10 production of DC. IL-10 production of OVA-fed DC after CD40 stimulation was higher than that of untreated DC, especially in PP (Fig. 3A). We further investigated the kinetics of IL-10 production in PP DC. When mRNA expression of IL-10 in PP DC after antigen administration for 0–7 days was analyzed, IL-10 mRNA expression was increased at 5–7 days (Fig. 3B). Our oral tolerance model using DO11.10 mice can amplify Ag-specific T cell–DC interaction. To check whether this IL-10 increase in DC is attributed to interaction with Ag-specific T cells, we examined IL-10 expression in OVA-fed BALB/c mice and BSA-fed DO11.10 mice. In these mice, IL-10 mRNA expression of OVA-fed DC was unchanged (Fig. 3C and D). Recently it was reported IL-27 induces IL-10 production in T cells. We detected IL-27p28 mRNA expression and production was transiently increased at 2–5 days (Fig. 3E). As in the case of IL- 10 expression, neither feeding BALB/c mice with OVA nor feeding DO11.10 mice with BSA increased IL-27p28 expression by PP DC (Fig. 3F and G). The secretion of IL-27p28 upon CD40 stimulation also increased by short term antigen-feeding (Fig. 3H). TGF-β has been shown to amplify generation of IL-10 producing T cells by IL- 27. Although we examined the TGF-β secretion by DC of untreated or OVA-fed DO11.10 mice, it was undetectable (detection limit: 30pg/ml). Subsequently, we examined whether these IL-10 producing DC or IL-27 producing DC can induce IL-10 production in T cells. $CD4^+$ T cells were cultured with IL-27 producing day 2 DC or IL-10 producing day 5 DC, and IL-10 production in T cells was determined by intracellular staining of IL-10. FACS analysis revealed both day 2 DC and day 5 DC induce IL-10 producing T cells (Fig. 3I). These results suggest that IL-10 or IL-27 production in DC is increased through interaction with Ag-specific T cells, and these DC may induce IL-10 producing cells.

$CD11b^+$ DC increased in oral tolerance

DC can be characterized by their surface molecule expression. To determine whether there

was any alteration in DC subset in oral tolerance, we examined the expression of several surface molecules of freshly isolated OVA-fed DC or untreated DC. We were especially interested in CD11b. Three distinct DC subsets have been identified in murine PP, $CD8\alpha^+CD11b^-$ DC, $CD8\alpha^-CD11b^+$ DC, and double negative DC (60), and it has been shown that oral tolerance cannot be induced in CD11b deficient mice (61).

As shown in Fig. 4A, $CD11b^+$ DC was increased in PP of OVA-fed mice. The proportion of $CD8\alpha^+$ DC was unchanged (data not shown). To confirm that this increase of $CD11b^+$ DC is also attributed to interaction with Ag-specific T cells, we examined $CD11b^+$ DC in OVA-fed BALB/c mice. In BALB/c mice, $CD11b^+$ DC proportion of OVA-fed DC hardly changed (data not shown). We further examined the kinetics of CD11b expression. $CD11b^+$ DC increased together with the term of oral antigen administration (Fig. 4C). Expression of CD86 and CD103 was also examined, since DC that have low expression of co-stimulatory molecules are considered as tolerogenic DC and $CD103^+$ DC can induce $Foxp3^+$ Treg by RA production. We found that CD86 expression was unchanged (Fig. 4A, B). On the other hand, the proportion of $CD103^+$ DC increased in PP. However, these DC could not induce $Foxp3^+$ Treg (Fig. 2B), so we have not yet determined whether increase of $CD103^+$ DC has biological effects or not.

Because it is reported $CD11b^+$ DC from PP has a higher capacity to produce IL-10 [38], this increase of $CD11b^+$ DC subset may be responsible for the induction of IL-10 production in DC. Thus we examined IL-10 expression of FACS-sorted, $CD11b^+$ and $CD11b^-$ DC. It was clearly shown that the $CD11b^+$ subset expressed high levels of IL-10 mRNA, while IL-10 expression in $CD11b^-$ DC was minimal (Fig. 4D). IL-27p28 mRNA was also dominantly expressed by $CD11b^+$ DC, but $CD11b^-$ DC expressed some IL-27. The levels of IL-10 and IL-27 in $CD11b^+$ DC did not differ greatly after antigen-feeding, but an approximately four-fold increase in IL-27 expression was observed in the case of $CD11b^-$ DC (Fig. 4D).

2.4. Discussion

It is shown here that in oral tolerance, both T cells and DC increased IL-10 production, and IL-10$^+$ T cells were induced by DC from orally tolerant mice. The results also demonstrate that CD11b$^+$ DC, as main producer of IL-10 and IL-27, increased in oral tolerance.

It has been shown that antigen-specific Tr1 cells were induced in PP by feeding exogenous proteins(62), and there is a large body of evidence showing that IL-10 plays a key role in modulating inflammatory responses in the intestine. IL-10$^{-/-}$ mice develop a severe form of enterocolitis as a result of the absence of Tr1 cells, and IL-10 produced by T cells can inhibit inflammation in the gut(9)(63)(64). Moreover, several studies have suggested that oral administration of self-antigens induce Tr1 cells that can suppress autoimmunity. For instance, oral administration of myelin basic protein suppresses experimental autoimmune encephalomyelitis by inducing peripheral tolerance, and oral co-administration of IL-10 enhance oral tolerance in autoimmune encephalomyelitis and diabetes. These reports suggest IL-10 controls the immune responses especially in the intestine, and IL-10$^+$ T cells play a key role in establishment of oral tolerance.

Ehirchiou et al showed CD11b$^{-/-}$ mice exhibit defective antigen-induced oral tolerance (65), suggesting CD11b$^+$ cells are essential in oral tolerance induction. As one mechanism of the induction of oral tolerance by CD11b$^+$ cells, we present in this study the possibility that CD11b$^+$ DC play a key role in inducing IL-10$^+$ T cells via IL-10 or IL-27. In PP, CD11b$^+$ DC were the dominant IL-10 producers compared to CD11b$^-$ DC, and the level of IL-10 expression in CD11b$^+$ DC and CD11b$^-$ DC hardly changed after oral antigen administration. These results suggest that IL-10 expression in whole DC depends on IL-10 expression in CD11b$^+$ DC subset, and the increase of IL-10 expression of OVA-fed DC results from the increase of CD11b$^+$ DC. It is also reported that Th17 immune deviation is promoted in CD11b$^{-/-}$ mice following feeding and immunization with antigen. IL-17 administration interferes with the establishment of oral tolerance(65), and IL-10 suppresses Th17 cytokines(66). We ascertained IL-10 production increased also in SPL and MLN, and CD11b$^+$ DC increased in these sites. Therefore IL-10$^+$CD11b$^+$ DC and/or IL-10$^+$ T cells induced by DC may possibly inhibit IL-17, resulting in establishment of oral tolerance.

IL-10 and IL-27 expression of DC from OVA-fed BALB/c mice (Fig. 3) and BSA-fed DO11.10 mice (data not shown) was not altered. Neither was the ratio of $CD11b^+$ DC population (data not shown). These results suggest that the increase in IL-10 and IL-27 production of DC, and $CD11b^+$ DC population were induced by interaction with antigen specific T cells. Onishi et al showed that naturally occurring Treg (nTreg) preferentially form aggregates on DC(67), so we suspected the possibility that DC-Ag-specific T cell interaction may be DC-Ag-specific nTreg interaction, because DO11.10 mice have nTreg which express OVA specific TCR, and such OVA-specific nTreg will scarcely be present in wild-type mice. To ascertain whether unconventional nTreg were involved in DC change, we used $Rag2^{-/-}$DO11.10 mice which lack OVA specific nTreg. The results that OVA-fed DC from $Rag2^{-/-}$ DO11.10 mice increased IL-10 expression and $CD11b^+$ DC population (data not shown) suggest these alteration of DC resulting from DC-Ag-specific T cell interaction was not due to interaction between DC and Ag-specific nTreg. On the other hand, Awasthi et al showed that TGF-β-induced Treg cells modified DC, and these IL-27-producing DC elicited the generation of Tr1-like cells that produced large amounts of IL-10(37). The modified DC had a plasmacytoid-like phenotype ($CD11c^{int}CD11b^{lo}CD8\alpha^-CD45RB^{hi}B220^{hi}$). However, in our system, $B220^-CD11c^{hi}$ DC (including the $CD11b^+$ DC subset) play a critical role in IL-27 production. $B220^{hi}CD11c^{int}$ DC express subtle IL-27 mRNA compared to $B220^-CD11c^{hi}$ DC in normal condition, and the level of IL-27 expression in $B220^{hi}CD11c^{int}$ DC and $B220^-CD11c^{hi}$ DC hardly changed after oral antigen administration for 7d (data not shown). In our model, IL-10 and IL-27 production of DC suggested to be mainly dependent on $CD11b^+$ DC. Nevertheless, we also found that $CD11b^-$ DC (containing $B220^{hi}$ DC and a part of $B220^-$ DC subsets) from OVA-fed (2-5d) mice tended to increase IL-27 expression (data not shown). Therefore it may be possible that $CD11b^-$ DC are also involved in IL-27 production, especially in the early phase.

Our results suggest that $CD11b^+$ DC, the main source of IL-10 and IL-27, is induced by interaction with Ag-specific non-Treg T cells. The significant increase of the $CD11b^+$ DC subset points to an important role of this subset in the establishment of oral tolerance. As a reason for the increase of $CD11b^+$ DC, it is possible that $CD11b^+$ DC migrated to the lymphoid tissue or $CD11b^-$ DC were induced to express CD11b, or $CD11b^-$ DC were induced to undergo apoptosis, but this has yet to be clarified. Although IL-10 can induce Tr1

differentiation, the resulting Tr1 cells do not proliferate because of the suppressive nature of IL-10, whereas IL-27 can induce and enhance Tr1. Therefore it is possible that $CD11b^+$ DC can keep optimum balance of $IL\text{-}10^+$ T cells in conjunction with IL-27 and IL-10 secretion in oral tolerance.

An increasing number of preclinical studies are focusing on the capacity of DC to induce antigen specific tolerance. For instance, transfer of IL-10-treated DC from $IL\text{-}10^{+/+}$ but not $IL\text{-}10^{-/-}$ donors can suppress airway hyperresponsiveness and inflammation(68), or IL-10 expressed DC can establish long-term antigen specific tolerance(69). Therefore $IL\text{-}10^+$ DC induced by oral tolerance may have the potential to manage allergy, transplantation, autoimmunity or chronic inflammatory diseases.

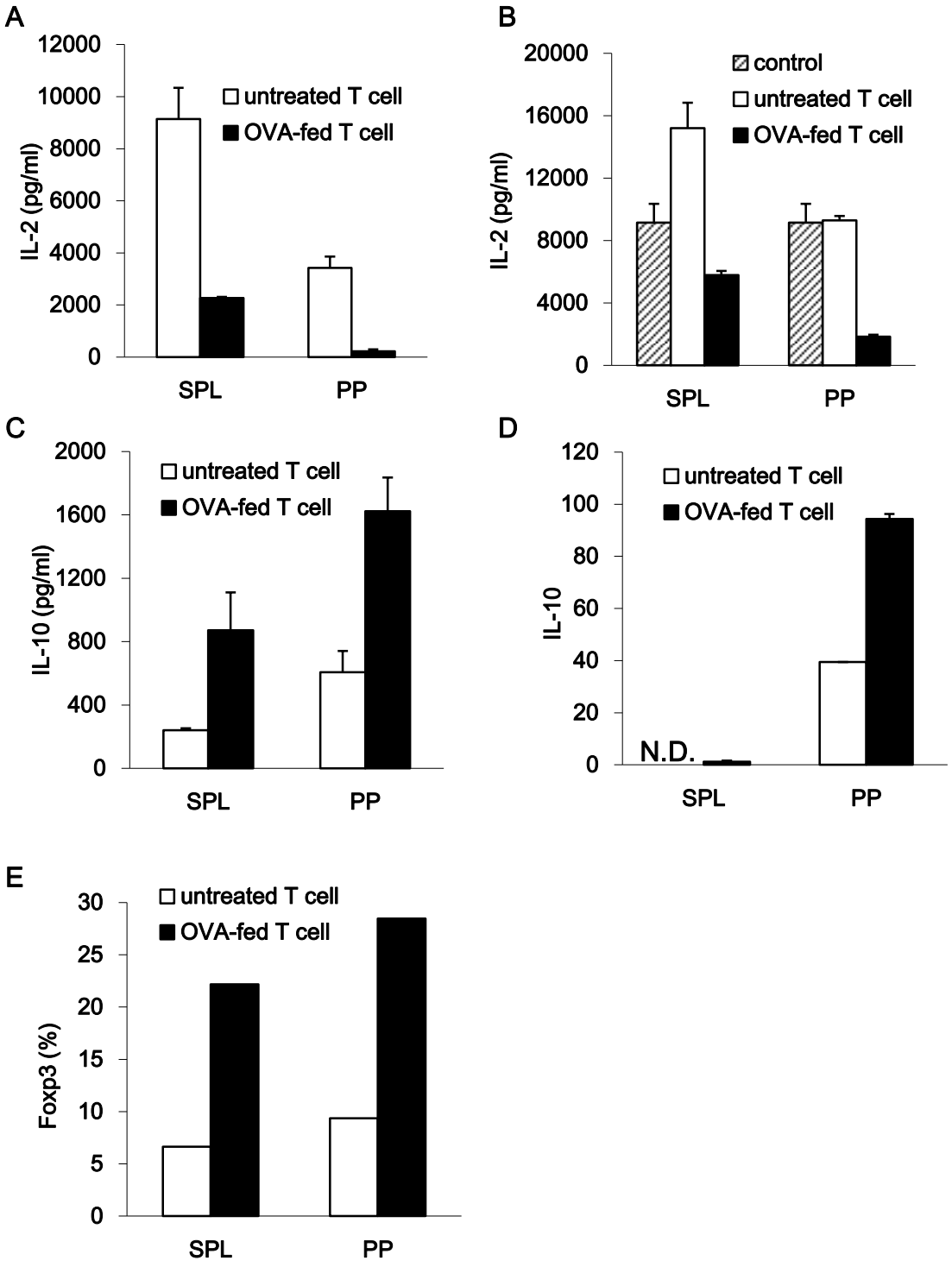

Figure 1. Characterization of orally tolerized T cells.
DO11.10 mice were given OVA in drinking water for 7 d. (A-C) SPL and PP $CD4^+$ T cells from OVA-fed or untreated mice were cultured with antigen presenting cells and 1 μM OVA peptide in the absence (A, C) or presence (B) of untreated splenic $CD4^+$ T cells. After 48 h, IL-2 (A, B) and IL-10 (C) in the supernatants were measured by ELISA (mean ± SD of triplicate cultures). (D) IL-10 mRNA of freshly isolated $CD4^+$ T cells were measured by quantitative RT-PCR. The mRNA level was expressed as ratio of that of OVA-fed SPL T cells (=1). (E) Foxp3 expression in freshly isolated T cells was analyzed by FACS. Bars show the percentage of $CD4^+$ cells expressing Foxp3. Data represent one of two independent experiments.

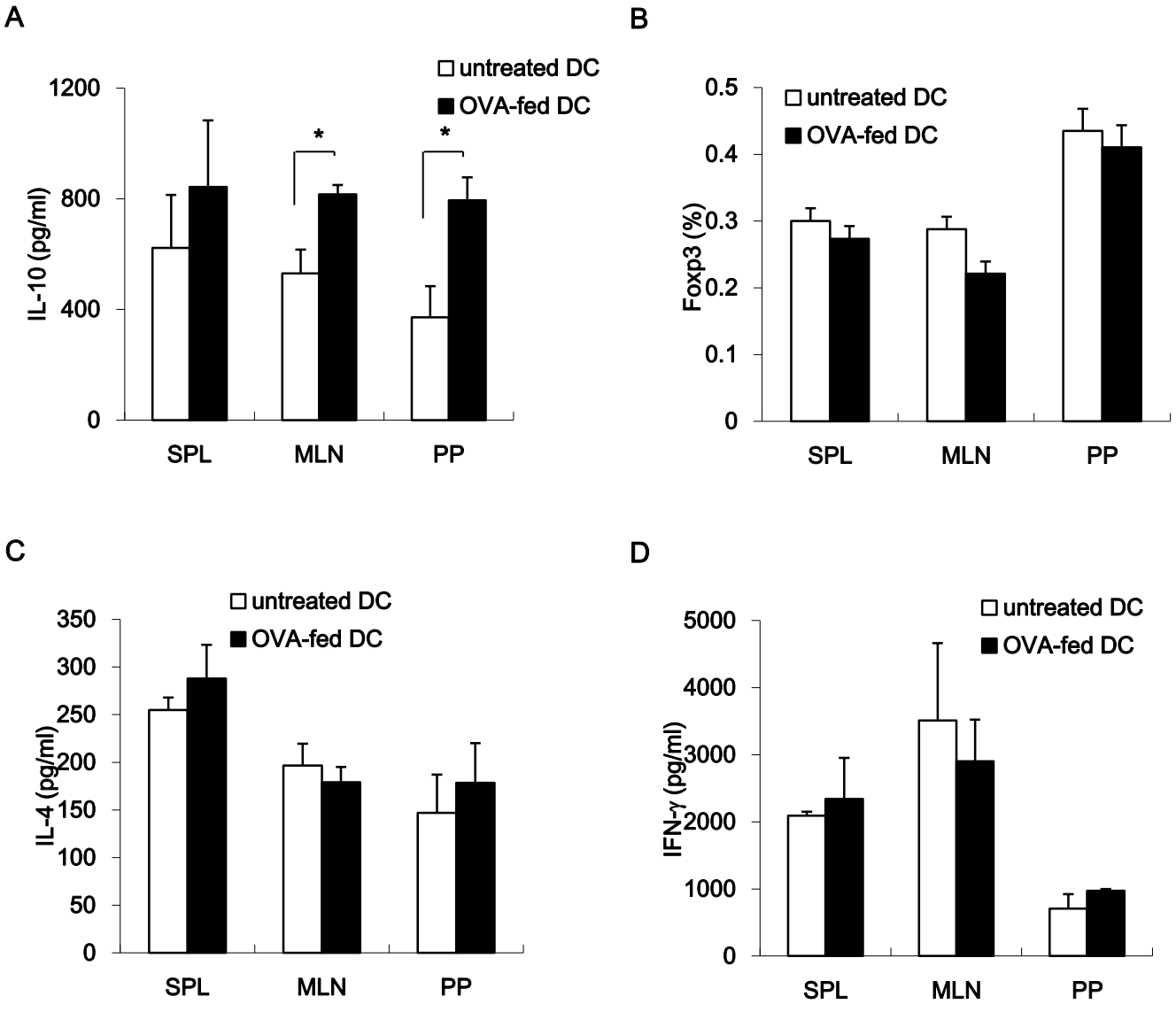

Figure 2. IL-10 production is increased in cultures of T cell and OVA-fed DC.
(A, C, D) SPL, MLN, and PP DC from OVA-fed or untreated mice were cultured with $CD11c^{-}CD4^{+}$ T cells from DO11.10 mice and 0.3 □M OVA peptide. After 72 h, IL-10 (A), IL-4 (C) and IFN-γ (D) in the supernatants were measured by ELISA (mean ± SD of triplicate cultures). (B) SPL, MLN, and PP DC from OVA-fed or untreated mice were cultured with $CD11c^{-}CD4^{+}$ T cells from $Rag2^{-/-}$DO11.10 mice and 0.3 μM OVA peptide. After 72 h, Foxp3 expression in cultured T cells was analyzed by FACS. Bars show the percentage of $CD4^{+}$ cells expressing Foxp3. *,$p<0.005$. Data represent one of nine (A), two (B) or three (C, D) independent experiments.

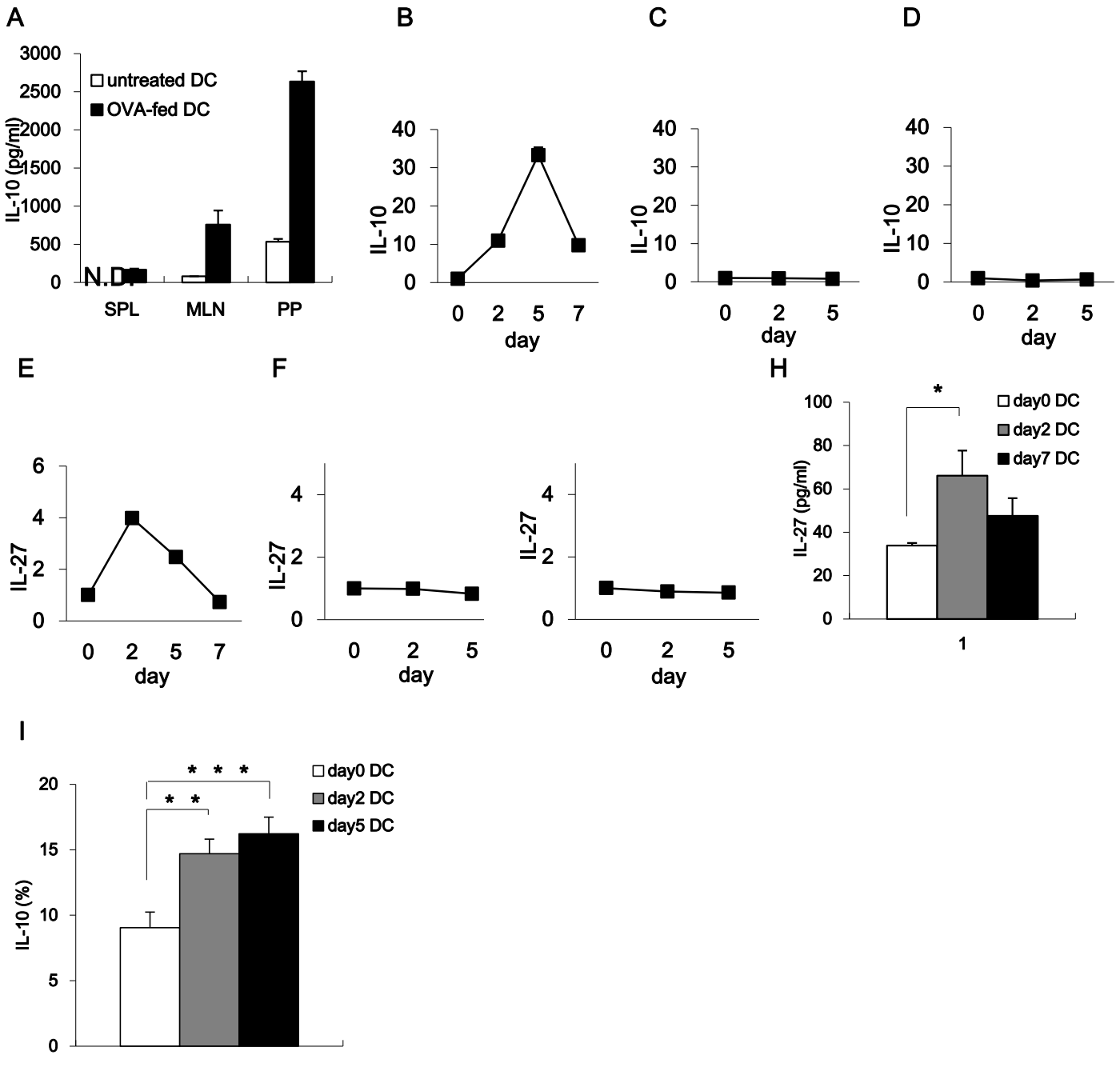

Figure 3. IL-10 and IL-27 producing OVA-fed DC induce IL-10 producing T cells.
(A) SPL, MLN, and PP DC from OVA-fed or untreated DO11.10 mice were stimulated with 5 μM anti-CD40 Ab. After 72h, IL-10 in the supernatants was measured by ELISA (mean±S.D. of triplicate cultures). (B–D) IL-10 mRNA of freshly isolated PP DC from OVA-fed DO11.10 mice (B), OVA-fed BALB/c mice (C), or BSA-fed DO11.10 mice (D) were measured by quantitative RT-PCR. (E–G) IL-27mRNA of freshly isolated PP DC from OVA-fed DO11.10 mice (E), OVA-fed BALB/c mice (F), or BSA-fed DO11.10 mice (G) were measured by quantitative RT-PCR. (H) PP DC from OVA-fed (0, 2, 7 days) DO11.10 mice were stimulated with 5 μM anti-CD40 Ab. After 72h, IL-27p28 in the supernatantswas measured by ELISA. (I) PP DC from OVA-fed (0, 2, 5 days) DO11.10 micewere cultured with splenic $CD11c^{-}CD4^{+}$ T cells from DO11.10 mice and 10 μM OVA peptide. After 48h, IL-10 production in $CD4^{+}$ T cellswas analyzed by FACS. Bars showthe percentage of $CD4^{+}$ cells expressing IL-10 (mean±S.D. of triplicate cultures, A, H, and I). *p<0.05; **p<0.005; ***p<0.001. Data represent one of three (A, B, D, E, and G), two (C and F) or six (I) independent

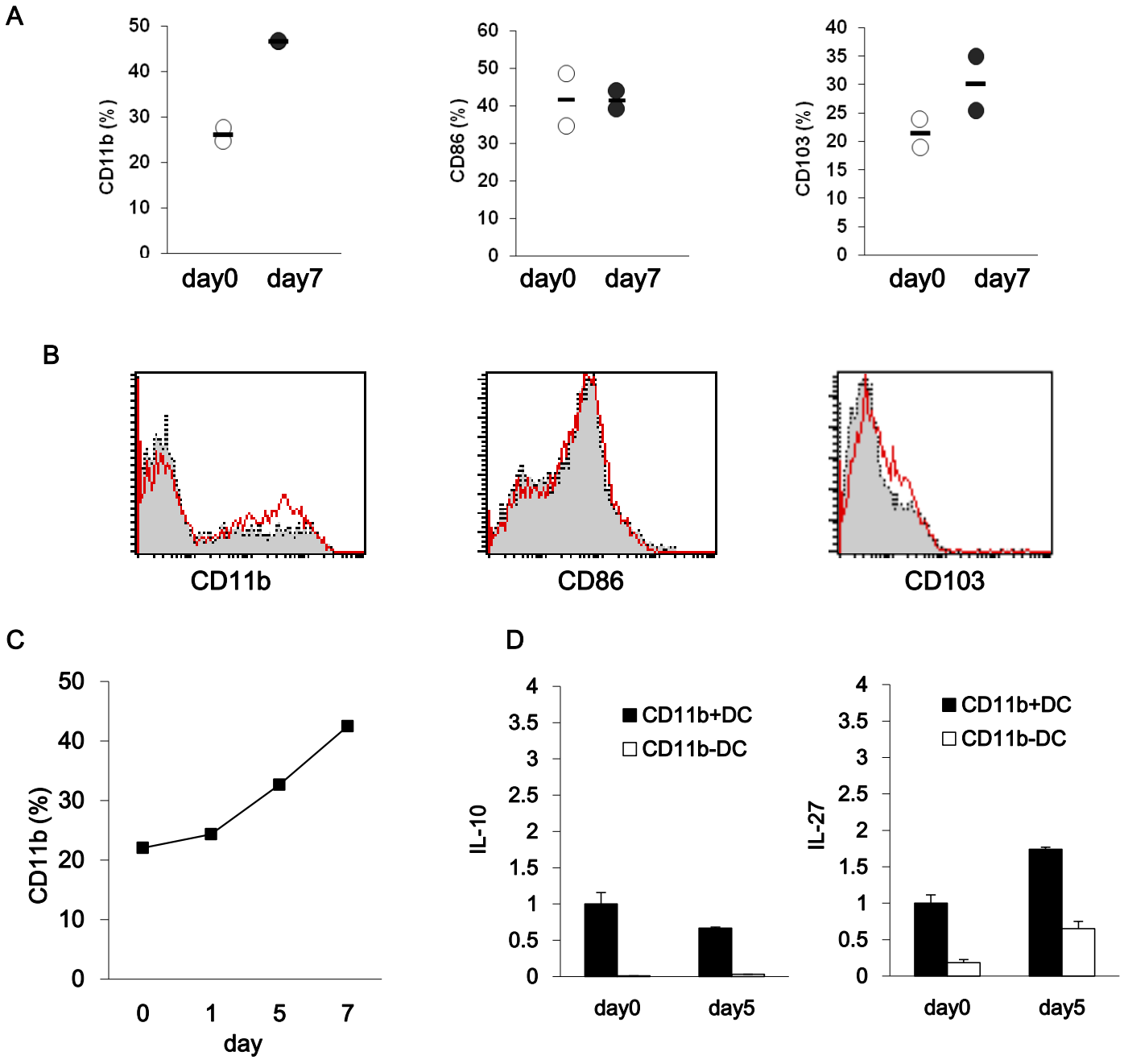

Figure 4. CD11b⁺ DC increase during oral tolerance induction.
(A) Surface expression of CD11b, CD86, and CD103 on freshly isolated PP DC from OVA-fed or untreated DO11.10 mice were analyzed by FACS. Plots show the percentage of $CD11c^+$ cells expressing CD11b, CD86, or CD103 of two independent experiments (bars show the means). (B) Histograms are gated on $CD11c^+$ cells. Shaded histograms are untreated DC and open histograms are OVA-fed DC. (C) CD11b expression of DC in freshly isolated PP cells from OVA-fed (0-7 d) DO11.10 mice was analyzed by FACS. Plots show the percentage of $CD3^-IgM^-CD11c^+$ cells expressing CD11b. (D) Expression of IL-10 or IL-27p28 mRNA in freshly isolated PP $CD11b^+$ DC or $CD11b^-$ DC from untreated or OVA-fed (5 d) DO11.10 mice were measured by quantitative RT-PCR. The mRNA level was expressed as ratio of that of day0 $CD11b^+$DC. Data represent one of two (C) or three (D) independent experiments.

Chapter 3

Th2 suppressive arginase 1 expressing CD11b$^+$ dendritic cells are induced in Peyer's patch after oral antigen administration

3.1. Introduction

Oral tolerance is the antigen (Ag)-specific suppression of immune responses to orally administrated Ag such as food proteins. Food allergies presumably result from either a failure to establish oral tolerance, or a breakdown in existing tolerance (43). Allergic immune responses are attributed to an excessive T helper (Th) 2-type immune response, characterized by IL-4, IL-13, and IL-5. Especially, IL-4 direct Th2 cell differentiation and trigger Ig class switching to IgE in B cells (70), and IL-4 is critical for induction and maintenance of allergic immune responses (71)(72). Foxp3$^+$ regulatory T cells (Treg) are essential for oral tolerance induction (7), however, IL-4 could abrogate Foxp3$^+$ Treg induction (73). Therefore, during oral Ag administration, suppression of excessive IL-4 production may be necessary to successfully establish oral tolerance.

In recent years, the perception of dendritic cells (DC) has shifted from inducers of immune reactivity to crucial regulators of immunity, namely DC contribute to T cell tolerance through co-stimulatory molecules or humoral factors (74)(75). DC are thought to play an important role in inducing oral tolerance (3), and several reports showed that particular DC subsets involved in inducing oral tolerance (8)(9)(10)(11)(12)(13). However, whether DC inducing oral tolerance are also capable of suppressing allergic responses remain understood. We hypothesized that DC in mice administrated oral Ag could suppress excessive Th2 response for successful induction of oral tolerance. This study therefore aimed to investigate the characteristics and mechanisms of DC suppressing Th2 response during oral tolerance induction by focusing on interactions with Ag-specific T cells. In order to expand the interaction with Ag-specific T cells, we used DO11.10 mice for oral tolerance induction. We investigated IL-4 suppressive function of DC in spleen, mesenteric lymph nodes, and Peyer's

patch (PP) from untreated or Ag-fed DO11.10 mice.

In this study, we determined the role of PP DC in IL-4 suppression during oral tolerance induction. Arginase 1 expressing PP $CD11b^+$ DC were induced by oral Ag administration, and were capable of suppressing the excessive Th2 responses

3.2. Materials and Methods

Mice

Balb/c mice were purchased from CLEA Japan (Tokyo, Japan). Balb/c mice and DO11.10 T cell receptor (TCR) transgenic mice (27) were used at 7–20 weeks old. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of The University of Tokyo. The protocol was approved by the Animal Use Committee of the Faculty of Agriculture at The University of Tokyo (approval number: P11-533).

Oral tolerance induction

Oral tolerance was induced as previously described (59). In brief, DO11.10 mice were fed with ovalbumin (OVA) (Wako) via drinking water (100 mg/ml) for 5 days. For control protein administration, DO11.10 mice were fed bovine serum albumin (BSA) (Wako, 013-15104) via drinking water (100 mg/ml).

Culture medium, antibodies, and reagents

Cells were cultured in RPMI 1640 medium supplemented with 5% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-mercaptoethanol. The following antibodies were used: FITC-labeled anti-CD11c (N418), PE-labeled anti-CD11b (M1/70, eBioscience), PE-Cy5-labeled anti-mouse CD4 (H129.19, BD Pharmingen), PE-labeled anti- IL-4, anti-CD16/32 (93, BioLegend), (BD Pharmingen), rIL-4 (PeproTech), anti-IL-12 (C17.8), anti-CD3ε (145-2C11), anti-CD28 (37.51, BD Pharmingen)

Preparation of DCs

DC were prepared from spleen (SPL), mesenteric lymph node (MLN) and PP in parallel. SPL, MLN and PP were incubated for 60 min at 37 ºC with collagenase (0.5 mg/ml; Wako, 032-10534) in RPMI containing 5 % FCS, and single cell suspensions were prepared. For

isolation of $CD11c^+$ cells, cells were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech) and selected on magnetic activated cell sorting (MACS) separation columns (Miltenyi Biotech). For isolation of DC subpopulations, cells selected on the basis of CD11c expression by MACS were stained with CD11c-FITC and CD11b-PE. Then $CD11b^+CD11c^+$ DC and $CD11b^-CD11c^+$ DC were sorted by fluorescence activated cell sorting (FACS) using a FACSVantage (BD Bioscience). For DC culture, MACS purified $CD11c^+$ DC were cultured with anti-CD40 (5 μg/ml) and without or with rIL-4 (2 ng/ml) for 48 h.

Preparation of $CD4^+$ T cells and Th2 induction

$CD4^+$ T cells were prepared from SPL, MLN, and PP of untreated DO11.10 mice. SPL, MLN, and PP was mashed and a single-cell suspension was prepared. The cells were incubated with anti-mouse CD4-coated magnetic beads and selected on MACS separation columns. For T cell transfer, SPL $CD4^+$ T cells (2×10^7 cells) from untreated DO11.10 mice were injected intravenously into Balb/c mice. For Ag-presenting cells (APC), splenocytes from BALB/c mice were treated with mitomycin C for 30 min at 37 °C. To generate Th2 cells, SPL $CD4^+$ T cells (2×10^6 cells) from untreated DO11.10 mice were cultured with APCs (1×10^6 cells), OVA peptide (1 μM: ISQAVHAAHAEINEGR; Biologica), rIL-4 (2 ng/ml), and anti-IL-12 (5 μg/ml) in 48 well plates. Cultures were maintained for 5 days and re-stimulated for 48 h by plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml).

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by using QIAshredder and RNeasy Mini Kits (both from Qiagen). Single-stranded cDNA was synthesized using Superscript II reverse transcriptase and Oligo dT primers (both from Invitrogen). Quantitative PCR reactions were performed using Quantitect Primer Assays with SYBR green PCR mastermix (Qiagen) with the following primers : arginase 1 forward, 5'-GCAACCTGTGTCCTTTCTCC-3'; reverse, 5'-GCAAGCCAATGTACACGATG-3': IL-4 forward, 5'-CGAAGAACACCACAGAGAGTGAGCT-3'; reverse, 5'-GACTCATTCATGGTGCAGCTTATC-3': HPRT forward,

5'-GAAGAGACTGGGGATCACTC-3'; reverse, 5'-CATGCCATCTTCCATATTGT-3' with the LightCycler (Roche). Gene expression levels for each individual sample were normalized to hypoxanthine phosphoribosyltransferase (HPRT).

Intracellular staining for IL-4

For Th2 suppression analysis, Th2 cells (2×10^5 cells) were co-cultured with splenic DC from Balb/c mice (2×10^4 cells), DC from OVA-fed DO11.10 mice (5×10^4 cells), and OVA peptide (1 μM) with or without the arginase inhibitor, Nor-NOHA (500 μM; Calbiochem) in 96-well flat-bottomed plates for 48 h. Goldistop (BD Pharmingen) was added to the cultures for the last 6 h. Cultured cells were harvested and stained with CD4-PE-Cy5 for IL-4 staining. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with saponin buffer (0.5% saponin; Sigma, 0.5% BSA, 0.2% NaN_3 in PBS) for 10 min at room temperature. After blocking Fcγ receptor with anti-CD16/32 for 20 min, cells were stained with IL-4-PE for 30 min. Data were acquired using a BD LSR flow cytometer (BD Bioscience) and analyzed using FlowJo software.

Statistical analysis

All experimental data were expressed as the mean ± standard deviation (SD). Statistical differences for all experimental data were analyzed by Student's *t*-tests.

3.3. Results

PP DC from OVA-fed mice suppress IL-4 production in Th2 cells

To induce oral tolerance, OVA was administrated to DO11.10 OVA-specific TCR transgenic mice in their drinking water for 5 days. In general, food Ag-induced allergic immune responses are characterized by the production of Th2-type cytokines. To examine whether DC after oral Ag administration could suppress IL-4 production in Th2 cells, $CD11c^+$ cells in SPL, MLN and PP from OVA-fed (OVA-fed DC) or untreated (untreated DC) DO11.10 mice were cocultured with Th2 cells. When cocultured with OVA-fed PP DC, IL-4 production in Th2 cells was decreased (Fig. 1). These results suggested that PP DC during oral tolerance induction have an ability to suppress excessive Th2 response.

$CD11b^+$ DC increase in PP after Ag-specific T cell response

It was previously reported that oral tolerance was induced in this system, and was shown that $CD11b^+$ DCs in PP after oral Ag administration increased in this model (20) (Fig. 2A). The percentage of $CD11b^+$ DC to whole PP cells, the number of $CD11b^+$ DC, and the percentage of $CD11b^+$ DC to whole PP DC increased after oral Ag administration (Fig. 2B). The increase of $CD11b^+$ DC was thought to be caused by Ag-specific T cell response. To investigate this possibility, DO11.10 mice were fed bovine serum albumin (BSA) as a non-specific Ag. No increase in $CD11b^+$ DC was observed in BSA-fed mice (Fig. 2C). To confirm the Ag specificity of the system, we adoptively transferred $CD4^+$ T cells from DO11.10 mice to Balb/c mice, and Balb/c mice were subsequently administrated OVA. $CD11b^+$ DC were also increased in this transfer model after oral Ag administration (Fig. 2D). These results suggested that the increase of $CD11b^+$ DC is caused by Ag-specific T cell response.

Arginase 1 expression in PP $CD11b^+$ DC increase after oral Ag administration

Figure 1 showed OVA-fed PP DC strongly suppress IL-4 production in Th2 cells compared to SPL DC or MLN DC. To determine tolerogenic factors of PP DC from OVA-fed DO11.10 mice, we analyzed the expression levels of several genes in SPL, MLN, and PP DC after oral Ag administration. We found that OVA-fed PP DC expressed higher arginase 1 than SPL DC or MLN DC, and arginase 1 expression in PP DC was greatly increased after oral Ag administration (Fig. 3A). To confirm the Ag specificity of the increase of arginase 1 expression, we adoptively transferred $CD4^+$ T cells from DO11.10 mice to Balb/c mice, and Balb/c mice were subsequently administrated OVA. Arginase 1 expression in DC was also increased in this transfer model after oral Ag administration (Fig. 3B). Figure 2 showed $CD11b^+$ DC were increased in PP after oral Ag administration, therefore we hypothesized that arginase 1 expression was correlated with $CD11b^+$ DC. As expected, further analysis revealed that prominent expression of arginase 1 was characteristic of the PP $CD11b^+$ DC subset (Fig. 3C). Arginase 1 expression was not remarkable in $CD11b^-$ DC nor $CD11b^+$ DC in SPL and MLN (Fig. 3C). The mechanism of arginase 1 upregulation in PP DC after oral Ag administration would be at least partly caused by the increase of $CD11b^+$ DC population in PP, but we further found that the mRNA expression level of arginase 1 was also increased in the $CD11b^+$ DC subset (Fig. 3D). These results suggested that the increase of arginase 1 expression in PP $CD11b^+$ DC are induced by Ag specific T cell response after oral Ag administration, both in quantity and quality.

PP $CD11b^+$ DC from OVA-fed mice suppress IL-4 production in T cells via arginase 1

As shown above, OVA-fed PP DC suppressed IL-4 and arginase 1 expressing $CD11b^+$ DC increased in PP after oral Ag administration. Therefore we hypothesized that arginase 1 activity in $CD11b^+$ DC was involved in IL-4 suppression. To examine whether PP $CD11b^+$ DC suppress IL-4 production, Th2 cells were cocultured with PP $CD11b^+$ DC from OVA-fed mice. To investigate arginase 1-mediated suppression, we performed *in vitro* assay by using arginase inhibitor Nω-hydroxy-nor-L-arginine, diacetate salt (Nor-NOHA). IL-4 production by Th2 cells was actually inhibited by PP $CD11b^+$ DC, and this IL-4 inhibition by PP $CD11b^+$ DC was abrogated by Nor-NOHA (Fig. 4). These results suggested that IL-4 suppressive function in PP DC was at least partly attributed to arginase 1 activation in $CD11b^+$ DC.

Arginase 1 expression in DC is induced by IL-4

Since the increase of arginase 1 expression in PP DC was caused by Ag-specific T cell response, we assumed that certain cytokine produced by PP T cells was key factor to induce arginase 1 expression. It is reported that arginase 1 expression in myeloid cells was induced by Th2 cytokine (76). Therefore we investigated IL-4 expression in SPL, MLN, and PP $CD4^+$ T cells from untreated mice. IL-4 was prominently expressed in PP $CD4^+$ T cells compared to SPL or MLN $CD4^+$ T cells (Fig. 5A). To test whether this cytokine was involved in arginase 1 upregulation, IL-4 was added to DC culture. As a result, arginase 1 expression in DC was increased by IL-4 (Fig. 5B). These observations suggested that after oral Ag administration, PP T cells abundantly produce IL-4, and increased arginase 1 expression in PP $CD11b^+$ DC. Then PP $CD11b^+$ DC suppress excessive IL-4 production by arginase 1, establishing IL-4-arginase 1 negative feedback loop.

3.4. Discussion

In this study, we determined the IL-4 suppressive mechanism of PP DC during oral tolerance induction. Arginase 1 expressing PP CD11b$^+$ DC were induced by oral Ag administration, and were capable of suppressing the excessive Th2 responses.

Arginase 1 expression represents part of the general phenotype of mouse myeloid cells, and M2 macrophages and MDSCs are well known to express arginase 1. Nevertheless, there are few reports about arginase 1 expression in DC. Arginase 1 expression in macrophage was induced by Th2 cytokines, such as IL-4 or IL-10 (77). We also showed arginase 1 expression in DC was increased by IL-4. Arginase 1 upregulation after oral Ag administration was specific for PP CD11b$^+$ DC, and did not significantly occur in CD11b$^-$ DC. For DC induction *in vitro*, GM-CSF and IL-4 are used in general, and these DC mostly express CD11b (78). It is possible that CD11b$^+$ DC are naturally sensitive for IL-4 signaling.

Arginase 1 is the enzyme to metabolize L-arginine, and its primary suppressive effect is to inhibit T cell proliferation by starving the cells of L-arginine (76). However, in this current study, arginase 1 inhibited IL-4 production, but not proliferation (data not shown). Arginase 1 also affects cytokine production (79)(80)(81), and suppressed colitis in a *Citrobacter rodentium* colitis model (82). So far, the role of arginase 1 in Th2 polarized immune responses has not been well known. Arginase 1-expressing macrophages function as suppressors of Th2 dependent inflammation and fibrosis (81), and LPS-induced lung F4/80$^+$Gr1^{int}CD11b$^+$ MDSCs suppress Th2 effector function via arginase 1 (79). The precise mechanism whereby arginase 1 inhibits excessive Th2 response remains unclear, but it may be metabolites of L-arginine. Arginase 1 converts L-arginine to L-ornithine and urea. L-ornithine is a precursor for the synthesis of polyamines (83). Polyamines such as putrescine, supernine, or spermidine might be assumed to be involved in Th2 suppression.

PP is a priming site to contact with foreign Ag. During Ag-specific T cell response, T cells in PP produced large amount of IL-4 compared to T cells in other secondary lymphoid organs such as SPL or MLN. Since excessive PP T cell activation may be a risk to induce allergic response, Th2 suppressive mechanism should be required especially in PP. Therefore IL-4-arginase 1 negative feedback loop may be a useful mechanism for suppressing excessive Th2 response in PP. The results presented in this study together with the previous

study (20) show that Ag recognition of T cells at PP induces $CD11b^+$ DC with high IL-10, IL-27 and arginase 1 expression, which regulate T cell responses, and presumably maintain immune homeostasis in the intestine.

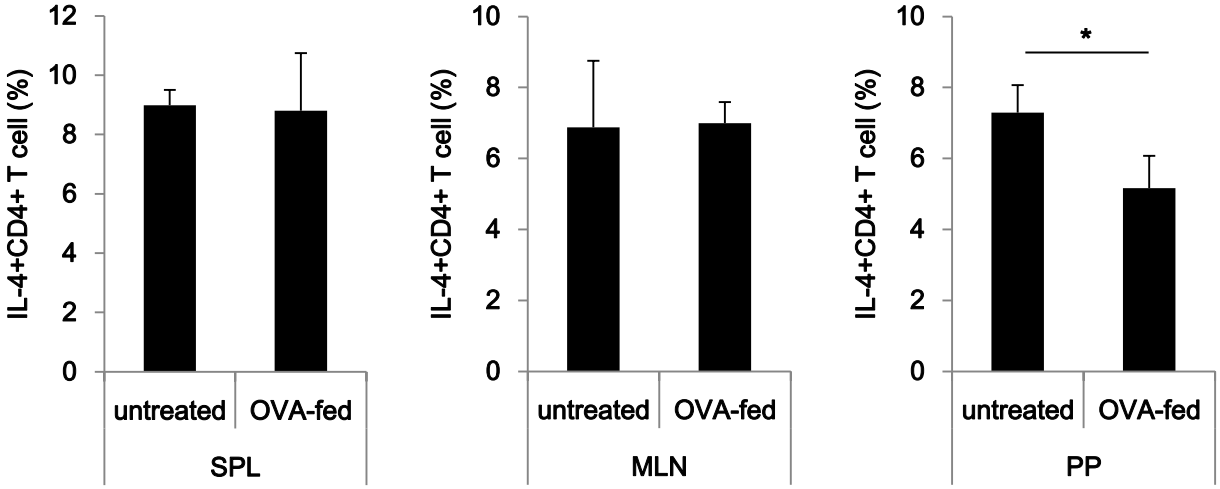

Figure 1. PP DC from OVA-fed mice suppress IL-4 production in Th2 cells.
DO11.10 mice were given OVA in drinking water for 5 days. *In vitro*-generated Th2 cells (2×10^5 cells) were cultured with SPL, MLN or PP DC (5×10^4 cells) from untreated or OVA-fed DO11.10 mice and OVA peptide (1 mM). After 48h, IL-4 production in $CD4^+$ T cells was analyzed by flow cytometry. Bars show the percentage of $CD4^+$ cells expressing IL-4. Bars represent mean±S.D. of triplicate cultures. *$p<0.05$ by the Student's *t*-test. Data are representative of three independent experiments.

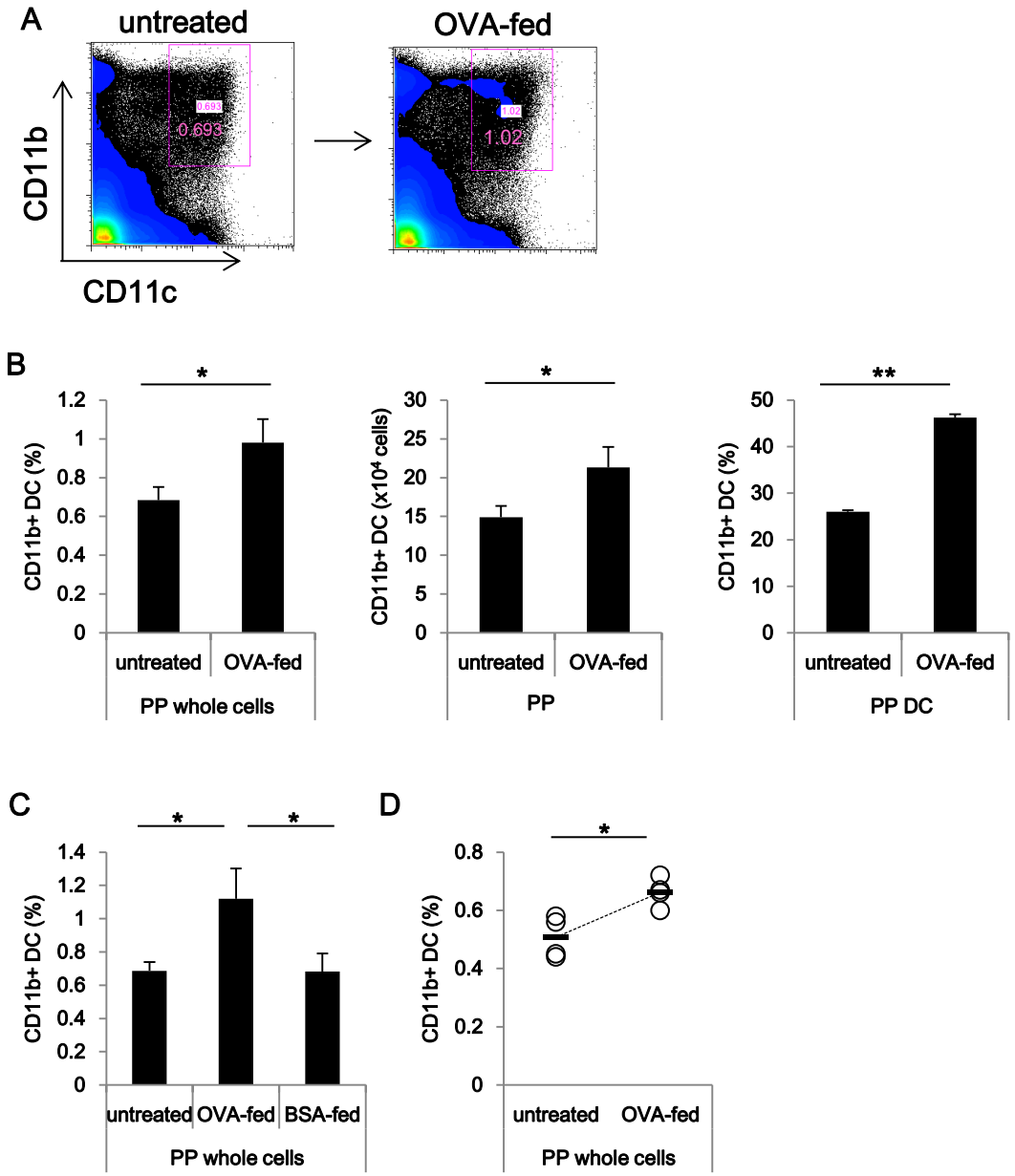

Figure 2. CD11b⁺ DC population increased after oral Ag administration in PP.
DO11.10 mice were given OVA in drinking water for 5 days. (**A**) PP whole cells from untreated or OVA-fed mice were stained with CD11c and CD11b, and analyzed by flow cytometry. Numbers adjacent to square areas indicate percentage of $CD11b^+CD11c^+$ population to whole cells. (**B**) Percentage of $CD11b^+$ DC to whole cells (left panel), absolute numbers of $CD11b^+$ DC in PP (middle panel), and percentage of $CD11b^+$ DC to all DC populations (right panel). n=3. Bars represent mean±S.D. of individual mice. (**C**) DO11.10 mice were given OVA or BSA in drinking water for 5 d, and percentage of $CD11b^+$ DC to whole cells was analyzed by flow cytometry. n=3. Bars represent mean±S.D. of individual mice. (**D**) Balb/c mice were adoptive transferred (i.v.) with splenic $CD4^+$ T cells from untreated DO11.10 mice. 24 h after transfer, Balb/c mice were maintained untreated or given OVA in drinking water for 5 d, and percentage of $CD11b^+$ DC to whole cells was analyzed by flow cytometry. n=3. Circles represent individual mice and horizontal lines represent the means for each group of samples. *p<0.05; **p<0.01 by the Student's *t*-test. Data are representative of nine (A and B) or three (C and D) independent experiments.

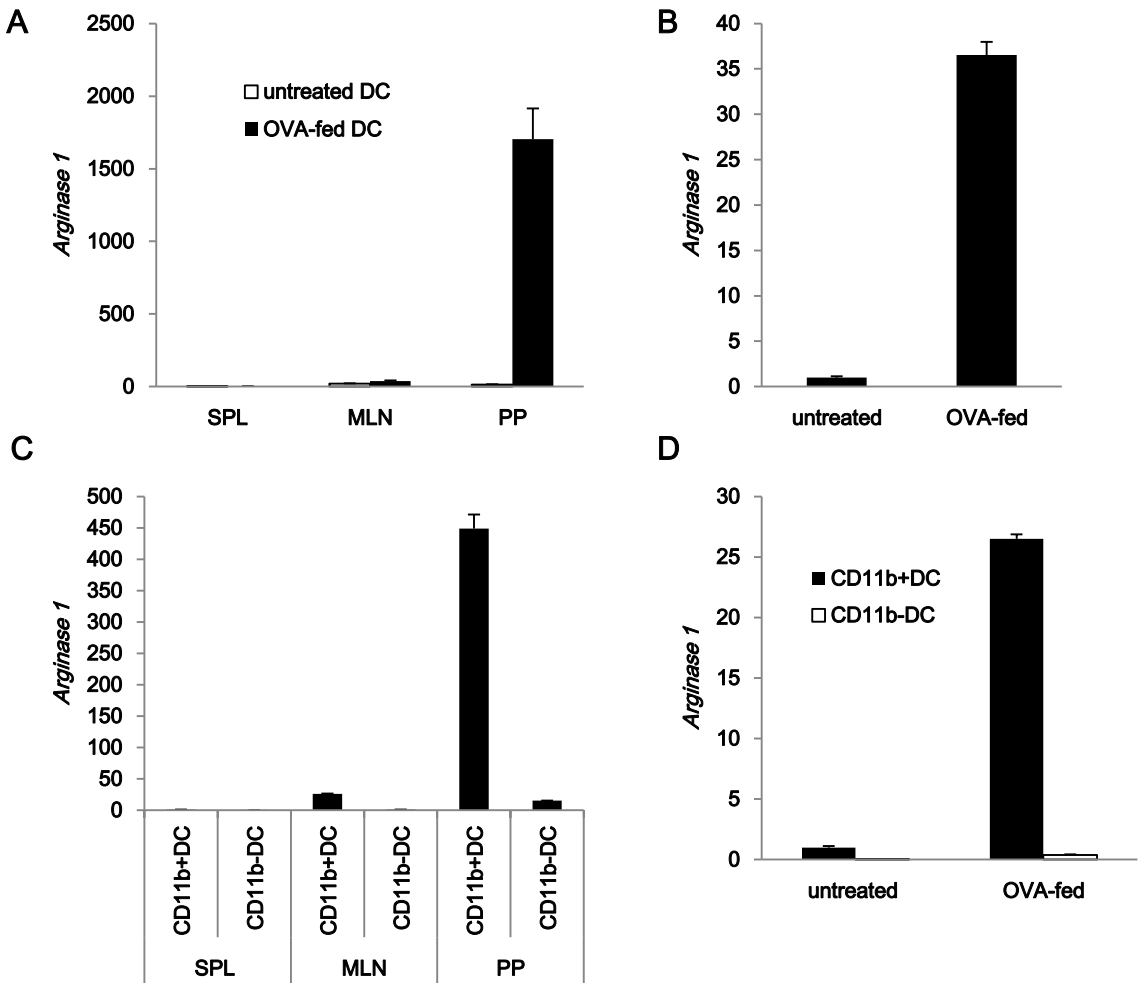

Figure 3. PP CD11b$^+$ DC from OVA-fed mice express arginase 1, which increased after oral Ag administration.

(**A**) Arginase 1 expression in SPL, MLN, PP DC from untreated or OVA-fed DO11.10 mice. The mRNA level of each sample was displayed as ratio to that of untreated SPL DC. (**B**) Balb/c mice were adoptive transferred (i.v.) with splenic CD4$^+$ T cells from untreated DO11.10 mice. 24 h after transfer, Balb/c mice were maintained untreated or given OVA in drinking water for 5 d, and arginase 1 expression in PP DC was measured. The mRNA level of OVA-fed DC was displayed as ratio to that of untreated DC. (**C**) Arginase 1 expression in CD11b$^+$ DC and CD11b$^-$ DC in SPL, MLN, and PP from OVA-fed DO11.10 mice. The mRNA level of each sample was displayed as ratio to that of SPL CD11b$^+$ DC. (**D**) Arginase1 expression in PP CD11b$^+$ DC and PP CD11b$^-$ DC from untreated or OVA-fed DO11.10 mice. The mRNA level of each sample was displayed as ratio to that of untreated CD11b$^+$ DC. Data are representative of five (A and D) or three (B and C) independent experiments.

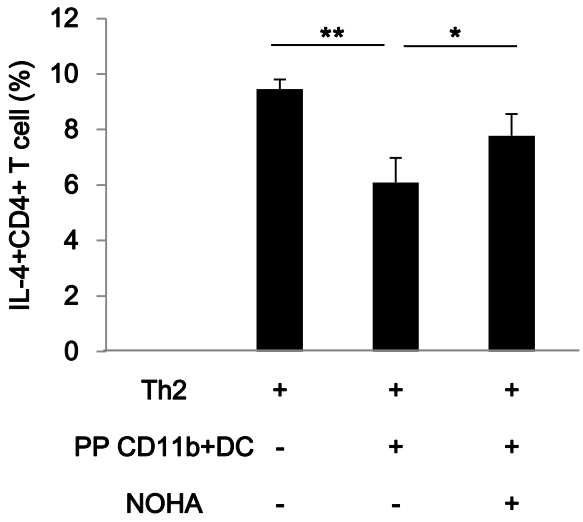

Figure 4. PP CD11b$^+$ DC from orally tolerized mice suppress excessive IL-4 production via arginase 1. *In vitro*-generated Th2 cells (2×10^5 cells) were were activated by splenic DC from Balb/c mice (2×10^4 cells) and OVA peptide (1 mM). In this culture (Th2), PP CD11b$^+$ DC from OVA-fed DO11.10 mice (5×10^4 cells) were added without or with Nor-NOHA (500 mM) as indicated. After 48 h, IL-4 production by Th2 cells was analyzed by flow cytometry. Bars show the percentage of CD4$^+$ cells expressing IL-4. Bars represent mean±S.D. of triplicate cultures. *p<0.05; **p<0.01 by the Student's *t*-test. Data are representative of four independent experiments.

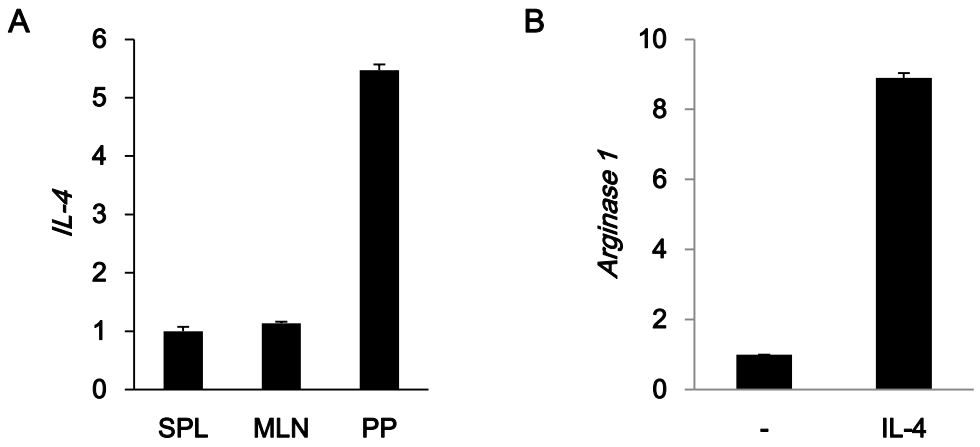

Figure 5. Arginase 1 expression in DC is induced by IL-4.
(**A**) IL-4 mRNA expressions in CD4 $^{+}$ T cells in SPL, MLN, PP from untreated DO11.10 mice. The mRNA level of each sample was displayed as ratio to that of SPL CD4 $^{+}$ T cells. (**B**) Arginase 1 expression in SPL DC from Balb/c mice cultured with anti-CD40 (5 mg/ml) in the absence or presence or of rIL-4 (2 ng/ml) for 48h. The mRNA level of IL-4 treated (IL-4) DC was displayed as ratio to that of untreated (-) DC. Bars represent mean±S.D. of triplicate cultures. Data are representative of three independent experiments.

Chapter 4

Th2 suppressive arginase 1 expressing neutrophils accumulating in Peyer's patch after oral Ag administration

4.1. Introduction

Neutrophils have long been viewed as the effetor cells of the innate immune responses, being recruited to inflamed tissue and clearing extracellular pathogens (84). The participation of neutrophils in adaptive immune responses has not been considered relevant until recently. Nevertheless, evidence is growing that neutrophils are involved in the activation, regulation and effector functions of adaptive immune cells as well as innate immune cells (85). For instance, neutrophils can participate in Ag presentation, and therefore could induce and modulate the T cell response (86). Neutrophils increased IFN-γ and IL-17 in activated T cells (87), and it was recently demonstrated that expression of MHC class II molecules in neutrophils can be induced by T cells *in vitro* and consequently promote the differentiation of Ag-specific Th1 and Th17 cells (88). In addition to the these immunoactive function, neutrophils can also have immunosuppressive functions (89) (90). However, it is not clear whether neutrophils are involved in Ag-specific immune tolerance. Therefore we examined that neutrophils involved in Ag-specific immune tolerance using a mouse model of oral tolerance, the Ag-specific suppression of immune responses to orally administrated Ag such as food proteins. In order to expand the response of Ag-specific T cells, we used DO11.10 mice for oral tolerance induction.

In this study, we determined the role of neutrophils in IL-4 suppression during oral tolerance induction. Neutrophils found to be accumulated in Peyer's patch (PP) of the small intestine, the main Ag sampling site. It was suggested that neutrophils played a role in oral tolerance induction through IL-4 suppression.

4.2. Materials and Methods

Mice

Balb/c mice were purchased from CLEA Japan (Tokyo, Japan). Balb/c mice and DO11.10 TCR ransgenic mice (27) were used at 7–20 weeks old. All work was performed in accordance with The University of Tokyo guidelines for animal care and use.

Oral tolerance induction

Oral tolerance was induced as in the previous study (59). In brief, DO11.10 mice were fed with ovalbumin (OVA) (Wako; Albumin, from Eggs, 012-09885) via drinking water (100 mg/ml) for 5 days. The daily intake of OVA was estimated to be around 100 mg/mouse. The endotoxin level of this material was tested by *Limulus* amoebocyte lysate assay using an ET-5000 Toxinometer (Wako) and was confirmed to be below approximately 1 endotoxin unit/mg. For control protein administration, DO11.10 mice were fed bovine serum albumin (BSA) (Wako, 013-15104) via drinking water (100 mg/ml).

Culture medium, antibodies, and reagents

Cells were cultured in RPMI 1640 medium supplemented with 5% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-mercaptoethanol. The following antibodies were used: anti-CD16/32 (93, BioLegend), FITC-labeled anti-CD11b (M1/70, BioLegend), biotinylated anti-Gr1 (RB6-8C5, BioLegend), PE-labeled anti-Ly6G (1A8, BioLegend), PE-labeled anti-CD115 (AFS98, BioLegend), biotinylated anti-gp38 (8.1.1, BioLegend), FITC-labeled anti-CD45 (30-F11, BioLegend), FITC-labeled anti-CD31 (390, BioLegend), FITC-labeled anti-CD35 (7E9, BioLegend), PE-labeled anti-gp38 (8.1.1, BioLegend), streptavidin-PE (BD Pharmingen), streptavidin-PE-Cy5 (BD Pharmingen), streptavidin-Texas Red (Beckman Coulter), PE-Cy5-labeled anti-mouse CD4 (H129.19, BD Pharmingen), PE-labeled anti-IL-4 (BD Pharmingen), rIL-4 (PeproTech), anti-IL-12 (C17.8), anti-CD3ε (145-2C11), anti-CD28 (37.51,

BD Pharmingen)

Preparation of neutrophils in secondary lymphoid organs (SLO)

Neutrophils were prepared from spleen (SPL), mesenteric lymph nodes (MLN) and PP in parallel. SPL, MLN and PP were incubated for 60 min at 37 ºC with collagenase (0.5 mg/ml; Wako, 032-10534) in RPMI containing 5 % FCS, and single cell suspensions were prepared. For isolation of neutrophils, firstly $CD11b^+CD11c^-$ cells were enriched. Cells were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech) and anti-mouse CD11b-coated magnetic beads (Miltenyi Biotech), and $CD11b^+CD11c^-$ cells were selected on magnetic activated cell sorting (MACS) separation columns (Miltenyi Biotech). $CD11b^+CD11c^-$ enriched cells were blocked Fcγ receptor with anti-CD16/32, and then stained with CD11b-FITC, Ly6G-PE, and Gr1-bio-Cy5. $Gr1^{hi}CD11b^{hi}Ly6G^+$ cells or $CD11b^{hi}Ly6G^+$ cells (for Th2 culture) were sorted by FACS using a FACSVantage (BD Bioscience).

Preparation of peritoneal neutrophils

Neutrophils are prepared using protocol to isolate peritoneal neutrophil preparation (91). In brief, 1 ml casein solution (9 g casein (from bovine milk, sodium salt; Sigma) in PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) was injected into the peritoneal cavity. Inflammatory response was allowed to develop overnight and injection of 1.0 ml casein solution was repeated the next day. 3 h after the second injection, peritoneal fluid was collected and incubated with biotin-labeled anti-Gr1. Then cells were incubated with streptavidin-coated magnetic beads and $Gr1^+$ cells were purified by MACS. $Gr1^+$ enriched cells were blocked Fcγ receptor with anti-CD16/32, and then stained with CD11b-FITC, Ly6G-PE, and Gr1-bio-Cy5. $Gr1^{hi}CD11b^{hi}Ly6G^+$ cells were sorted by FACS using a FACSVantage or FACSAria.

Preparation of bone marrow (BM) cells and blood cells

BM cells were isolated from hind leg bones. The resulting cell suspension was passed

through a nylon mesh filter, and erythrocytes were removed using RBC lysis buffer (0.01M Tris-HCl, 0.15M NH_4Cl). Blood cells were isolated by heart puncture of anesthetized mice, and erythrocytes were removed using RBC lysis buffer. For CFSE labeling, BM cells from Balb/c mice, at a maximum concentration of 5×10^7 cells/ml, were incubated in 5 μM CFSE (Molecular Probes) in PBS for 10 min at room temperature.

Immunohistochemical staining

For immunofluorescence studies, PPs were dissected from mouse small intestine, and frozen in Tissue-Tek OCT compound (Sakura Finetechnical). Cryosections (6-μm thick) were prepared with a cryostat, HM505E (Microm). The sections were fixed in cold acetone for 10 min. Nonspecific binding was blocked with 10% normal rat serum and anti-CD16/32. Each antibody was stained for 3 h. For nuclear staining, DAPI (0.4 μg/ml, DOJINDO, D523) was stained for 15 min. After staining, slides were mounted with malinol (Muto Pure Chemicals). The stained slides were analyzed under a fluorescence microscope (BZ-8100; Keyence, Tokyo, Japan).

Preparation of FRC and neutrophil migration assay

For isolation of FRC from PP, PP cells were incubated with biotinylated anti-gp38 for 20 min at 4 ºC. Cells were incubated with streptavidin-coated magnetic beads (Miltenyi Biotech) and selected by MACS. $Gp38^+$ enriched cells were blocked Fcγ receptor with anti-CD16/32, and then stained with CD45-FITC, CD31-FITC, CD35-FITC and gp38-PE. $CD45^-CD31^-CD35^-gp38^+$ cells were sorted by FACS using a FACSVantage (BD Bioscience) and used as FRC. Sorted FRC (1×10^5 cells) were cultured for 5d in RPMI containing 10 % FCS. Supernatants (235 μl) from FRC culture or medium (RPMI containing 10 % FCS incubated for 5d) were added to the lower chamber of a transwell plate (Corning HTS 96 well transwell, 3.0 μm pore size). Peritoneal neutrophils (1×10^5 cells) were added to the upper chamber of the transwell plate (75μl). After 24h, the number of neutrophils which had migrated to the lower chamber was counted.

Preparation of CD4+ T cells and Th2 induction

$CD4^+$ T cells were prepared from SPL, MLN, and PP of untreated DO11.10 mice. SPL, MLN, and PP was mashed and a single-cell suspension was prepared. The cells were incubated with anti-mouse CD4-coated magnetic beads (Miltenyi Biotech) and selected on MACS separation columns (Miltenyi Biotech). For APCs, splenocytes from BALB/c mice were treated with mitomycin C for 30 min at 37°C. To generate Th2 cells, $CD4^+$ T cells (2×10^6 cells) from DO11.10 mice were cultured with APCs (1×10^6 cells), OVA peptide (1 μM: ISQAVHAAHAEINEGR; Biologica), rIL-4 (2ng/ml), and anti-IL-12 (5 μg/ml) in 48 well plates. Cultures were maintained for 5 days and re-stimulated for 48 h by plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml).

Adoptive cell transfer

For T cell transfer, SPL $CD4^+$ T cells (2×10^7 cells) from DO11.10 mice were injected intravenously into Balb/c mice. For BM cell transfer, CFSE-labeled BM cells (1×10^8 cells) from Balb/c mice were injected intravenously into untreated DO11.10 mice.

Measurement of cytokine secretion

Cytokine levels in the culture supernatants were assayed by specific sandwich ELISAs. IL-4 and IFN-γ were measured as previously described (28). An OPTEIA ELISA set (BD Biosciences) was used for IL-10 measurement.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using QIAshredder and RNeasy Mini Kits (both from Qiagen). Single-stranded cDNA was synthesized using Superscript II reverse transcriptase and Oligo dT primers (both from Invitrogen). Quantitative PCR reactions were performed using Quantitect Primer Assays with SYBR green PCR mastermix (Qiagen) with the following primers : arginase 1 forward, 5'-GCAACCTGTGTCCTTTCTCC-3'; reverse,

5'-GCAAGCCAATGTACACGATG-3': IL-4 forward, 5'-CGAAGAACACCACAGAGAGTGAGCT-3'; reverse, 5'-GACTCATTCATGGTGCAGCTTATC-3': HPRT forward, 5'-GAAGAGACTGGGGATCACTC-3'; reverse, 5'-CATGCCATCTTCCATATTGT-3' with the LightCycler (Roche). Gene expression levels for each individual sample were normalized to hypoxanthine phosphoribosyltransferase (HPRT).

Intracellular staining for IL-4

For IL-4 producing analysis, Th2 cells (2×10^5 cells) were stimulated by plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml) and neutrophils (5×10^4 cells) were added with or without the arginase inhibitor, Nor-NOHA (500 μM; Calbiochem) in 96-well flat-bottomed plates for 48 h. Goldistop (BD Pharmingen) was added to the cultures for the last 6 h. Cultured cells were harvested and blocked Fcγ receptor with anti-CD16/32, and then stained with CD4-PE-Cy5 for intracellular IL-4 staining. The cells were then fixed with 4% paraformaldehyde in PBS and permeabilized with saponin buffer (0.5% saponin; Sigma, 0.5% BSA, 0.2% NaN_3 in PBS). After blocking Fcγ receptor, cells were stained with IL-4-PE. Data were acquired using a BD LSR flow cytometer (BD Bioscience) and analyzed using FlowJo software.

Statistical analysis

All experimental data were expressed as the mean ± standard deviation (SD). Statistical differences for all experimental data were analyzed by Student's *t*-tests.

4.3. Results

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Neutrophils are increased in PP upon CD4+ T cell response to orally administrated Ag

To induce oral tolerance, OVA was fed to DO11.10 OVA-specific TCR transgenic mice in their drinking water for 5 days. $Gr1^{hi}CD11b^{+}$ cells were increased in PP of small intestine, the site of antigen sampling and induction of immune response after oral Ag administration (Fig. 1A, left). Anti-Gr-1 antibodies (clone RB6-8C5) recognize both Ly6G and Ly6C, and the latter are expressed by monocytes and dendritic cells (DC). We verified that $Gr1^{hi}CD11b^{+}$ cells accumulated in PP highly expressed Ly6G and did not express CD115/M-CSF receptor (Fig. 1A, right). Moreover, morphology showed they are neutrophils (Fig. 1B). The percentage of neutrophils to whole PP cells and the absolute number of neutrophils increased after oral Ag administration (Fig. 1C). Since the response of Ag-specific $CD4^{+}$ T cells can be amplified in this DO11.10 OVA-specific TCR transgenic mouse model, the increase of neutrophils was assumed to be caused by Ag-specific $CD4^{+}$ T cell response. To investigate this possibility, DO11.10 mice were fed bovine serum albumin (BSA) as a non-specific Ag. No increase in neutrophils was observed in these mice (Fig. 1D). To confirm the Ag specificity of the system, we adoptively transferred $CD4^{+}$ T cells from DO11.10 mice to Balb/c mice, which were subsequently administrated OVA. Neutrophils were also increased in this transfer model after oral Ag administration (Fig. 1E). These results suggest that neutrophils are increased in PP after oral Ag administration due to the Ag-specific $CD4^{+}$ T cell response.

Neutrophils are attracted by FRC to PP after oral Ag administration

We then analyzed the localization of neutrophils in PP. Tissue-section analysis revealed that $Ly6G^{+}$ neutrophils were localized in the T cell–rich interfollicular region (IFR) of PP after oral Ag administration (Fig. 2A). Neutrophils are produced solely in the bone marrow and recruited to inflamed tissues via the blood (92), and a number of studies have shown that neutrophils can also traffic to lymph nodes (93)(94)(95). Similarly, the proportion of neutrophils in the blood increased after oral Ag administration (Fig. 2B). We assumed that neutrophils and/or their precursors in BM accumulate in PP via the blood after oral Ag

administration as well as under an inflamed condition. To test this possibility, we adoptively transferred CFSE-labeled BM cells to untreated DO11.10 mice, which were subsequently administrated OVA. As expected, BM-derived neutrophils in PP were increased after oral Ag administration (Fig. 2C). In SLO such as PP, T cell regions are known to be constituted by FRC (96), which are classified as $gp38^{+}CD31^{-}CD45^{-}$ cells, and also negative for CD35 (follicular dendritic cell marker). Since Figure 2A showed that neutrophils and FRC were placed at the same region, we assumed that FRC are involved in the neutrophil accumulation. To elucidate whether FRC attract neutrophils after oral Ag administration, FRC from untreated or OVA-fed mice were cultured for 5d. Supernatant of FRC culture was added to the bottom of transwells and assayed for their ability to recruit neutrophils. Supernatant of FRC from OVA-fed mice significantly stimulate neutrophil migration compared to FRC from untreated mice or medium alone (Fig. 2D). Above results suggest a stepwise neutrophil attraction; Ag-specific $CD4^{+}$ T cell response activates FRC to secrete certain chemokines, which subsequently promote neutrophil migration from blood to T cell region in PP.

PP neutrophils suppress IL-4 production in $CD4^{+}$ T cells

To examine whether the accumulated neutrophils after oral Ag administration affected $CD4^{+}$ T cell response, PP neutrophils from OVA-fed mice were cocultured with naïve $CD4^{+}$ T cells, and measured cytokine secretion. When cocultured with neutrophils, IL-4 secretion in supernatant of cultures and IL-4 mRNA expression in $CD4^{+}$ T cells was decreased (Fig. 3A and B), whereas IFN-γ and IL-10 secretion was not affected (Fig. 3A). Consistently, IL-4 production of PP $CD4^{+}$ T cells decreased after oral Ag administration (Fig. 3C). These results suggest that neutrophils accumulated in PP have the ability to suppress IL-4 during oral tolerance induction.

Arginase 1 upregulated in PP neutrophils contributes to the suppression of IL-4 production in $CD4^{+}$ T cells after oral Ag administration

We next examined whether neutrophils in SLO other than PP after oral Ag administration

also have IL-4 suppressive function. Th2 cells were cocultured with neutrophils from SPL, MLN or PP, and IL-4 production in Th2 cells was measured. Besides PP neutrophils, MLN but not SPL neutrophils also suppressed IL-4 production in Th2 cells (Fig. 4A). To determine factors of IL-4 suppression in neutrophils, we analyzed the expression levels of several genes in SPL, MLN, and PP neutrophils after oral Ag administration. PP neutrophils markedly expressed arginase 1, which was also expressed in MLN neutrophils, but only very slightly in SPL neutrophils (Fig. 4B). As arginase 1 expression pattern of neutrophils was correlated with IL-4 suppressive function, we tested whether arginase 1 activity suppressed IL-4. To investigate arginase 1-mediated suppression, we performed *in vitro* assay by using arginase inhibitor Nω-hydroxy-nor-L-arginine, diacetate salt (Nor-NOHA). The suppression of IL-4 production in Th2 cells induced by PP neutrophils was abrogated by Nor-NOHA (Fig. 4C). These results suggest that IL-4 suppressive function of PP neutrophils is due to high arginase 1 expression in those cells.

IL-4 stimulation increases arginase1 expression in neutrophils

To examine whether arginase 1 expression in PP neutrophils is an acquired function or not, we measured the gene expression in PP neutrophils from untreated or OVA-fed mice. OVA-fed PP neutrophils prominently expressed arginase 1, whereas untreated neutrophils did only slightly (Fig. 5A). Previous studies demonstrated that human neutrophils constitutively express arginase1, whereas murine neutrophils from C57BL/6 mice did not express arginase1, unless stimulated by IL-4 (97)(98). Similar to the previous study (97), arginase 1 expression in neutrophils from Balb/c mice was increased by IL-4 (Fig. 5B). Therefore we investigated IL-4 production in SPL, MLN, and PP $CD4^+$ T cells from untreated mice. IL-4 was prominently produced in PP $CD4^+$ T cells compared to SPL or MLN $CD4^+$ T cells after antigenic stimulation (Fig. 5C). Furthermore, the IL-4 treated neutrophils prominently suppressed IL-4 production in $CD4^+$ T cells compared to non-treated neutrophils (Fig. 5D). These observations suggest that after oral Ag administration, PP $CD4^+$ T cells abundantly produce IL-4 which subsequently increased arginase 1 expression in PP neutrophils. Then the upregulated arginase 1 activity in PP neutrophils suppress excessive IL-4 production in $CD4^+$ T cells, establishing IL-4-arginase1 negative feedback

loop which may contribute to oral tolerance induction.

4.4. Discussion

In this study, we found a role for neutrophils during oral tolerance induction. First, after oral Ag administration, neutrophils are attracted by PP FRC after Ag-specific $CD4^+$ T cell response. Second, PP accumulated neutrophils suppressed excessive Th2 responses through arginase 1. Third, arginase 1 expression in neutrophils would be induced by IL-4 producing PP $CD4^+$ T cells.

Food allergies are attributed to an excessive Th2-type immune response, and especially IL-4 is critical for induction and maintenance of allergic immune responses (71)(72). IL-4 could abrogate induction of $Foxp3^+$ regulatory T cells (Treg) (73), which are essential for oral tolerance induction (7). Food allergies presumably result from either a failure to establish oral tolerance, or a breakdown in existing tolerance. Therefore, during oral Ag administration, suppression of excessive IL-4 production may be necessary to successfully establish oral tolerance and prevent food allergy. We show here a role of neutrophils to be a player for successful oral tolerance induction by suppressing excessive Th2 response via arginase 1.

IL-4 induced arginase 1expression in neutrophils. Oral administration of antigen enhanced IL-4 expression in neutrophils, and neutrophils from Ag-fed mice were capable of inhibiting T-cell IL-4 response. Finally, IL-4 production in CD4 T cells was decreased by Ag feeding. These results suggest the presence of a IL-4- arginase 1 negative feedback loop established by $CD4^+$ T cells and neutrophils. It has been reported that arginase 1 expression is induced by IL-10 as well as IL-4 (77)(97). We and others have reported that during oral tolerance induction, IL-10 production is increased in PP (62)(20). Therefore, in addition to IL-4, IL-10 could be another inducer of arginase 1.

Arginase 1 is the enzyme to metabolize L-arginine (83)(76). Arginase 1 expression in neutrophils has been well investigated in human neutrophils but not so in murine neutrophils. Whereas human neutrophils constitutively express arginase 1, this expression in murine neutrophils has been reported to be inducible (97)(98). Some reports have shown that arginase 1 in neutrophils suppress T cell proliferation (99)(100). In general, the suppressive effect of arginase1 in neutrophils was focused on suppression of proliferation. On the other hand, our study firstly shows Th2 suppressive function by arginase 1 in

neutrophils. Such Th2 suppressive function of arginase 1 has been previously reported in macrophages (81) and myeloid-derived suppressor cells (79), but has not yet been reported in neutrophils. The detailed Th2 suppressive mechanism by arginase 1 remains unknown, but L-arginine metabolites such as polyamines might function as a mediator of this suppression.

Although it has been reported that neutrophils acquired Ag presenting function in certain conditions (101)(86)(88), we verified that accumulated PP neutrophils only slightly expressed MHC class II and could not efficiently present Ag presentation *in vitro* (data not shown). Nevertheless, it is possible that neutrophils cooperated with other APC, such as DC, in Ag-specific responses. It has been reported that neutrophils induce TGF-β producing tolerogenic DC (102)(90). Moreover, neutrophils interfere with Ag presentation of DC, which leads to poor DC-T cell interaction, resulting in suppression of T cell response *in vivo* (103). Therefore neutrophils accumulated in PP might cooperate with DC to establish oral tolerance.

Our results also suggest that certain chemokine production by FRC was upregulated after Ag-specific T cell response, contribute to attract neutrophils from blood to PP. In SLO, FRC residing in T cell region are well known to produce CCL19 and CCL21, and retain T cells and DC in CCR7 dependent manner (96). It has also been reported that neutrophils migrate to secondary lymph nodes in CCR7 dependent manner (94). Therefore CCL19 and CCL21 production in PP FRC could to be involved in neutrophil attraction. In addition, CXCL8 might also be a possible chemokine involved in neutrophil attraction by FRC. FRC are assumed to be differentiated from mesenchymal stem cells (MSC) (104), so FRC are assumed to function similarly to MSC. Itwas reported that activated MSCs secreted large amounts of inflammatory cytokines and recruited neutrophils in a partly CXCL8 dependent manner (105), and MSC controlled neutrophil function (106). Further investigation is needed for detect neutrophil attracting chemokine during oral tolerance induction.

In summary, we identified suppressive function of neutrophil establishing IL-4-arginase 1 negative feedback loop during oral tolerance induction.

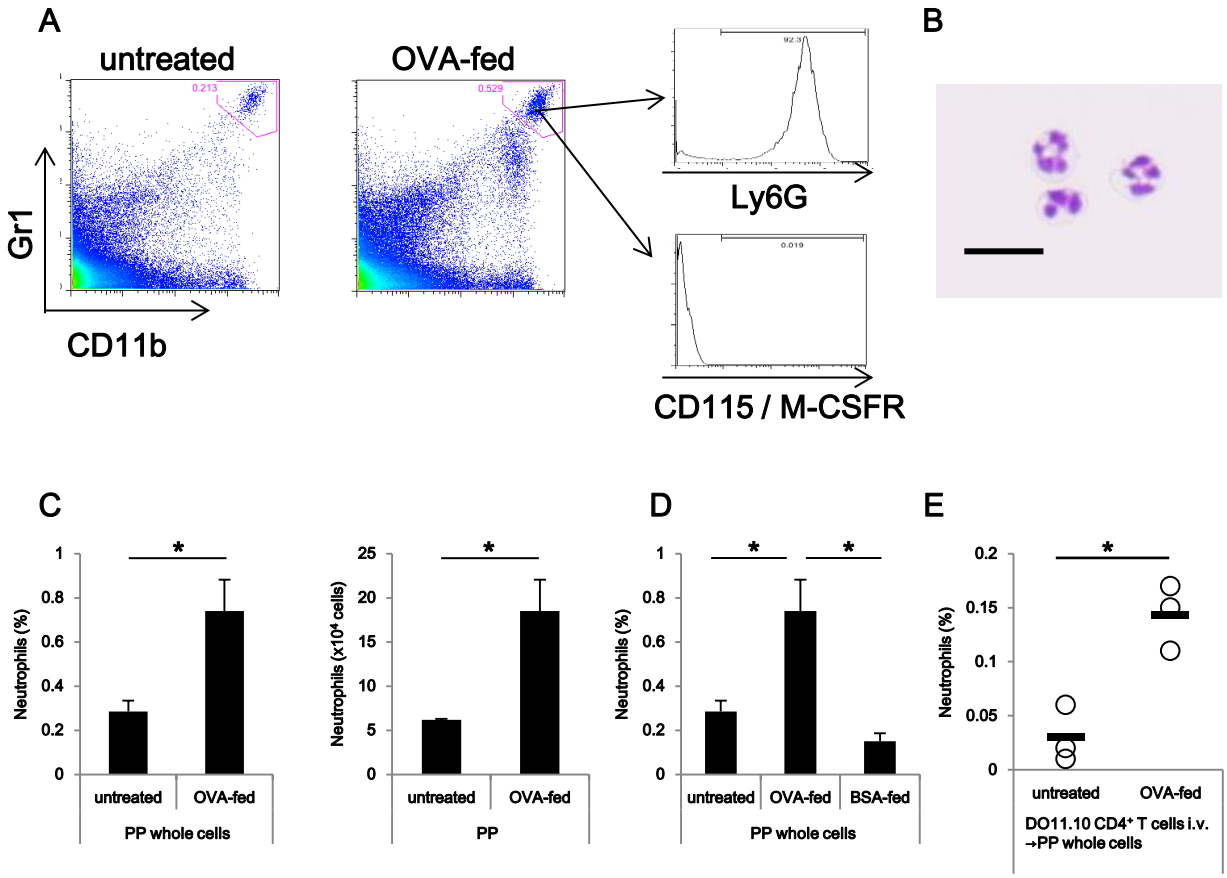

Figure1. Neutrophils increased in PP after oral Ag administration.
DO11.10 mice were given OVA in drinking water for 5 d. (**A**) PP whole cells from untreated or OVA-fed mice were stained with anti-CD11b and anti-Gr1 and analyzed by flow cytometry (left panels). $Gr1^{hi}CD11b^{hi}$ cells from OVA-fed mice were further analyzed for the expression of Ly6G and CD115/M-CSFR (right panel). (**B**) FACS sorted $Gr1^{hi}CD11b^{hi}$ cells were dispersed by cytospin and stained with Diff-Quick. Scale bar shows 50mm. (**C**) Percentages of neutrophils to whole cells, and absolute number in Figure 1A. n=3. Bars represent mean±S.D. of individual mice. (**D**) DO11.10 mice were given OVA or BSA in drinking water for 5 d, and percentages of neutrophils to whole cells analyzed by flow cytometry. n=3. Bars represent mean±S.D. of individual mice. (**E**) Balb/c mice were adoptive transferred (i.v.) with splenic $CD4^+$ T cells (1×10^6 cells) from untreated DO11.10 mice. 24 h after transfer, Balb/c mice were maintained untreated or given OVA in drinking water for 5 d, and percentages of neutrophils to whole cells analyzed by flow cytometry. n=3. Circles represent individual mice and horizontal lines represent the means for each group of samples. *p<0.05 by Student's *t*-test. Data are representative of nine (A and C) or three (B, D and E) independent experiments.

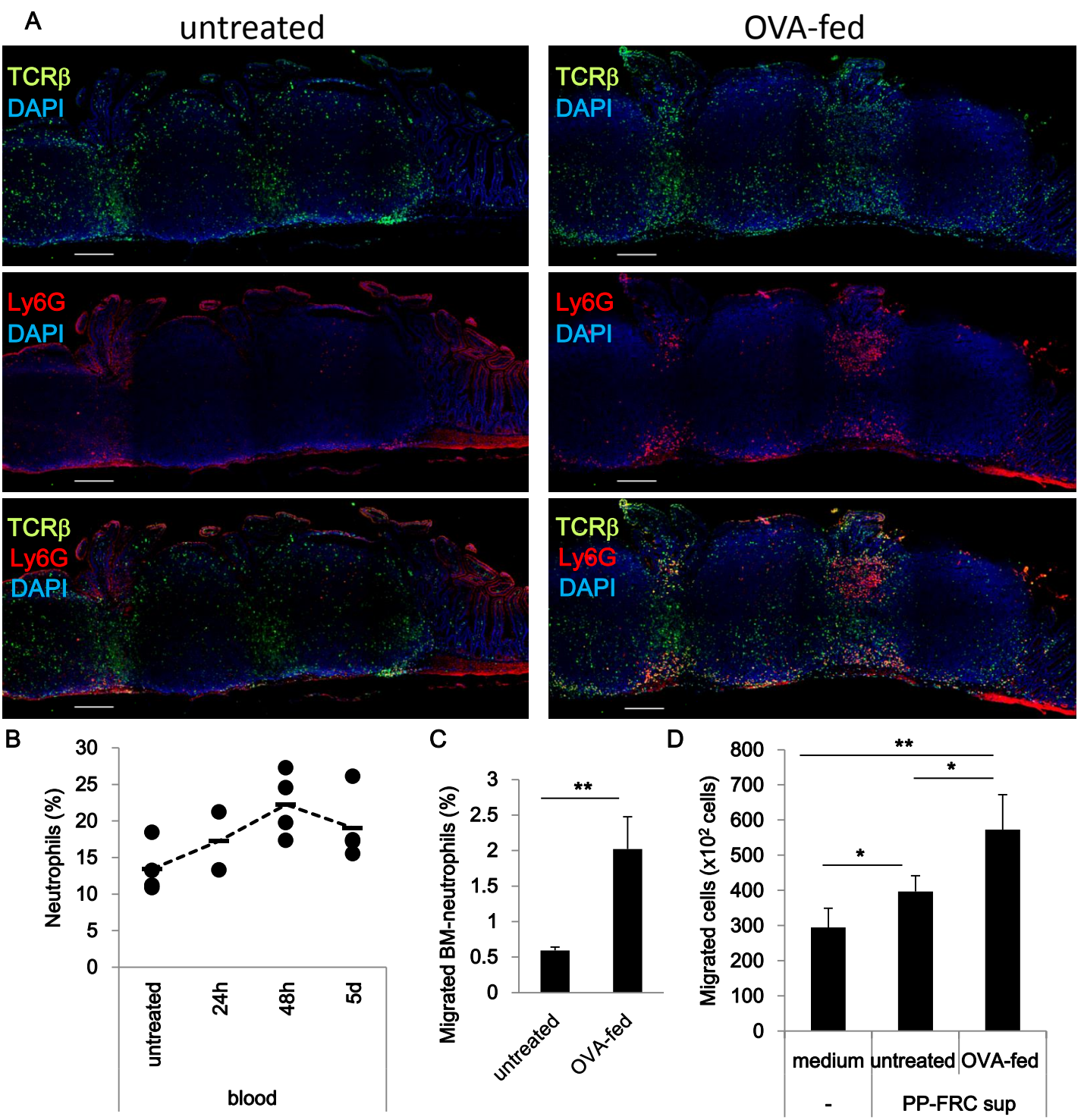

Figure 2. FRC residing in PP chemoattract BM-derived neutrophils after oral Ag administration.
(**A**) Frozen sections of PP tissues from untreated or OVA-fed (5d) mice were stained with anti-TCRb (FITC; green), anti-Ly6G (TxRD; red), and DAPI (blue). Scale bar indicates 200 mm. (**B**) Percentage of neutrophils to whole cells in blood of untreated or OVA-fed (24 h, 48 h, and 5 d) mice were analyzed. n=3-4. Circles represent individual mice and horizontal lines represent the means for each group of samples. (**C**) Untreated DO11.10 mice were adoptive transferred (i.v.) with CFSE-labeled BM cells ($1x10^8$ cells) from Balb/c mice. 24 h after transfer, DO11.10 mice were maintained untreated or given OVA in drinking water for 48 h. neutrophils in PP $CFSE^+$ cells were analyzed by flow cytometry. Percentage of neutrophils in $CFSE^+$ cells was shown. n=4. Bars represent mean±S.D. of individual mice. (**D**) PP-FRC from untreated or OVA-fed mice were cultured for 5 d. Supernatants of FRC culture were added to bottom of transwell plate, and numbers of chemoattracted neutrophils were counted after 24 h. Bars represent mean±S.D. of triplicate cultures. *p<0.05; **p<0.01 by the Student's *t*-test. Data are representative of seven (A) or three (B, C and D) independent similar experiments.

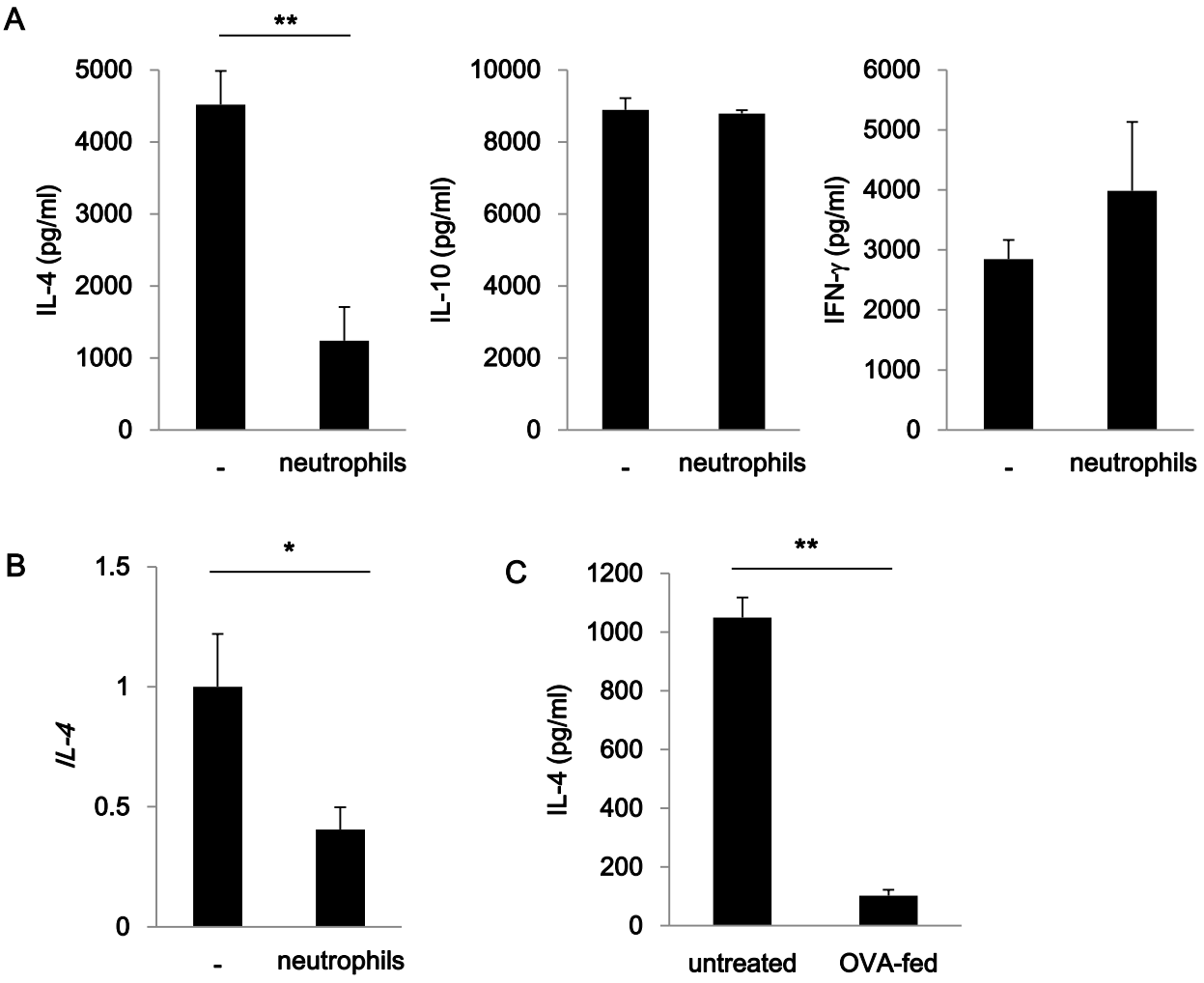

Figure 3. PP neutrophils from OVA-fed mice suppress IL-4 production in T cells.
(**A**) Neutrophils were sorted from OVA-fed mice. $CD4^+$ T cells (2×10^5 cells) were stimulated by plate bound anti-CD3 and CD28 without or with neutrophils (2×10^4 cells) for 72h. IL-4, IL-10, and IFN-g in supernatants were measured by ELISA. Bars represent mean±S.D. of triplicate cultures. (**B**) $CD4^+$ T cells were cultured as described in Figure 3A. IL-4 mRNA in cultured $CD4^+$ T cells was measured by quantitative RT-PCR. The mRNA level of $CD4^+$ T cells cultured with neutrophils was displayed as ratio to that of without neutrophils. Bars represent mean±S.D. of pooled samples. (**C**) $CD4^+$ T cells in PP were prepared from untreated or OVA-fed mice. $CD4^+$ T cells (1×10^5 cells) were cultured with APC (2×10^5 cells) and OVA peptide (0.3 mM) for 72h. IL-4 in supernatants was measured by ELISA. Bars represent mean±S.D. of triplicate cultures. *p<0.05; **p<0.01 by the Student's *t*-test. Data are representative of three independent experiments.

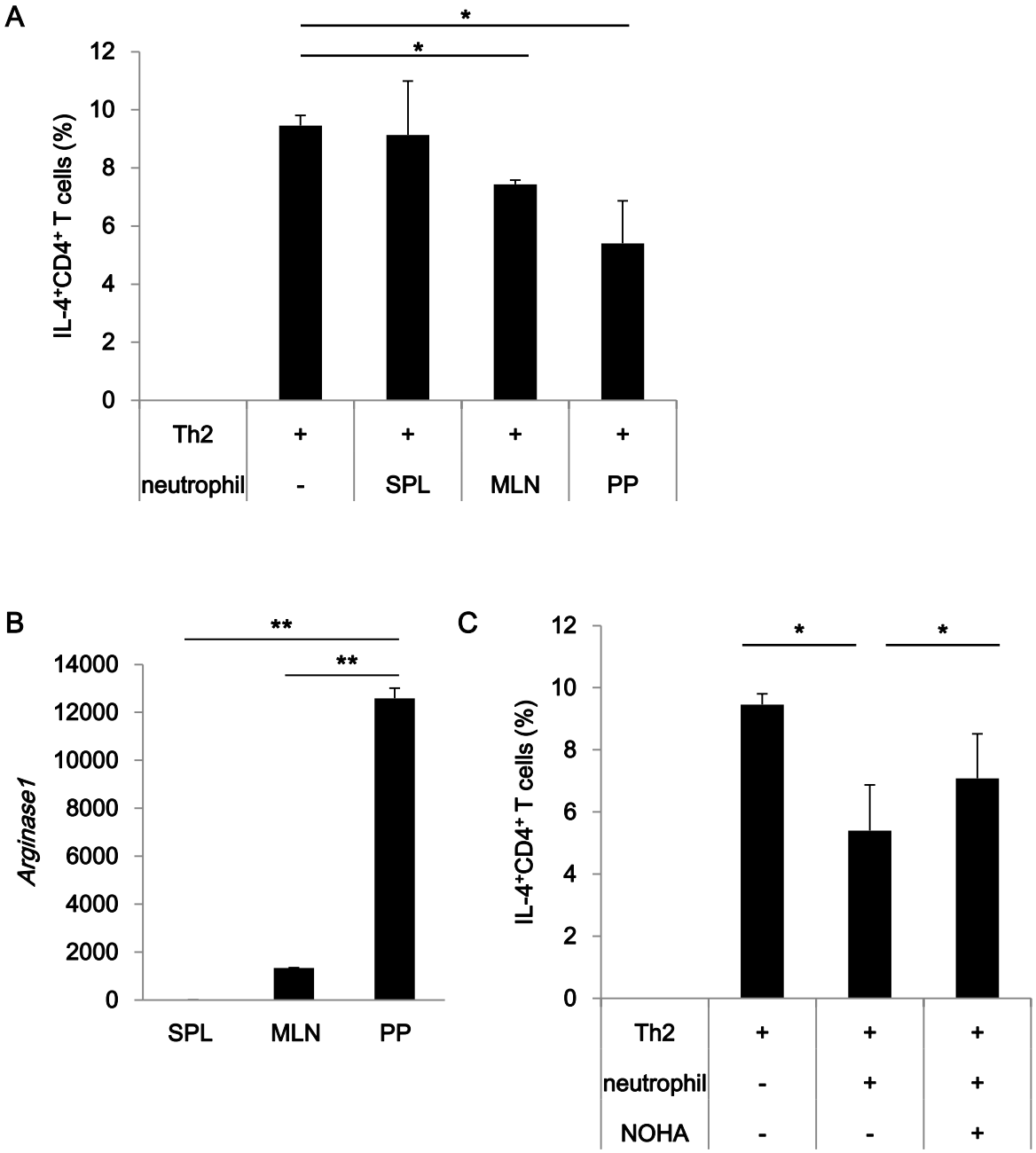

Figure 4. PP neutrophils from OVA-fed mice suppress excessive IL-4 production via arginase1.
Neutrophils were sorted from OVA-fed mice. (**A**) *In vitro*-generated Th2 cells (2×10^5 cells) were activated by plate bound anti-CD3 and anti-CD28. In this culture (Th2), neutrophils from SPL, MLN or PP (5×10^4 cells) were added. After 48 h, IL-4 production by Th2 cells was analyzed by FACS. Bars show the percentage of $CD4^+$ cells expressing IL-4. Bars represent mean±S.D. of triplicate cultures. (**B**) Arginase 1 expression in neutrophils from SPL, MLN or PP was measured by quantitative RT-PCR. The mRNA expression level of each sample was displayed as ratio to that of SPL neutrophils. Bars represent mean±S.D. of pooled samples. (**C**) *In vitro*-generated Th2 cells (2×10^5 cells) were activated by plate bound anti-CD3 and anti-CD28. PP neutrophils (5×10^4 cells) were added without or with Nor-NOHA (500 mM) as indicated. After 48 h, IL-4 production by Th2 cells was analyzed by FACS. Bars represent mean±S.D. of triplicate cultures. *p<0.05; **p<0.01 by the Student's *t*-test.
Data are representative of three independent experiments.

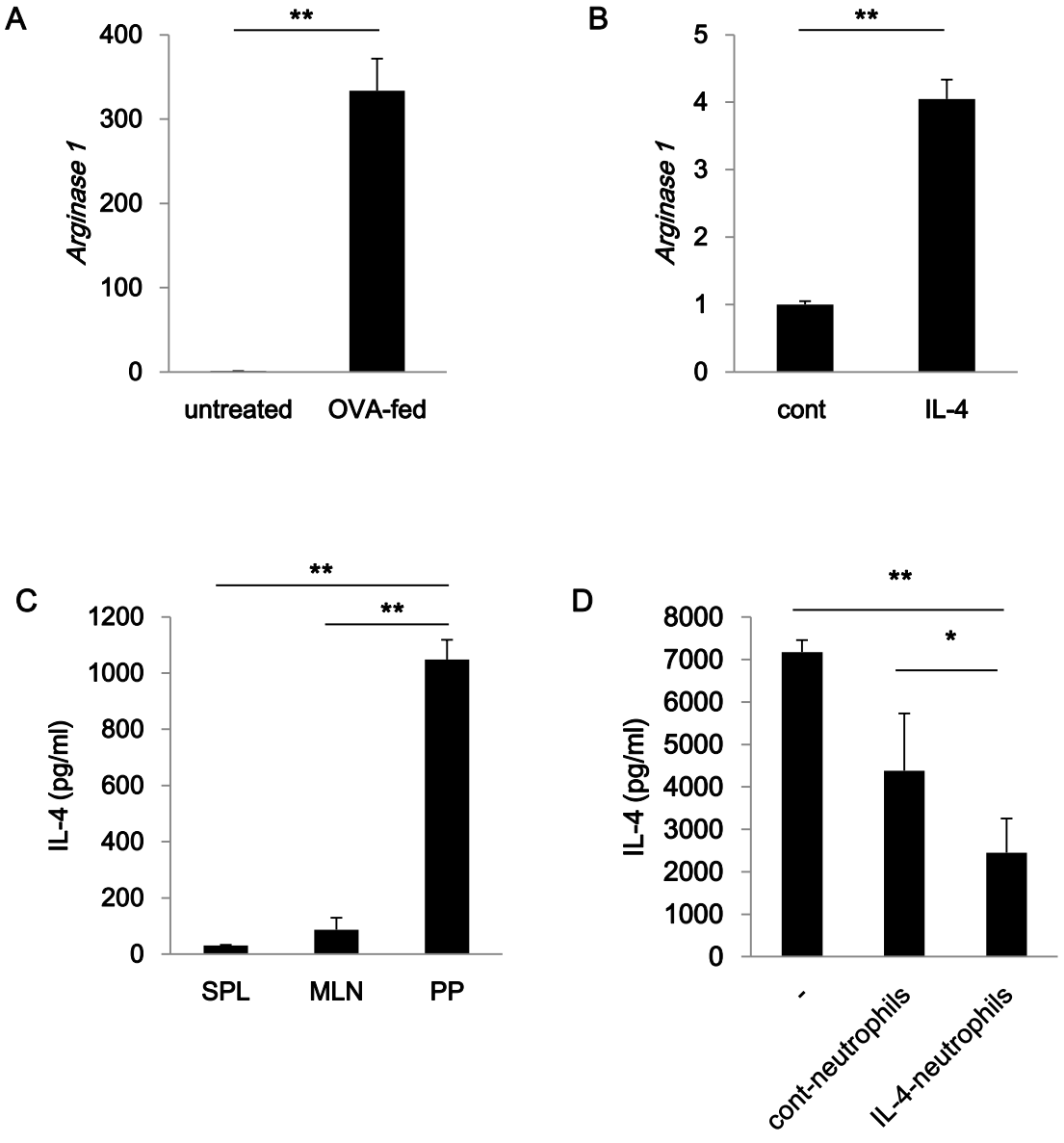

Figure 5. IL-4 primed neutrophils increased the expression of arginase 1.
(**A**) Arginase 1 expression in neutrophils from untreated or OVA-fed DO11.10 mice. The mRNA expression level of each sample was displayed as ratio to that of untreated neutrophils. Bars represent mean±S.D. of pooled samples. (**B**) Arginase 1 expression in peritoneal neutrophils cultured in the absence or presence or of rIL-4 (2 ng/ml) for 48h. The mRNA expression level of IL-4 primed neutrophils was displayed as ratio to that of untreated neutrophils. Bars represent mean±S.D. of pooled samples. (**C**) $CD4^+$ T cells in SPL, MLN, PP were prepared from untreated DO11.10 mice. $CD4^+$ T cells (1×10^5 cells) were cultured with APC (2×10^5 cells) and OVA peptide (0.3 mM) for 72 h. IL-4 in supernatants was measured by ELISA. Bars represent mean±S.D. of triplicate cultures. (**D**) Peritoneal neutrophils were precultured in the absence (cont-neutrophil) or presence (IL-4-neutrophils) of rIL-4 (2 ng/ml) as in Figure 5B. Plate bound anti-CD3 and anti-CD28 activated $CD4^+$ T cells (4×10^5 cells) were cultured without (-) or with precultured neutrophils (1×10^5 cells) for 72h. IL-4 in supernatant was measured by ELISA. Bars represent mean±S.D. of triplicate cultures. *p<0.05; **p<0.01 by the Student's t test. Data are representative of nine (A) or three (B, C and D) independent experiments.

Chapter 5

Stromal cells in mesenteric lymph nodes and Peyer's patch have distinct immunoregulatory function

5.1. Introduction

Hematopoietic cells are also strongly influenced by the surrounding nonhematopoietic cells (96)(107). Stromal cells are nonhematopoietic cells present within secondary lymphoid organs (SLO) and have long been viewed as cells that simply provide structural support to the lymphoid organs and that create different microdomains for T and B lymphocytes. However, several reports revealed that stromal cells are crucial for normal functioning of the immune system and regulate immune responses.

Stromal cells are difficult to study for their few cell numbers in SLO. Expression of the glycoprotein podoplanin (gp38) and the adhesion molecule CD31 (PECAM-1) distinguishes the following lymph node stromal cell subsets: $gp38^{+}CD31^{-}$ fibroblastic reticular cells (FRC), $gp38^{+}CD31^{+}$ lymphatic endothelial cells (LEC), $gp38^{-}CD31^{+}$ blood endothelial cells (BEC) and $gp38^{-}CD31^{-}$ double-negative cells (DNC). The T cell zone is delineated by FRC, construct and regulate a specialized reticular network of fibers used by lymphocytes and DCs as a scaffold on which to migrate and interact. FRC also secrete survival factors and chemokines that attract T cells and dendritic cells (DC) (108)(109)(110). Moreover, several reports recently showed that FRC and LEC inhibited T cell proliferation through a tightly regulated mechanism dependent on nitric oxide synthase 2 (NOS2) (111)(112)(113). Follicular dendritic cells (FDC) are the main stromal cell type in the B cell follicles, however, the function, localization or surface markers of DNCs are still poorly understood (114).

Previous studies show phenotypes of stromal cells in mesenteric lymph nodes (MLN) are distinct from peripheral lymph nodes (PLN), and stromal cells shape tissue specific immune responses (115)(4)(116)(117)(118) Uniquely, MLN but not PLN stromal cells express high levels of retinoic acid (RA) producing enzymes and support induction of CCR9 on

activated T cells, imprinting gut tropism (119)(120).

cyclooxygenase-2 (COX-2), an enzyme that synthesizes prostaglandins from arachidonic acid, have been identified as another unique factor of intestinal stromal cells. COX-2 is constitutively expressed in intestinal stromal cells (121). COX-2-dependent arachidonic acid metabolite prostaglandin E2 (PGE2) is the most abundant prostanoid found in the human body. PGE2 suppresses T cell proliferation, or induces $Foxp3^+$ regulatory T cells (Tregs), and has regulatory roles in immune responses (122). PGE2 has suppressive role for intestinal inflammation (123), and important for inducing $Foxp3^+$ Tregs and establishment of tolerance to dietary antigen (124)(125). These results suggested stromal cells in the intestine have unique phenotype.

Although the precise relationship between embryonic lymphoid tissue organizer cells and such different types of stromal cells in adult SLOs remain unclear, FRC are assumed to be different from precursors in stromal organizer cells (96). A previous report suggested organizer cells in MLN and Peyer's patch (PP) are distinct (126). So we assumed that MLN and PP FRC have also different phenotypes. As above mentioned, phenotypes of stromal cells in MLN have been revealed, however, stromal cells in PP remains very poorly understood.

The aim of this chapter was to determine stromal cell phenotype specific to intestinal tissue. So subsets and functions of stromal cells in MLN and PP, important sites for intestinal immune regulation, were investigated.

The current study showed FRC in MLN and PP showed distinct mRNA expression, and T cell function. MLN-FRC strongly suppress T cell proliferation, whereas PP-FRC prominently induce $Foxp3^+$ Tregs.

5.2. Materials and Methods

Mice

Balb/c mice were purchased from CLEA Japan (Tokyo, Japan). Balb/c mice and DO11.10 TCR transgenic mice (27) were used at 7–20 weeks old. All work was performed in accordance with The University of Tokyo guidelines for animal care and use.

Culture medium, antibodies, and reagents

Cells were cultured in RPMI 1640 medium supplemented with 10 % heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-mercaptoethanol. The following antibodies were used: FITC-labeled anti-CD45 (30-F11, BioLegend), biotinylated anti-CD45 (30-F11, BioLegend), FITC-labeled anti-CD31 (390, BioLegend), biotinylated anti-CD31 (390, BioLegend), biotinylated anti-gp38 (8.1.1, BioLegend), PE-labeled anti-gp38 (8.1.1, BioLegend), FITC-labeled anti-CD35 (7E9, BioLegend), streptavidin-PE-Cy5 (BD Pharmingen), FITC-labeled anti-CD4 (H129.19, BD Pharmingen), PE-Cy5-labeled anti-mouse CD4 (H129.19, BD Pharmingen), PE-labeled anti-Foxp3 (FJK-16s, eBioscience), anti-CD3ε (145-2C11, purified in our lab.), anti-CD28 (37.51, BioLegend)

Preparation of stromal cells

Stromal cells were prepared from spleen (SPL), MLN and PP in parallel. SPL, MLN and PP were incubated for 60 min at 37 ºC with collagenase P (0.2 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche) in RPMI containing 5% FCS, and single cell suspensions were prepared.
For isolation of $CD45^-$ cells, cells were incubated with anti-mouse CD45-coated magnetic beads (Miltenyi Biotech) and negative selected on magnetic activated cell sorting (MACS) separation columns (Miltenyi Biotech). For isolation of FRCs, cells were incubated with biotin labeled anti-mouse gp38 for 20min at 4 ºC. Cells were incubated with streptavidin-coated magnetic beads (Miltenyi Biotech) and selected on magnetic activated

cell sorting (MACS) separation columns (Miltenyi Biotech). For isolation of stromal cells, cells negative selected on the basis of CD45 expression by MACS were stained with CD45-bio-Cy5, CD31-bio-Cy5, CD35-FITC and gp38-PE. $gp38^{+}CD31^{-}CD35^{-}CD45^{-}$ cells and $gp38^{-}CD31^{-}CD35^{-}CD45^{-}$ cells were sorted by FACS using a FACSVantage (BD Bioscience). For isolation of FRC in some case, cells selected on the basis of gp38 expression by MACS were stained with CD45-FITC, CD31-FITC, CD35-FITC and gp38-PE. $gp38^{+}CD31^{-}CD35^{-}CD45^{-}$ cells were sorted by FACS using a FACSVantage (BD Bioscience).

Preparation of $CD4^{+}$ T cells and APC

$CD4^{+}$ T cells were prepared from SPL of DO11.10 mice. SPL was mashed, and single cell suspension was prepared. The cells were incubated with anti-mouse CD4-coated magnetic beads (Miltenyi Biotech) and selected on MACS separation columns (Miltenyi Biotech). For antigen presenting cells, CD4 negative cells prepared as above were treated with mitomycin C for 30 min at 37 °C.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from MACS-purified $CD11b^{+}$ cells and FACS-sorted $CD11b^{+}$ and DC subpopulations using QIAshredder and RNeasy Mini Kits (both from Qiagen). Single-stranded cDNA was synthesized using Superscript II reverse transcriptase and Oligo dT primers (both from Invitrogen). Quantitative PCR reactions were performed using Quantitect Primer Assays with SYBR green PCR mastermix (Qiagen) with the following primers : Raldh2 forward, 5'-GACTTGTAGCAGCTGTCTTCACT -3'; reverse, 5'-TCACCCATTTCTCTCCCATTTCC-3': Raldh1 forward, 5'-ATGGTTTAGCAGCAGGACTCTTC-3'; reverse, 5'-CCAGACATCTTGAATCCACCGAA-3': COX-2 forward, 5'- ACCCGGACTGGATTCTAT-3'; reverse, 5'-GCTTCCCAGCTTTTGTAA-3': IDO1 forward, 5'-TCCAGTGCAGTAGAGCGTTCA-3'; reverse, 5'-GAAAAACGTGTCTGGGTCCA-3': NOS2 forward, 5'- ACATTGAAGAAGCTGGTGGC-3'; reverse, 5'-GGAAAAGACTGCACCGAAGA-3': Foxp3 forward, 5'-GAGTTCTTCCACAACATGGA-3'; reverse, 5'-TTTCATTGAGTGTCCTCTGC-3': HPRT

forward, 5'-GAAGAGACTGGGGATCACTC-3'; reverse, 5'-CATGCCATCTTCCATATTGT-3' with the LightCycler (Roche). Gene expression levels for each individual sample were normalized to hypoxanthine phosphoribosyltransferase (HPRT).

FRC-T cell cocultures

FRC (2×10^4 cells) were plated and allowed to rest overnight. The next day, splenic $CD4^+$ T cells (1×10^5 cells) from DO11.10 mice were added to the plates, together with CD4-APC (1×10^5 cells) and OVA peptide (0.3 μM). Indomethacin (100 μM), L-NMMA (500 μM), Nor-NOHA (500 μM), anti-IFN-γ (10 μg/ml), 1-MT (1 mM), rIL-2 (10 ng/ml), or 0.01% DMSO were added in some cases. After 48 h, 37 kBq of ^{3}[H] thymidine was added per well, and the plates were cultured for an additional 24 h. Subsequently, cells were harvested using a March III harvester (Tomtec, Hamden, CT), and incorporated ^{3}[H] thymidine was counted using a Trilux1450 Microbeta counter (Wallac, Gaithersburg, MD) and Microbeta 270.004 software (Wallac).

For $Foxp3^+$ Treg induction, FRC (2×10^4 cells) were plated and allowed to rest overnight. The next day, splenic $CD4^+$ T cells (1×10^5 cells) from DO11.10 mice were added to the plates, together with $CD4^-$ APC (1×10^5 cells) and OVA peptide (0.3 μM) for 5 days. Retinol (50 nM) or LE540 (1 μM) ware added were added to culture wells in some cases. Cultured cells were harvested and stained for CD4-Cy5 and/or intracellular Foxp3, and $CD4^+$ cells were sorted by FACS using a FACSVantage. Sorted $CD4^+$ T cells were measured for Foxp3 and CCR9 mRNA expression by quantitative RT-PCR.

Intracellular staining for Foxp3

For Foxp3 staining, cells were washed in FACS buffer (1%FCS, 0.1% NaN3 in PBS) and incubated with mAb 2.4G2 to block Fcγ receptors for 15min. Surface CD4 were stained with CD4-FITC for 20 min at 4 ºC. Intracellular Foxp3 staining was performed with Foxp3- PE and Foxp3 staining buffer set (eBioscience) in accordance with manufacturer's instructions. Data were acquired using a BD LSR flow cytometer (BD Bioscience) and analyzed using FlowJo software.

Statistical analysis

All experimental data were expressed as the mean ± standard deviation (SD). Statistical differences for all experimental data were analyzed by Student's *t*-tests.

5.3. Results

Phenotypes of stromal cells in SPL, MLN and PP are very different

We analyzed the phenotype of stromal cells in MLN and PP where intestinal immune response is regulated, and stromal cells in SPL where systemic immune responses are regulated as a control. First, we checked the percentage of stromal cells in these organs. Percentages of $CD45^-$ stromal cells were very different in SPL, MLN, and PP (Fig. 1A). We then analyzed expression of several mRNA in $CD45^-$ cells. mRNA expression of several genes was distinct between SPL, MLN and PP, especially COX-2 expression was remarkable in MLN $CD45^-$ cells (Fig. 1B). The $CD45^-$ cells contains several non-hematopoietic subsets, and expression of the glycoprotein podoplanin (gp38) and the adhesion molecule CD31 (PECAM-1) distinguishes the following lymph node stromal cell subsets: $gp38^+CD31^-$ FRC, $gp38^+CD31^+$ LEC, $gp38^-CD31^+$ BEC and $gp38^-CD31^-$ DNC. When we analysed stromal cell subsets, percentages of stromal cell subsets were very different in SPL, MLN, and PP (Fig. 1C). In this study, we focused on adaptive immunity. Therefore we further analyzed the phenotype of FRC. $gp38^+CD31^-CD45^-$ cells also containe FDC which express CD35, therefore we excluded contamination of FDC by CD35 staining. Moreover, DNC were abundant in PP, so DNC were also examined. Through the use of a combination of antibodies to the surface markers CD45, CD31, CD35 and gp38, FRC ($gp38^+CD31^-CD35^-CD45^-$ cells) and DNC ($gp38^-CD31^-CD35^-CD45^-$ cells) were sorted from MLN and PP. mRNA expression levels of several genes in FRC or DNC were different in MLN and PP (Fig. 1D). MLN-FRC have been reported to express RALDHs(119)(127) , however, we verified that Raldh1 and Raldh2 expression in PP-FRC were higher than MLN-FRC. As other features, MLN-FRC markedly expressed COX-2, and PP-DNC markedly express indoleamine-pyrrole 2,3-dioxygenase 1(IDO1). These results suggested that phenotypes of stromal cells subsets are very distinct in SPL and intestinal organs, moreover intestinal MLN and PP have also distinct features.

MLN-FRC suppresses $CD4^+$ T cell proliferation via COX-2

We examined whether FRC and DNC in MLN and PP suppress $CD4^+$ T cell proliferation.

FRC and DNC were sorted from MLN and PP. Stromal cells were rested overnight, and $CD4^+$ T cells from DO11.10 mice, APC and OVA were added on those stromal layers. The proliferation of T cells was suppressed in the presence of stromal cells, especially in the presence of MLN-FRC (Fig. 2A). To test whether suppressive function requires cell-contact, supernatants from stromal cell cultures were added to $CD4^+$ T cells, APC, and OVA cultures. Supernatant from stromal cell cultures also suppressed T cell function, especially in the case of supernatant of MLN-FRC culture (Fig. 2B), suggesting MLN-FRC suppressed T cell proliferation without cell-contact mechanism. Lukacs-Kornek et al. suggested MLN-FRC suppressed proliferation of OT-I T cell via NOS2 *in vivo* (112)(128), and we demonstrated that MLN-FRC significantly expressed COX-2. To investigate NOS2 or COX-2 mediated suppression, we performed *in vitro* assay by using competitive inhibitor of all NOS isoforms (L-NMMA) or COX-1/2 inhibitor indomethacin (INDO). T cell proliferation was inhibited by MLN-FRC, and this inhibition by MLN-FRC was only slightly abrogated by L-NMMA and partly abrogated by INDO (Fig. 2C). It was reported that IFN-γ was also a key factor in FRC mediated T cell proliferation suppression (112)(111)(112), and IFN-γ increased IDO and NOS2 expression in stromal cells. NOS as well as arginase (ARG) are enzymes to metabolise L-arginine, regulating T cell proliferation (76). To determine whether these molecules are involved in the mechanism underlying the suppression of T cells by FRC, we included specific inhibitors of arginase 1 (Nor-NOHA) and IDO (1-MT), anti-IFN-γ Ab and recombinant IL-2. Neither arginase 1 inhibitor Nor-NOHA, anti-IFN-γ Ab, IDO inhibitor 1-MT, nor IL-2 had a significant effect on MLN-FRC mediated inhibition of T cell proliferation (Fig. 2D). These results suggested that MLN-FRC significantly suppressed $CD4^+$ T cell proliferation which was partly due to COX-2 activation.

PP-FRC induces Foxp3 expression in $CD4^+$ T cells via RA production

For immune suppression, $Foxp3^+$ Treg induction is an important mechanism. We next examined whether FRC and DNC in MLN and PP induced $Foxp3^+$ Treg. FRC and DNC were sorted from MLN and PP. Stromal cells were rested overnight, and $CD4^+$ T cells from DO11.10 mice, APC and OVA were added on those stromal layers. Foxp3 mRNA expression in $CD4^+$ T cells was significantly increased when cultured with PP-FRC (Fig. 3A). RA is

known to induce Foxp3$^+$ Treg in the presence of transforming growth factor-β (TGF-β). PP-FRC highly expressed Raldh1 and Raldh2, which enzyme produce RA. Therefore we assumed that PP-FRC mediated Foxp3$^+$ Treg induction was due to RA production. To test whether Foxp3$^+$ Treg induction by PP-FRC is dependent on RA production, RA receptor antagonist LE540 was added to T cell and PP-FRC culture. In the presence of TGF-β, the percentage of Foxp3$^+$ Treg to CD4$^+$ T cells was increased by PP-FRC, and this increase by PP-FRC was abrogated by LE540 (Fig. 3B). RALDH enzymes are needed to convert retinol into RA. For further analysis of RA mediated Foxp3 expression, retinol was added as a substrate for RA production by PP-FRC. As expected, Foxp3 expression in CD4$^+$ T cells was increased by PP-FRC and further increased in addition to retinol, whereas LE540 abrogate Foxp3 expression (Fig. 3C). These results suggested that PP-FRC could induce Foxp3$^+$ Treg via RA production.

5.4. Discussion

In this study, we demonstrated the different function of stromal cells in MLN and PP. MLN-FRC strongly suppressed T cell proliferation via COX-2, whereas PP-FRC induced $Foxp3^+$ Treg by producing RA.

Previous study showed FRC in PLN ans MLN suppressed T cell proliferation by iNOS, but this study indicated that freshly isolated MLN-FRC have only slight suppressive function by iNOS. This discrepancy may be caused by the condition of stromal cells and difference of mouse strain. We used FRC and T cells from Balb/c mice background, but previous report used C57BL/6 mice background. We verified mRNA expression of several genes in freshly isolated FRC and cultured FRC was different, and COX-2 expression of MLN-FRC was diminished after culture (data not shown). It was also reported that iNOS expression required IFN-γ signaling in activated T cells (112)(113)(111). In general, T cells from Balb/c mice background favor producing Th2 type cytokines, whereas T cells from C57BL/6 mice favor producing Th1 type cytokines (129). It might be possible that in C57BL/6 mice background, effect of IFN-γ signaling for inducing iNOS expression was more significant than in Balb/c mice background.

Suppressive function by COX-2 was also reported in FRC cell lines (113), and some reports in mesenchymal stem cells (MSC) (130)(131). In most cases, COX-2 associated suppression was dependent on PGE2 synthesis. PGE2 has a variety of functions to regulate T cells (122)(132). PGE2 acts upon binding to one of four subtypes of receptors, E prostanoid 1 (EP1), EP2, EP3 or EP4, alone or in combination. T cells expressed EP2 and EP4, and their function is regulated through these receptors (133)(134). PGE2 primarily exerts its inhibitory effect on lymphocyte proliferation through an inhibition of IL-2 production by targeting AP-1 and NF-AT transcription factors (135). PGE2 signaling produce cAMP, and increases in cAMP were also found to inhibit expression of IL-2 receptors (136). PGE2-mediated cAMP also regulate transcription factors that control growth, survival and differentiation of T cells. We verified that adding extra IL-2 did not recover proliferation, therefore in our study, COX-2 dependent PGE2-cAMP pathway might be the inhibitory mechanism. However, suppression was not completely dependent on COX-2. Since supernatant of FRC culture strongly suppressed T cell proliferation, the main suppressive

function was humoral factors. We tested other possible mediators to suppress T cells known in MSC, but we could not identify other suppressive factors. Further studies are needed to determine suppressive function of MLN-FRC.

Raldh2 expression was reported in MLN-FRC, but we showed PP-FRC exhibited significantly higher Raldh2 expression than MLN-FRC. Actually, RA dependent $Foxp3^+$ Treg induction was observed for PP-FRC. In the intestine, vitamin A metabolized RA is a critical regulator of intestinal immune response. In addition to Raldh2 expression in MLN-FRC, Raldh2 expression in MLN DC and LP DC, Raldh1 expression in PP DC, and Raldh1 expression in IEC are known (137). We previously reported that in oral tolerance induction, $Foxp3^+$ Treg was significantly increased after oral Ag administration, but PP-DC could not induce $Foxp3^+$ Treg. Therefore we presumed that $Foxp3^+$ Treg inducer cells other than DC could be presence in PP, and current study showed that the $Foxp3^+$ Treg inducer cell would be FRC.

We also identified that MLN- and PP-DNC significantly expressed iNOS, and IDO1 expression was expressed in PP-DNC but not in MLN-DNC. iNOS and IDO1 expression in stromal cells was induced by IFN-γ (113)(112)(138) In addition to IFN-γ, we identified some TLR ligand stimulation also induced NOS2 and IDO expression in bone marrow stromal cells containing MSC which are progenitors of tissue stromal cells (data not shown). MLN and PP reside in intestine, PP being adjacent to intestinal lumen. MLN and PP stromal cells, in particular PP, could be exposed more abundantly to TLR ligands from commensal bacteria than other tissues. It might be possible that NOS2 and IDO1 expression in intestinal DNC are induced by intestinal bacteria.

Transcriptional analysis revealed that DNCs most closely resembled FRCs in terms of global gene expression and production of cytokines, chemokines and growth factors (114). Extrathymic AIRE-expressing stromal cells (eTAC) (139)(140) are included in $gp38^-CD31^-$ stromal cell population, which we referred as DNC. As its name suggested, a feature of eTAC is expression of autoimmune regulator (Aire). Aire gene regulates the expression of a wide array of tissue specific self antigens. Expression of Aire was well known in thymic stromal cells and critical for central tolerance. We analyzed expression of Aire to determine whether $gp38^-CD31^-$ stromal cell population in MLN and PP contains eTACs. However, $gp38^-CD31^-$ stromal cells did not express Aire (data not shown), suggesting that NOS2 expressing DNC

would be different from eTACs.

In summary, we showed stromal cells in MLN and PP affected immune response in a different manner. We could not verify whether COX-2 in MLN-FRC or Raldh2 in PP-FRC were functional *in vivo*, but our *in vitro* study mimics Ag-specific T cell response. COX-2 and Raldh2 expressing cells were reported as inducers of oral tolerance induction (125)(141), and intestinal stromal cells are important for inducing oral tolerance (117). Therefore MLN-FRC and PP-FRC could also be involved in oral tolerance via COX-2 and Raldh2, respectively. For further understanding of intestinal immune regulation, involvement of stromal cells needs to be taken into consideration.

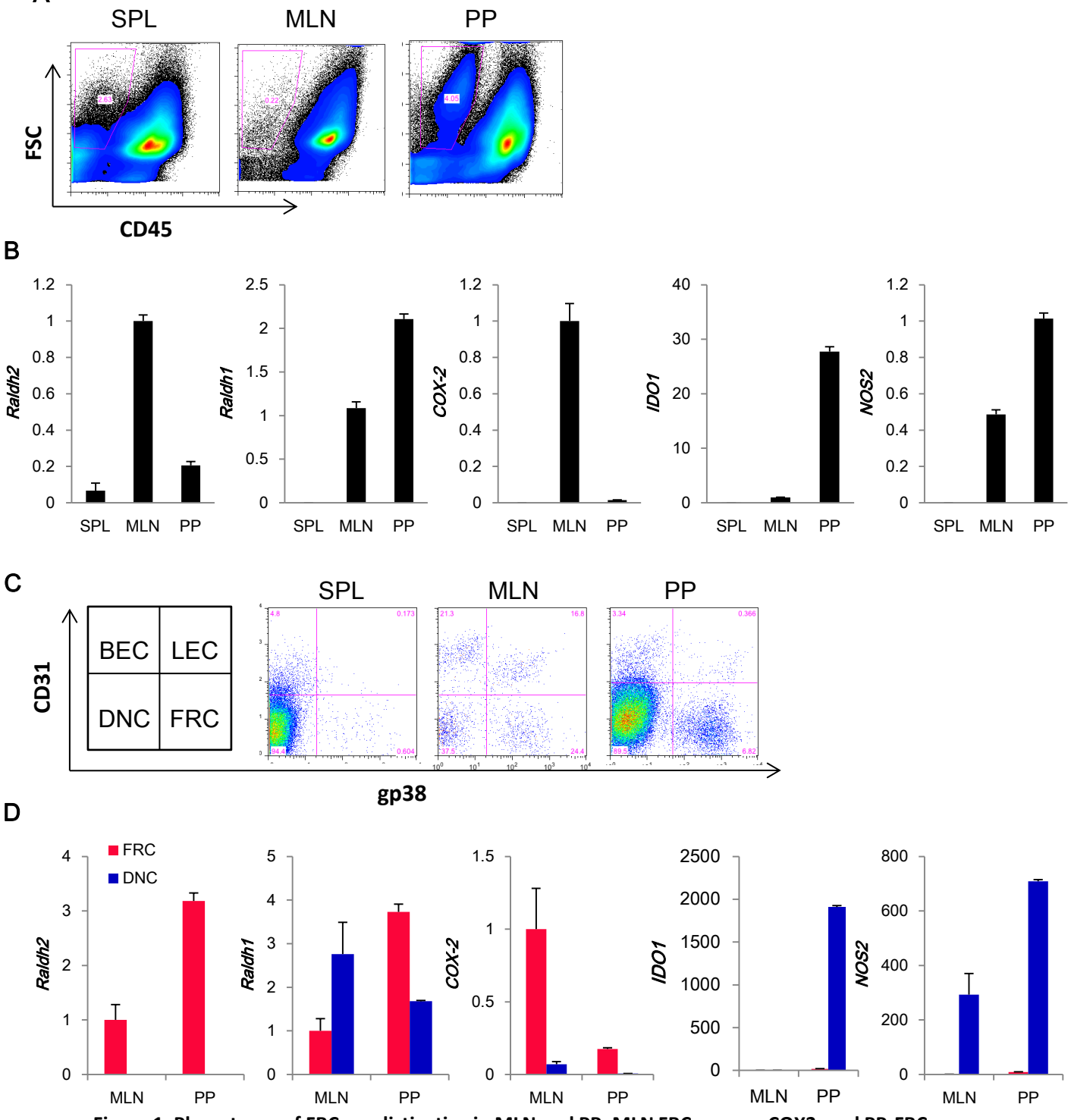

Figure 1. Phenotypes of FRC are distinctive in MLN and PP; MLN FRC express COX2, and PP-FRC express raldh

(A) SPL, MLN, and PP whole cells were stained with anti-CD45 and analyzed by flow cytometry. Numbers adjacent to outlined areas indicate percentage of cells in the gated population (outlined): $CD45^-$ cells among live gated cells. FSC, forward scatter. (B) mRNA expression in $CD45^-$ cells in SPL, MLN, and PP were measured by quantitative RT-PCR. The mRNA level of each sample was displayed as ratio to that of MLN $CD45^-$ cells. (C) Flow cytometry of SPL, MLN, and PP stromal cell populations stained with anti-CD45, anti-CD31, anti-gp38. Numbers in quadrants indicate percent of each stromal cell subset among $CD45^-$ cells. (D) mRNA expression in FRC ($gp38^+CD31^-CD35^-CD45^-$ cells) and DNC ($gp38^-CD31^-CD35^-CD45^-$ cells) of MLN and PP were measured by quantitative RT-PCR. The mRNA level of each sample was displayed as ratio to that of MLN-FRC.

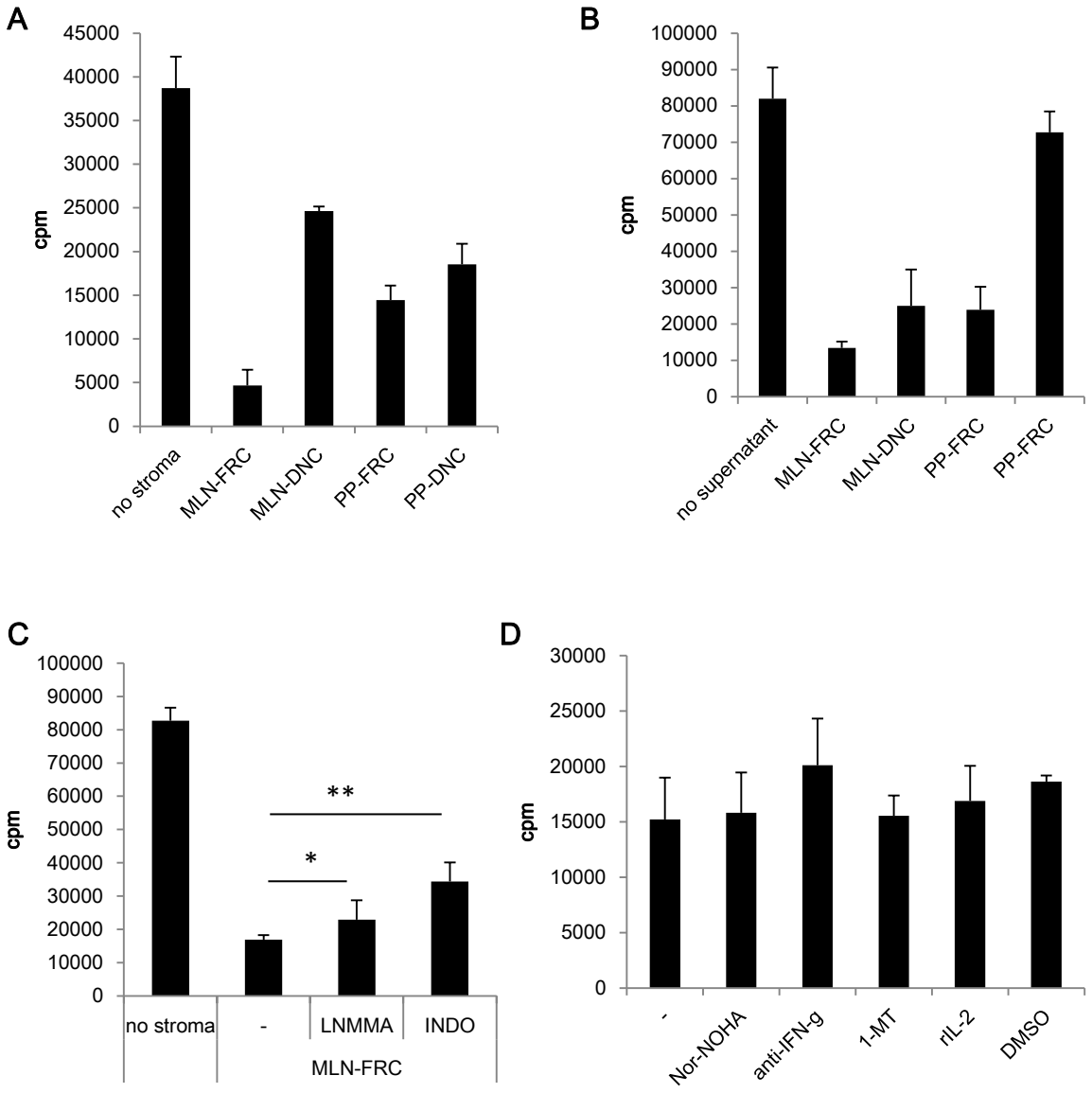

Figure 2. MLN FRC suppress T cell proliferation via COX2 and iNOS

FRC or DNC were sorted from MLN or PP. Cells (2×10^4 cells) were plated and allowed to rest overnight. (A) DO11.10 CD4+ T cells (1×10^5 cells) were cultured with OVAp (0.3 μM) and APC (1×10^5 cells) without stromal cells (no stroma) or with sorted stromal cell subset (2×10^4 cells) for 72h. ^{3}[H] thymidine was pulsed for the last 24 h, and proliferative response was determined by ^{3}[H] thymidine uptake. (B) FRC or DNC (2×10^4 cells) were cultured 5 d Proliferation of DO11.10 $CD4^+$ T cells (1×105 cells) cultured with OVAp (0.3 μM) and APC (1×10^5 cells) in addition without (no supernatant) or with supernatant of FRC or DNC culture (100 μl). (C) Proliferation of DO11.10 $CD4^+$ T cells (1×10^5 cells) cultured with OVAp (0.3 μM) and APC (1×10^5 cells) without FRC (no stroma) or with MLN-FRC (2×10^4 cells) in the presence or absence of the pharmacological inhibitors against iNOS (L-NMMA ;500 μM) or COX-1/2 (INDO; 100 μM) for 72h. (D) Proliferation of DO11.10 $CD4^+$ T cells ($1x10^5$ cells) cultured with OVAp (0.3 μM) and APC (1×10^5 cells) with MLN-FRC ($2x10^4$ cells) in the absence (-) or presence of the arginase 1 inhibiror (Nor-NOHA, 500 μM), anti-IFN-γ (10 μg/ml), IDO1 inhibitor (1-MT; 1 mM), rIL-2 (10 ng/ml), and 0.01% DMSO for 72h. *p<0.05; **p<0.01 by the Student's t test

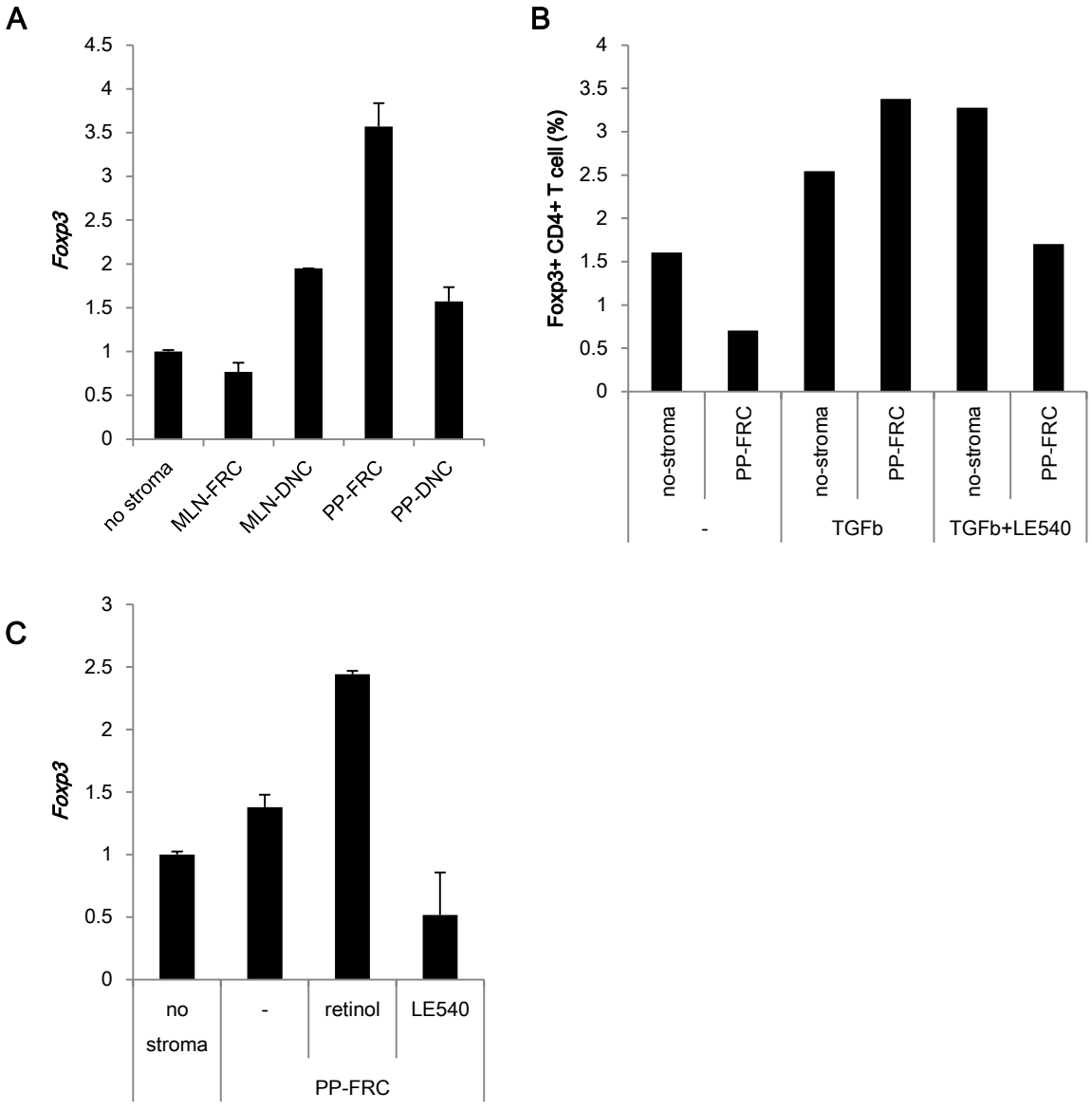

Figure 3. PP-FRC induce Foxp3+ Treg via retinoic acid production

FRC or DNC were sorted from MLN or PP. Cells (2×10^4 cells) were plated and allowed to rest overnight. (A) DO11.10 $CD4^+$ T cells (1×10^5 cells) were cultured with OVAp (0.3 μM) and APC (1×10^5 cells) without (no stroma) or with FRC or DNC ($2x10^4$ cells) for 5d. Cultured cells were harvested and $CD4^+$ T cells were sorted. Foxp3 mRNA expression in sorted $CD4^+$ T cells was analyzed by quantitative RT-PCR. The mRNA level of each sample was displayed as ratio to that of no stroma T cells. (B) DO11.10 $CD4^+$ T cells (1×10^5 cells) were cultured with OVAp (0.3 μM) and APC (1×10^5 cells) without (no stroma) or with PP-FRC (2×10^4 cells) for 5d. LE540 (1 μM) and/or TGF-β (2 ng/ml) were added to this culture. Foxp3 expression in $CD4^+$ T cells was analyzed by Flow cytometry. (C) DO11.10 $CD4^+$ T cells (1×10^5 cells) were cultured with OVAp (0.3 μM) , TGF-β (2 ng/ml) and APC (1×10^5 cells) without (no stroma) or with PP-FRC (2×10^4 cells) for 5d. Retinol (50 nM) or LE540 (1 μM) were added to this culture. Cultured cells were harvested and $CD4^+$ T cells were sorted. Foxp3 mRNA expression in sorted $CD4^+$ T cells was analyzed by quantitative RT-PCR. The mRNA level of each sample was displayed as ratio to that of T cells with no stroma.

General discussion and perspective

This study was started as analysis of dendritic cells (DC) as Ag presenting cells in oral tolerance induction. During the investigation, it was noticed that more cells were involved in oral tolerance induction than initially assumed. This study first showed neutrophil involvement in oral tolerance, and suggested stromal cell function in intestine. The distinct functions of DC subsets were also revealed. It is suggested that the immune suppression in the intestine is regulated by the network of these cell populations. In Peyer's patch (PP), $CD11b^+$ DC induce IL-10 producing T cells by IL-10, IL-27, and also suppress IL-4 production in T cells via arginase 1. Neutrophils in PP also suppress IL-4 production via arginase1. Stromal cells in PP could be involved in inducing $Foxp3^+$ Treg by produce retinoic acid (RA). On the other hand, in mesenteric lymph nodes (MLN), three $CD103^+$ DC subsets cooperate through Ag presentation and $Foxp3^+$ Treg induction. Stromal cells in MLN could suppress excessive T cell activation via COX-2.

The phenotypes of immune cells or results of immune response are different in different tissues. Therefore, the microenvironment was critical in immune response *in vivo*. In the intestine, previous studies on the effect of environment was largely about commensal bacteria or intestinal epithelial cells. In fact, it is known that regional lymph nodes and their components are important for tolerance (5), but such studies have not progressed for difficulty of cell preparation.

Here, the dynamics and differentiation of these cells after oral Ag administration are discussed further, focusing on the important role of stromal cells.

1. Effect of stromal cells on DC subset differentiation

In chapter 1, functions of MLN-DC subsets were shown. The different phenotypes in DC subsets are at least in part likely to be because the origins of some DC subsets are different.

Several studies demonstrated that transcriptional factors and chemokines were different in DC subpopulation development(142)(19)(143). $CD8\alpha^+$ DC, $CD103^+$ DC, and $CD11b^+$ DC are known to go through broadly different developing steps. In brief, $CD103^+$ DC development require transcriptional factors: *IRF8, Nfil3, Id2, Batf3* and cytokine: FLT3L, from pre-DC.

CD8α$^+$ DC development require transcriptional factors: Id2, E4B4, Batf3, and cytokine: FLT3L, from pre-DC. CD11b$^+$ DC development require transcriptional factors: RelB, IRF2, IRF4, and cytokines: GM-CSF, M-CSF, from pre-DC. Some CD11b$^+$ DC develop from monocytes, but the transcription factor(s) involved remain unclear (144).

The phenotypical characteristics such as cytokine expression is also different in same DC subsets (as determined by cell surface marker expression), and this is assumed to be affected by certain cytokines produced in response to stimulation from the environment. However, strict regulation of the quantity of each DC subset cannot be well explained solely by cytokines. More precisely, non-variable factors are needed. One possibility is regulation of the number of precursor migration, and this is regulated by blood endothelial cells. But it is unlikely that the blood vessel mediates tissue-specific function. For explanation of tightly regulated differentiation, the concept of "Niche" might be useful. It is well known about hematopoietic stem cells (HSC) niche, and the definition of a niche cell consists of 3 points. 1) Local microenvironment. 2) Contact with target cell. 3) Essential for target cell(145)(146). The number and function of HSC shown to be strictly regulated and maintained by some HSC niche cells of different cell type and different localization (CAR cell, Nestin$^+$ cell, or osteoblast)(147)(148)(145). Likewise, functional differences of DC might be also determined by stromal cells. in fact, certain phenotypes of DC was at least partly defined by stromal cells(149)(150)(151).

It may also be hypothesized that FRC are heterogeneous; for example, GM-CSF producing FRC and FLT3L producing FRC are different populations. If so, the numbers of FRC subpopulation may determine the ratio of DC population development. In addition to such growth factor production, certain cell-contact signaling may be involved in commitment of DC differentiation. For example, Notch signaling was reported as a DC differentiation factor. In mammals, there are four Notch receptors, Notch1-4, and five Notch ligands, Delta-like-1 (Dll1), Dll3, Dll4, Jagged 1 and Jagged 2. As for thymic DC development, previous *in vitro* studies suggested that high Jagged 1 levels supported the development of DC precursors from hematopoietic progenitors, whereas Dll1-expressing stroma favored the maturation of committed DC precursors (152). High levels of Dll1 or Dll4 were inhibitory to DC development, whereas medium levels of Dll4 allowed DC development but not myeloid development. Although the role of Notch signal in peripheral DC development remain

unclear, Notch 2 and 4 are expressed in SPL-DC (152). In is also possible that Notch ligand expression pattern and expression intensity in stromal cells regulate commitment to DC subpopulations in SLO.

2. Regulation of neutrophil dynamics after oral administration of Ag; from BM to the periphery

In chapter 4, results of BM cell transfer suggested that neutrophils were derived from the BM, and then migrate to PP via the blood. Although we showed that PP-FRC chemoattract blood neutrophils to PP, a question remains; how did the neutrophils move from BM to blood?

It is reported that granulocyte colony-stimulating factor (G-CSF) is an essential regulator of neutrophil release from the BM (153). In addition, CXCR2 and CXCR4 also regulate neutrophil mobilization in BM(154).

We observed the increase of neutrophils in blood and PP within 24h after oral Ag administration, and this was observed even at 12 h after feeding (PP, data not shown). Therefore certain Ag-specific signals should be induced in BM within 12h after administration. The possible initiation mechanisms of Ag-specific signal in BM were as follows. 1) Ag. in blood flow directly flowed into BM, and Ag-specific T cells in BM were activated. 2) Ag-specific T cells activated in the intestine migrated to BM. 3) Ag presenting cells having captured Ag, such as DC migrated to BM, and activated Ag-specific T cells in BM. 4) Soluble factors such as G-CSF were produced in association with activation of Ag-specific T cells in the intestine migrated into BM.

Concerning the time point of Ag-presentation in intestine, it is reported that orally administrated Ag was detected in DC of the PP and MLN 4 h after administration(155). T cell activation initially occurred after 24h in PP, and Ag-specific T cells in both MLN and PP had undergone approximately four divisions at 48 h(156). On the other hand, by *in vivo* analysis, T cells in MLN also started proliferation at 24 h(157). Therefore activation of T cells in intestine and subsequent migration to BM within 12 h (possibility 2) seems to be less likely. As for DC, Ag capture was very fast, but they require additional emigration to LN.

Therefore, possibility 3 might also be less likely. Taken together, it may be predicted that if activation signal was derived from intestinal response, possibility 4 is the most likely. BM is abundant of HSC or progenitors of several hematopoietic and non-hematopoietic cells, but mature T cells and DC also reside around the blood vessel (158). Where neutrophils localize in BM remain unclear, but it is likely that Ag in blood stream initiate Ag-specific T cell response in BM, and induce signaling to emigrate neutrophils (possibility 4).

If neutrophils emigrate from BM, the number in BM must be reduced. However, we also observed that the number of neutrophils in BM were not reduced, but tended to increase. This suggested that neutrophils expanded in BM, in addition to exiting from BM. Concerning this, expansion mechanism of neutrophils may be proliferation of precursors (159)(160)(161). It is possible that neutrophil expansion was regulated at the HSC level (see below for further details).

3. Regulation of DC dynamics and differentiation during oral Ag administration; from BM to the periphery

In chapters 2 and 3, it was shown that $CD11b^+$ DC also increased in PP, but the mechanism remains unclear. Several possible mechanisms could be responsible for the increase in $CD11b^+$ DC, including increased proliferation, migration, differentiation, or CD11b expression. First, we tested if $CD11b^+$ expression was increased after oral Ag administration. We adoptively transferred PP $CD11b^-$ cells to untreated DO11.10 mice, and then administered OVA or sterile water. $CD11b^-$ cells did not express CD11b after oral Ag administration (data not shown). Next, we investigated $CD11b^+$ DC proliferation in PP. When BrdU was injected for 48h, its uptake in $CD11b^+$ DC was not different from that in CD11b- DC after oral Ag administration (data not shown). This result suggested that the increase of $CD11b^+$ DC number was not caused by increase of $CD11b^+$ DC proliferation. Therefore, the increase of $CD11b^+$ DC in PP might be regulated by differentiation and/or migration.

As factors for BM cells migration, it may be speculated that some specific chemokines are involved. CCL2, the ligand of CCR2, has been reported to induce oral tolerance. $CCL2^{-/-}$ mice

and CCR2$^{-/-}$ mice are defective in their induction of high-dose oral tolerance (162), as are CCR5$^{-/-}$ mice, and CCR5 signaling has been suggested to induce CCL2 (163). Because both CCR2 and CCR5 are highly expressed in CD11b^{+} DC compared to CD11b- DC (data not shown), the abrogation of oral tolerance in CCR2$^{-/-}$ and CCR5$^{-/-}$ mice may be partly explained by the failure of CD11b^{+} DC (and possibly also neutrophils) to accumulate.

Furthermore, we investigated the change of MLN-DC subsets after oral Ag administration. CD103^{+}CD11b^{+}PD-L1^{+} DC migrated from LP, so we predicted this DC subset should increase after Ag administration. Actually CD103^{+}CD11b^{+}PD-L1^{+} DC increased, but CD103^{-}CD11b^{+}PD-L1^{+} DC increased even more. It may be considered that the increase of CD103^{-}CD11b^{+}PD-L1^{+} DC was caused by progenitor migration via blood like the case of PP. On the other hand, CD103^{+}CD11b^{+}PD-L1^{+} DC may be migrated from LP. Most of CD11b^{+} DC increased in PP were CD103-CD11b^{+}PD-L1^{-} DC (data not shown). The discrepancy of PD-L1 expression remains, but collectively CD103^{-}CD11b^{+} DC increased in MLN and PP after oral Ag administration.

It is reported that TLR signaling induces DC precursor migration from BM (164). As described in section 1, CD11b^{+} DC are differentiated from monocytes, or GM-CSF dependent pre-DC and these pathways might be regulated after oral Ag administration.

4. HSC and progenitor regulation by immune response

HSC are the progenitors of all blood and immune cells, yet their role in immunity was not well understood. Recent studies, however, have indicated that HSC also proliferate in response to systemic infection and supplement effector immune cells (160)(161). HSC might directly respond to inflammatory signaling such as interferons, tumor necrosis factor-α and Toll-like receptors are essential to the HSC response (165)(166)(167)(168)(169).

We discussed above that the increase of neutrophils and CD11b^{+} DC might be regulated in BM. For the simultaneous regulation of these different cell types (neutrophils and DC), it may be speculated that the common progenitor for these cells are regulated. As above referred, G-CSF is the most important cytokine regulating neutrophil production and release from the BM (153). G-CSF is produced by macrophages, activated T cells or stromal cells (endothelial

cells or fibroblasts). The mobilization of HSC into the peripheral blood is induced through several mechanisms (170)(171). Therefore, several mediators are assumed to be involved in neutrophil and $CD11b^+$ DC regulation during oral tolerance induction, and G-CSF may be predicted as one of the key cytokines.

5. Regulation of immune response by stromal cells; from the view of architecture

In chapter 5, it was suggested that FRC regulate immune cell response by functional mediators. In addition, another FRC function may be regulation of immune cell response by formation of special microenvironment through tissue structure. The number of DC is small compared to T cells, therefore the chance for engagement of T cells and DC are limited. CCL19 and CCL21 chemokines, produced by FRC are effective in guiding the migration of DCs and T cells, and retaining DC in the T cell zone. Therefore FRC microenvironments provide the structural basis for the induction of T-cell responses (172)(104). Moreover, accumulation of immune cells in lymph nodes is paralleled by extensive changes to the stromal cell network (173)(174). FRC proliferation is likely required to accommodate the increase in lymph node size and may be a limiting factor in regenerating the reticular network structure. It is known and we observed that lymph nodes enlarge as a consequence of immunization. During oral tolerance, we also observed PP and MLN gradually enlarged. This would be partly caused by accumulation of immune cells such as DC or neutrophils, and Ag-specific T cell proliferation. In addition, LN enlargement might be also caused by stromal cell expansion. We detected enlargement of LN in oral tolerized mice, however, more enlargement of LN was detected in food allergic mice. These results implied that retaining appropriate LN size was important for appropriate accumulation of immune cells and maintenance of immune tolerance.

It was reported that after influenza virus infection, temporary disruption of FRC structure could account for the transient immune suppression (173). It is also reported that after disruption, resolution of FRC depends on lymphoid tissue inducer cells and on $LT\alpha1\beta2$ dependent signaling (173). However, whether FRC expansion was due to proliferation of FRC or their progenitor cells remain unknown. As for progenitors, MSCs could be isolated

from some peripheral tissues in addition to BM (175). Moreover, MSC are circulating in peripheral blood (176). Taken together, it is possible that differentiation of MSC might be involved in FRC expansion. For modulation of immune response, regulation of stromal architecture would be also important.

6. Further perspective

Although the importance of microenvironment has been assumed, the precise mechanism has not been highlighted. And so far, the role of progenitor regulation in immune tolerance has been overlooked. Recently, it was reported that intestinal T cells affect BM progenitors, and affect colitis (177). Therefore, intestinal immune regulation may be closely related with BM regulation. Recent studies and this study suggest that stromal cells play a principal role in immune regulation. Nevertheless the mechanism of regulation of progenitor cell or stromal cell function during intestinal tolerance *in vivo* was not elucidated in this study. To study their functions must be important for understanding of intestine-originated systemic immune regulation.

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論 文 の 内 容 の 要 旨

応用生命化学　専　攻
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論文題目　Studies on immunosuppressive mechanisms in the intestine
（腸管における免疫抑制機構に関する研究）

The intestine is continually exposed to antigens from food proteins and from commensal or pathogenic bacteria. Strict regulation is thus required in the intestine, and oral tolerance represents a unique aspect of the intestinal immune system. Oral tolerance has been classically defined as the specific suppression of cellular or humoral immune responses to an antigen (Ag) by means of prior administration of Ag through the oral route. The response likely evolved as an analog of self-tolerance to prevent hypersensitivity reactions to food proteins. Oral tolerance results in antigen-specific T cell deletion, anergy, or induction of regulatory T cells (Tregs). Food hypersensitivity presumably results from either a failure in establishing oral tolerance or a breakdown in existing tolerance. Furthermore, since oral tolerance is a potent way of inducing regulatory cells towards specific Ag, the idea of using the oral route to induce tolerance to Ag involved in autoimmune diseases becomes an important clinical application of the phenomenon.

Chapter 1. Three different CD103$^+$ dendritic cells (DC) subsets in MLN have distinct function in intestinal immune regulation

DC have been revealed as important regulators in oral tolerance induction. It has been proposed that oral tolerance requires mesenteric lymph nodes (MLN), and CD103$^+$ DC and PD-L1$^+$ DC in the MLN are suggested to be critical for the induction of oral tolerance. However, the relationship of these DC subsets remains unclear. Therefore we aimed to clarify the phenotypes and functions of MLN DC subsets in relation to oral tolerance

induction. Flow cytometric analysis demonstrated that $CD103^+$ DC in MLN are divided into distinct three populations by CD11b and PD-L1 expression. $CD103^+CD11b^+PD\text{-}L1^+$ DC and $CD103^+CD11b^-PD\text{-}L1^+$ DC prominently expressed CCR7, which is the chemokine receptor required to migrate to MLN from the lamina propria. $CD103^+CD11b^+PD\text{-}L1^+$ DC presented orally administrated Ag to $CD4^+$ T cells and strongly induced T cell proliferation. On the other hand, $CD103^+CD11b^-PD\text{-}L1^+$ DC prominently expressed retinaldehyde dehydrogenase 2 (Raldh2) compared to other $CD103^+$ DC subsets, and strongly induced Foxp3 expression in $CD4^+$ T cells by producing retinoic acid. $CD103^+CD11b^-PD\text{-}L1^-$ DC could not present orally administrated Ag, but promptly induced IFN-γ production in $CD4^+$ T cells via IL-12 independent mechanism *in vitro*. These results suggested that the three $CD103^+$ DC subsets have distinctive functions, and may play different roles in inducing oral tolerance.

Chapter 2. IL-10 and IL-27-producing DC capable of enhancing IL-10 production of T cells are induced in oral tolerance [1)]

In addition to MLN, Peyer's patch (PP) is also an important site for establishing oral tolerance. PP DC from tolerized mice induced IL-10 production but not Foxp3 expression in co-cultured T cells. The number of $CD11b^+$ DC increased after ingestion of Ag, and $CD11b^+$ DC prominently expressed IL-10 and IL-27 compared with $CD11b^-$ DC. These results suggested that IL-10 and IL-27 producing $CD11b^+$ DC are increased by interaction with antigen specific T cells in PP, and these PP $CD11b^+$ DC act as inducers of IL-10 producing T cells in oral tolerance.

Chapter 3. Th2 suppressive arginase 1 expressing $CD11b^+$ DC are induced in PP after oral Ag administration

Food allergies presumably result from either a failure to establish oral tolerance, or a breakdown in existing tolerance. Allergy results in an excessive Th2-type immune response, characterized by IL-4, IL-13, and IL-5. Therefore, during oral Ag administration, suppression of excessive IL-4 production may be necessary to establish oral tolerance and prevent the onset of food allergy. In addition to the role of PP DC for inducing IL-10 producing T cells, we also found that PP DC from tolerized mice could suppress excessive IL-4 production in T cells. PP DC from tolerized mice prominently expressed arginase 1, and suppressed IL-4 secretion by $CD4^+$ T cells via arginase 1. Arginase 1 expression in PP DC was increased after oral Ag administration, and the expression was restricted to $CD11b^+$ DC. PP $CD4^+$ T cells prominently expressed IL-4 compared to SPL or MLN $CD4^+$ T cells, and arginase 1 expression in DC was induced by IL-4 *in vitro*. These observations suggested that after oral Ag administration, PP T cells abundantly produce IL-4, and IL-4 induced arginase

1 expression in PP CD11b$^+$ DC. Then PP CD11b$^+$ DC suppress excessive IL-4 production by arginase 1, establishing IL-4-arginase 1 negative feedback loop.

Chapter 4. Th2 suppressive arginase 1 expressing neutrophils are accumulated in PP after oral Ag administration

During examination of CD11b$^+$ DC, we also found that neutrophils were increased in PP after oral Ag administration, and accumulated around T cells in the intrafollicular region (IFR). Numbers of neutrophils in blood was also increased after oral Ag administration, and it was suggested that fibroblastic reticular cells (FRC) in OVA-fed PP could chemoattract neutrophils. These results suggested the possibility of stepwise attraction of neutrophils; Ag-specific CD4$^+$ T cell response promotes certain chemokine secretion from FRC, which promotes neutrophil migration from blood to PP IFR. Similar to CD11b$^+$ DC, accumulated PP neutrophils prominently expressed arginase 1, and suppressed production of IL-4 via arginase 1. Arginase 1 expression in neutrophils was induced by IL-4 *in vitro*. These results suggested that after oral Ag administration, FRC in PP recruited neutrophils, and these recruited neutrophils also were involved in establishing the IL-4-arginase 1 negative feedback loop.

Chapter 5. Stromal cells in gut-associated lymphoid tissue have distinct immunoregulatory function

Non-hematopoietic stromal cells provide structural support to the lymphoid organs. Recent studies have shown that stromal cells also have a crucial role in tolerance induction in the periphery. T cell zone of lymphoid tissue is delineated by FRC and forms a scaffold to provide essential guidance cues to cells of the immune system. It is suggested that stromal cells play important roles in shaping tissue-specific immune responses; however, intestinal tissue-specific phenotypes of stromal cells remain unclear and immunoregulatory function of PP-stromal cells have not been reported. Therefore, the characteristics of stromal cells in MLN and PP, in particular FRC (gp38$^+$CD31$^-$CD45$^-$ cells) were examined, focusing on T cell response. For comparison, double-negative cells (DNC; gp38$^-$CD31$^-$CD45$^-$ cells) were isolated from mouse MLN and PP. MLN-FRC prominently expressed cyclooxygenase-2 (COX-2) compared with PP-FRC or DNC. MLN-FRC strongly suppressed CD4$^+$ T cell proliferation but PP-FRC showed only weak suppression. MLN-FRC suppressed CD4$^+$ T cell proliferation depending partly on COX-2 activation. It was reported that cultured lymph node-FRC suppressed T cell proliferation dependent on nitric oxide synthase 2 (NOS2). However, we found that NOS2 expression in freshly isolated MLN- and PP-FRC was extremely lower than that in DNC, and that NOS2 was not essential for suppressive function of T cell

response by MLN-FRC. PP-FRC prominently expressed Raldh2 compared with MLN-FRC, and PP-FRC induced Foxp3 expression in $CD4^+$ T cells via producing retinoic acid. These results suggested that phenotypes and functions of FRC are distinct between MLN and PP, and they are involved in intestinal immune response in different manners.

Conclusion

DC have been thought to critical for inducing intestinal tolerance, and this study showed distinct DC subsets have distinct immunoregulatory function in different tissues. In addition to DC, it was shown that neutrophils and stromal cells are also involved in immune regulation. This study suggested that more types of cells than previously assumed communicate with each other, and are involved in intestinal immune tolerance.

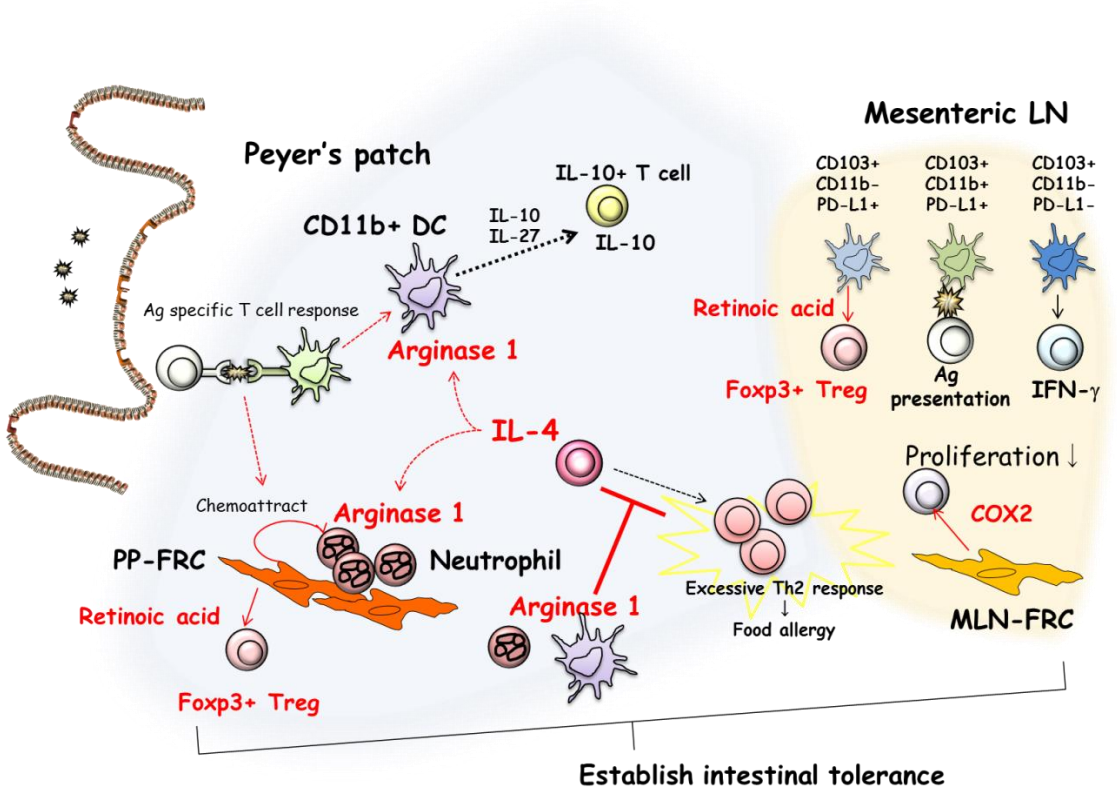

Figure 1. Suggested model of cellular network in intestinal immune regulation

1) Shiokawa A, Tanabe K, Tsuji NM, Sato R, Hachimura S. *Immunol Lett* 2009;125(1):7-14.

論 文 の 内 容 の 要 旨

応用生命化学　専　攻
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論文題目　Studies on immunosuppressive mechanisms in the intestine
（腸管における免疫抑制機構に関する研究）

腸管は常に様々な抗原(食餌由来や腸内細菌由来)に曝露されている。そのため腸管特有の免疫制御機構が備わっており、その一つに経口免疫寛容が挙げられる。経口免疫寛容とは、経口摂取した抗原に対して過剰な細胞性・液性免疫応答を抑制することで定義され、食物に対する過剰な免疫応答から自己を守るために発達したと考えられる。経口免疫寛容は、抗原特異的 T 細胞の除去、不応答化、制御性 T 細胞（Treg）の誘導の結果成立することが知られている。食物に対する過剰な免疫応答（食物アレルギー）は経口免疫寛容の破綻により生じると考えられる。経口免疫寛容は抗原特異的に免疫寛容が誘導されることから、自己免疫疾患の原因となる抗原を経口摂取して抗原に対する免疫寛容を誘導する、という考えは臨床治療へ応用されつつある。

第一章　腸間膜リンパ節における 3 つの CD103$^+$ 樹状細胞サブセットは腸管免疫制御において異なる性質を有する

樹状細胞(DC)は経口免疫寛容を制御する重要な細胞として知られている。経口免疫寛容の誘導には、腸間膜リンパ節 (MLN) が必要であり、そして MLN に存在する CD103$^+$ DC や PD-L1$^+$ DC が経口免疫寛容誘導において重要な働きをすると考えられている。しかしながら、これら CD103$^+$ DC と PD-L1$^+$ DC の関係は明らかでない。そのため、本研究では MLN における DC サブセットの性質や機能を経口免疫寛容に関連して明確にすることを目的とした。フローサイトメーターによる解析結果から、MLN における CD103$^+$ DC はさらに CD11b 分子と PD-L1 分子の発現パターンにより 3 つのサブセットに細分されることが示された。これらの CD103$^+$ DC サブセットを解析した結果、CD103$^+$CD11b$^+$PD-L1$^+$ DC と CD103$^+$CD11b$^-$PD-L1$^+$ DC は、小腸粘膜固有層から MLN へ遊走するために必要なケモカインレセプターである CCR7 を高発現していることが明らかになった。CD103$^+$CD11b$^+$PD-L1$^+$ DC は 経口摂取した抗原を T 細胞に提

示する能力が高く、T 細胞の増殖を強く誘導した。一方で CD103$^+$CD11b$^-$PD-L1$^+$ DC はレチノイン酸合成酵素である Raldh2 の発現が他の CD103$^+$ DC サブセットよりも顕著に高く、T 細胞における Foxp3 発現（Treg のマーカー遺伝子）を強く誘導した。 CD103$^+$CD11b$^-$PD-L1$^-$ DC は経口摂取した抗原を提示することは確認されなかったものの、*in vitro* における T 細胞との共培養により IL-12 非依存的に IFN-γ産生を強く誘導することが確認された。これらの結果より、3つの CD103$^+$ DC サブセットはそれぞれ異なる性質を持っており、経口免疫寛容誘導においても異なる働きで寄与する可能性が示された。

第二章　経口免疫寛容誘導において樹状細胞は IL-10, IL-27 を高産生し、T 細胞の IL-10 産生を亢進する [1)]

MLN に加えて、パイエル板(PP)もまた経口免疫寛容誘導において重要な場である。 経口免疫寛容を誘導したマウス由来の PP DC は T 細胞との共培養において免疫抑制性サイトカインである IL-10 の産生を誘導したが、Foxp3 発現は誘導しなかった。抗原の経口摂取により PP において CD11b$^+$ DC の数が増加し、これら CD11b$^+$ DC は CD11b$^-$ DC と比較して、T 細胞の IL-10 誘導を誘導するサイトカインとして知られる IL-10 および IL-27 を高発現していた。これらの結果より、PP において経口抗原特異的 T 細胞との相互作用の後、IL-10 および IL-27 を産生する CD11b$^+$ DC が増加し、これらの CD11b$^+$ DC が T 細胞の IL-10 産生を亢進して経口免疫寛容誘導に寄与している可能性が示された。

第三章　抗原の経口摂取後、パイエル板において Th2 反応を抑制する arginase 1 発現 CD11b$^+$ DC が増加する

食物アレルギーは経口免疫寛容の破綻により生じると考えられる。アレルギーは過剰な Th2 細胞の免疫反応が起因となり、これらは IL-4, IL-13 や IL-5 の産生を伴う。それゆえ、抗原の経口摂取時には過剰な Th2 細胞応答、すなわち IL-4 産生を抑制して、経口免疫寛容を成立させ、食物アレルギーを回避することが必要となる。Th2 細胞と DC の共培養の結果、経口免疫寛容を誘導したマウス由来の PP DC は T 細胞の IL-10 産生を誘導することに加えて、T 細胞の IL-4 産生を抑制することが明らかになった。これら経口免疫寛容を誘導したマウス由来の PP DC は arginase 1 を高発現しており、arginase 1 活性を介して T 細胞の IL-4 産生を抑制することが示された。arginase 1 は CD11b$^+$ DC にのみ発現しており、抗原の経口摂取後に上昇した。PP における T 細胞は脾臓や MLN における T 細胞と比較して IL-4 を高発現しており、*in vitro* の結果から DC の arginase 1 発現は IL-4 の添加により誘導されることが示された。これらの結果より、抗原の経口摂取後、PP において T 細胞は抗原に反応して IL-4 を大量に産生するが、この IL-4 は CD11b$^+$ DC における arginase 1 発現を誘導することが示唆された。そして arginase 1 発現を高発現した CD11b$^+$ DC は arginase 1 依存的に T 細胞の IL-4 産生を抑制し、PP においては IL-4-arginase 1 の負のフィードバックループを形成することで過剰な Th2 応答を回避している可能性が考えられた。

第四章　抗原の経口摂取後、パイエル板において Th2 反応を抑制する arginase 1 発現好中球が増加する

CD11b$^+$ DC に加えて好中球も抗原の経口摂取後 PP において増加することを見出した。また PP において増加する好中球は、T 細胞が多く存在する濾胞間領域に集積していた。血中における好中球数も抗原の経口摂取後増加しており、好中球は血中から PP へ流入することが示唆された。また PP の濾胞間領域には線維芽細網細胞(FRC)が局在するが、経口免疫寛容を誘導したマウス由来の FRC は未感作マウス由来の FRC と比較して好中球を遊走させる能力が高いことが示された。これらの結果を合わせると、抗原特異的 T 細胞の活性化により FRC における何らかの細胞遊走因子の産生が促進され、これが血中の好中球を PP の濾胞間領域に遊走させるという好中球遊走メカニズムが示唆された。さらに CD11b$^+$ DC と同様に、PP に増加した好中球は arginase 1 を高発現しており、arginase 1 活性を介して T 細胞の IL-4 産生を抑制することが示された。また好中球の arginase 1 発現は IL-4 により上昇した。これらの結果から、抗原の経口摂取後 PP における FRC は血中から好中球を誘引し、PP に遊走した好中球は CD11b$^+$ DC と同様に IL-4-arginase 1 の負のフィードバックループを形成することで過剰な Th2 応答を回避している可能性が考えられた。

第五章　腸間膜リンパ節およびパイエル板におけるストローマ細胞は異なる免疫制御機能をもつ

非造血系細胞のストローマ細胞はリンパ組織の支持構造を構成する細胞である。しかし近年の研究によりストローマ細胞は免疫反応にも関与し、特に免疫寛容に寄与していることが明らかになってきた。リンパ組織における T 細胞領域は FRC により構造が支持されており、免疫担当細胞の足場となっている。またストローマ細胞は組織特異的な免疫反応に重要な役割を担うと考えられているものの、腸管組織特異的なストローマ細胞（特に PP 由来）の性質は明らかとなっていない。そのため本研究では T 細胞応答におけるストローマ細胞の免疫制御機構に焦点をあて、MLN と PP における FRC（gp38$^+$CD31$^-$CD45$^-$ 細胞）の性質を解析した。また FRC の比較となるストローマ細胞として double-negative cells (DNC; gp38$^-$CD31$^-$CD45$^-$ 細胞) を用いた。遺伝子発現を調べた結果 MLN-FRC は PP-FRC や DNC と比較して cyclooxygenase-2 (COX-2) を高発現していた。また MLN-FRC は T 細胞増殖を強く抑制したのに対し、PP-FRC や DNC はその増殖抑制能が弱く、MLN-FRC の T 細胞増殖抑制は、一部 COX-2 の活性に依存することが示された。 これまでに培養した末梢リンパ節由来の FRC は nitric oxide synthase 2 (NOS2) 依存的に T 細胞の増殖を抑制することが報告されている。しかしながら本研究で使用した初代培養 MLN-FRC の T 細胞増殖抑制における NOS2 活性の寄与は非常に低く、さらに FRC における NOS2 の発現も DNC と比較して非常に低いことが示された。また、PP-FRC は MLN-FRC と比較して Raldh2 を高発現しており、レチノイン酸産生を介して T 細胞の Foxp3 発現を誘導することが示された。これらの結果より、MLN と PP における FRC はその性質や機能が異な

り、これらの異なるメカニズムで腸管の免疫反応調節に寄与している可能性が示された。

まとめ

DC は経口免疫寛容誘導に重要な役割を果たすと考えられているが、本研究では異なる組織で異なる DC サブセットが異なる免疫制御機能を持つことを示した。さらに DC に加えて、好中球やストローマ細胞も免疫制御に寄与していることを示した。本研究により、これまでの想定以上に様々な細胞がお互い関与し腸管の免疫寛容に関与していることが示唆された。

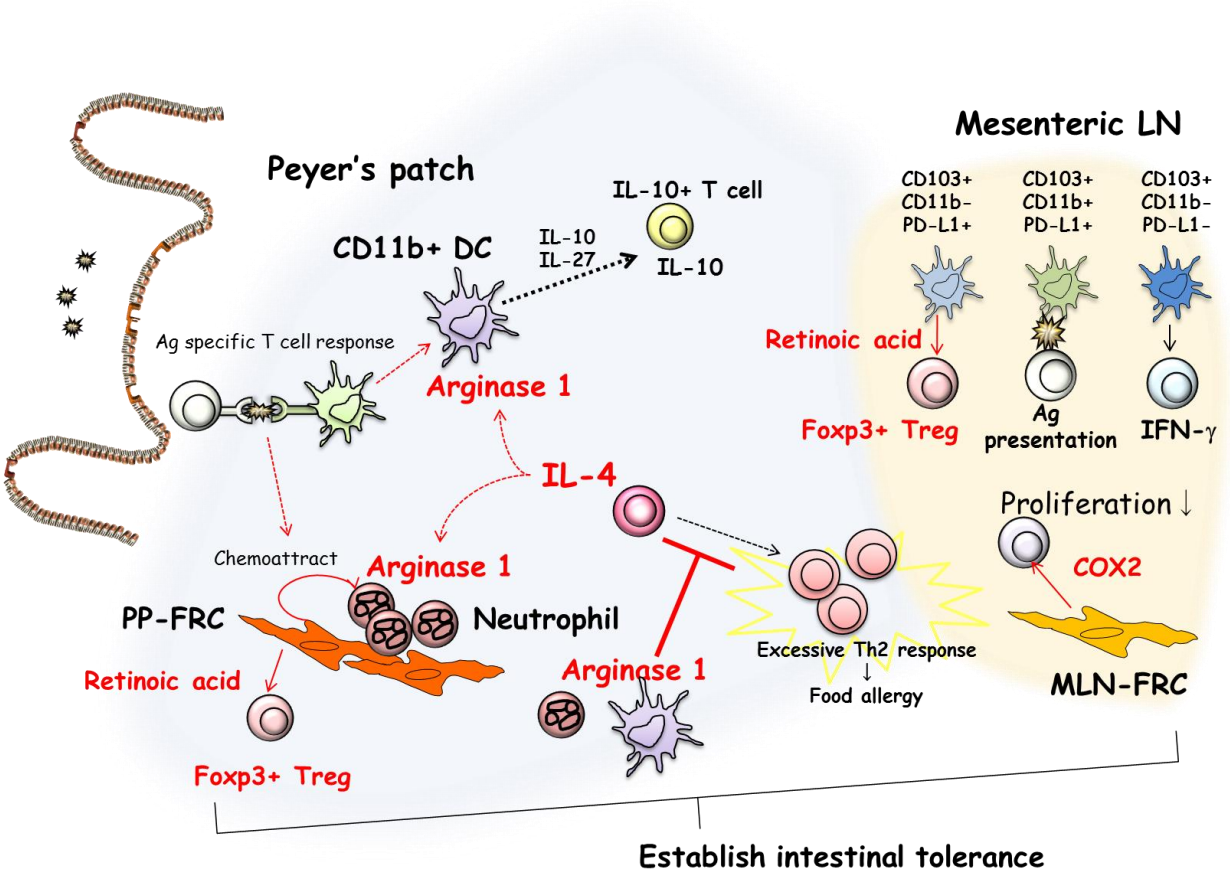

図１．腸管免疫制御における細胞ネットワークモデル

1) Shiokawa A, Tanabe K, Tsuji NM, Sato R, Hachimura S. *Immunol Lett* 2009;125(1):7-14.

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