Immune Netw. 2023 Oct;23(5):e37
https://doi.org/10.4110/in.2023.23.e37
pISSN 1598-2629·eISSN 2092-6685

IMMUNE NETWORK

Original Article

Combined Treatment With TGF-β1, Retinoic Acid, and Lactoferrin Robustly Generate Inducible Tregs (iTregs) Against High Affinity Ligand

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Received: Mar 20, 2023
Revised: Aug 25, 2023
Accepted: Sep 6, 2023
Published online: Sep 22, 2023

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ABSTRACT

Forkhead box P3-positive (Foxp3$^+$)-inducible Tregs (iTregs) are readily generated by TGF-β1 at low TCR signaling intensity. TGF-β1–mediated Foxp3 expression is further enhanced by retinoic acid (RA) and lactoferrin (LF). However, the intensity of TCR signaling required for induction of Foxp3 expression by TGF-β1 in combination with RA and LF is unknown. Here, we found that either RA or LF alone decreased TGF-β1–mediated Foxp3 expression at low TCR signaling intensity. In contrast, at high TCR signaling intensity, the addition of either RA or LF strongly increased TGF-β1–mediated Foxp3 expression. Moreover, decreased CD28 stimulation was more favorable for TGF-β1/LF–mediated Foxp3 expression. Lastly, we found that at high signaling intensities of both TCR and CD28, combined treatment with TGF-β1, RA, and LF induced robust expression of Foxp3, in parallel with powerful suppressive activity against responder T cell proliferation. Our findings that TGFβ/RA/LF strongly generate high affinity Ag-specific iTreg population would be useful for the control of unwanted hypersensitive immune reactions such as various autoimmune diseases.

Keywords: Lactoferrin; Transforming growth factor beta; Retinoic acid; Regulatory T cells; forkhead box P3 protein; T cell receptor; CD28 antigen

INTRODUCTION

Tregs are a T cell subset generated in the thymus and peripheral lymphoid organs, which maintains immune tolerance and homeostasis. Additionally, Tregs can be generated from naïve CD4$^+$ T cells in the presence of TGF-β and IL-2 *in vitro* (1), designated inducible Tregs (iTregs) (2). Tregs are characterized by upregulation of the master transcriptional factor forkhead box P3 (Foxp3) (3,4). TGF-β–mediated Foxp3 expression can be enhanced by retinoic acid (RA) (5,6), and we recently showed that lactoferrin (LF) causes naïve CD4$^+$ T cells to differentiate into Foxp3$^+$ Tregs and synergizes with TGF-β1 (7). As a rising field, the therapeutic applications

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
Akt, protein kinase B; APC, Ag-presenting cell; Foxp3, forkhead box P3; iTreg, inducible Treg; LF, lactoferrin; OVA, ovalbumin; pSmad3, phosphorylated Smad3; RA, retinoic acid; WT, wild-type.

Author Contributions
Conceptualization: Jang YS, Kim PH; Data curation: Jang YS, Park SH; Formal analysis: Jang YS, Park SH; Funding acquisition: Jang YS, Kim PH; Investigation: Jang YS; Project administration: Kim PH; Supervision: Kim PH; Validation: Kang SG, Lee JS, Ko HJ; Writing - original draft: Jang YS, Kim PH; Writing - review & editing: Kang SG, Kim PH.

and modulation of iTregs are documented for the treatment of autoimmune diseases, transplantation, cancer, as well as colitis and other inflammatory diseases (8). Thus, for the clinical application, it is essential to obtain the conditions by which maximum yield of iTregs against high affinity ligands are generated. With regard to iTreg differentiation, it is well known that TCR signaling intensity has a significant impact on the differentiation of $CD4^+$ T cell subsets. In the case of iTregs, it was shown that low doses of Ag or polyclonal TCR activators induce Foxp3 expression, whereas high doses of these stimuli ameliorate Foxp3 upregulation (9). Consistently, we and others have shown that inhibition of downstream mediators of TCR/CD28 signaling, such as PI3K, protein kinase B (Akt), and mTOR, significantly augments TGF-β1–induced Foxp3 expression (10,11). However, it is not known how TCR/CD28 signaling intensities (high or low) affect TGF-β-mediated Foxp3 expression in the presence of RA and LF.

We were particularly interested in the effects of RA and LF on differentiation of TGF-β1–mediated $Foxp3^+$ Tregs at a high TCR/CD28 signaling intensity. We found that, under conditions of high TCR/CD28 signaling intensity, combined treatment with TGF-β1, RA, and LF generates robust Foxp3 expression, leading to powerful T cell suppressor function.

MATERIALS AND METHODS

Animals

BALB/c and C57BL/6 wild-type (WT) mice were obtained from Orient Bio, Inc. (Seongnam, Korea). Ovalbumin (OVA)-specific TCR-transgenic OT-II mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were fed Purina Laboratory Rodent Chow 5001 *ad libitum*. Mice 8–12 wk of age were used in this study. Animal care was performed in accordance with the institutional guidelines established by Kangwon National University (approval No. KW-190515-1 and KW-150619-2).

Naïve T cell isolation and *in vitro* differentiation

Naïve $CD4^+CD25^-$ T cells from the spleens of 8- to 12-week-old mice were isolated by selection, using naïve $CD4^+$ T cell isolation kits and magnetic cell sorting (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions (purity >90%). For Treg differentiation, cells were activated by plate-bound anti-CD3e Ab, soluble anti-CD28 Ab (BD Pharmingen, San Diego, CA, USA), and IL-2 (100 IU/ml; Peprotech, Rocky Hill, NJ, USA). Cells were then treated with TGF-β1 (0.5 ng/ml; R&D Systems, Minneapolis, MN, USA) and RA (50 nM; Sigma–Aldrich, St. Louis, MO, USA). For the induction of Ag-specific Tregs, responder $CD4^+$ T cells (1×10^5) from OT-II transgenic mice were co-cultured with $OVA_{323–339}$-pulsed, irradiated splenoblasts (2×10^5) in the presence of TGF-β1 (1 ng/ml), RA (100 nM), and bovine LF (200 μg/ml; Morinaga Milk Co., Ltd, Zama, Japan) for 3 days. Irradiation (2,000 rad) was performed using a Gammacell 40 Exactor (Best Theratronics Ltd., Ottawa, ON, Canada). In some experiments, naïve $CD4^+$ T cells were cultured with anti-CD28 Ab (2 μg/ml) or CTLA-4 Ig (4 μg/ml) under the conditions for OVA-specific Treg induction.

Antibodies and flow cytometry

Cell surface staining was performed in phosphate-buffered saline with 2% fetal bovine serum, using the following Abs: anti-mouse CD4-v450, CD25-PE, CD44-PE, or CD62L-Ag-presenting cells (APC; eBioscience, San Diego, CA, USA). Intracellular staining was performed using Fixation and Permeabilization Buffer Sets with anti-Foxp3-PE/Cy7 (eBioscience), according to the manufacturer's instructions. Flow cytometry data were collected on a FACSVerse flow

cytometer (BD Biosciences, San Diego, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, MA, USA), using unstained controls to determine gating.

Quantification of T cell suppression by LF/RA/TGF-β1–induced Foxp3⁺ T cells

For preparation of iTregs, activated CD4⁺ T cells were cultured with $OVA_{323–339}$-pulsed, irradiated splenoblasts ($2×10^5$) in the presence of TGF-β1 (1 ng/ml), RA (100 nM), and LF (200 μg/ml) for 3 days. Cells were violet-labeled and co-cultured with CFSE-labeled responder $CD4^+CD25^-$ T cells ($1×10^5$) under activation by $OVA_{323–339}$-pulsed splenoblasts for 3 days. Dilution of CFSE was measured by flow cytometry gating of 10,000 viable cells.

Statistical analysis

Statistical significance of differences between experimental groups was determined by ANOVA or the unpaired, two-tailed Student's *t*-test, and values of $p<0.05$ were considered significant.

RESULTS

Robust generation of TGF-β1/RA–mediated Foxp3⁺ T cell population at higher TCR signal intensity

It is well established that TGF-β1 promotes iTreg differentiation, and RA further enhances this process (12,13). Subsequently, it was demonstrated that TGF-β1 readily promotes increased Foxp3 induction at low TCR signaling intensity (9). However, a TCR signaling intensity favorable for TGF-β1/RA–mediated iTreg differentiation has not been elucidated. Therefore, we examined the effects of different TCR signaling intensities on Foxp3 expression induced by both TGF-β1 and RA. As shown in **Fig. 1A**, we found that TGF-β1–induced Foxp3 expression increased as TCR signaling intensity weakened, and RA had little effect on Foxp3 expression. In contrast, RA markedly augmented TGF-β1–induced Foxp3 expression at a high TCR signaling intensity. We further examined the effect of CD28 (a costimulatory molecule) stimulation on Foxp3 expression under a high TCR signal strength (**Fig. 1B**). Results show an increased level of TGF-β1–induced Foxp3 expression in the absence of CD28 stimulation. Further, RA increased TGF-β1–induced Foxp3 expression regardless of CD28 signaling intensity. These results suggest that at lower TCR signaling intensity, TGF-β1 alone is sufficient for differentiation of Foxp3⁺ T cells. In contrast, at higher TCR signal intensity, RA is required for robust TGF-β1–mediated Foxp3⁺ T cell differentiation.

Ag-specific Foxp3+ T cell population are strongly generated by combined treatment with TGF-β1 and LF at high TCR signaling intensity

We recently demonstrated that LF enhances both Ag-nonspecific and Ag-specific Foxp3⁺ iTreg differentiation and synergizes with TGF-β1 to induce expression of Foxp3 (7). Here, to better identify the functional role of LF, we performed kinetic analyses measuring Ag-specific Foxp3⁺ iTreg differentiation induced by TGF-β1 and LF. To this end, naïve CD4⁺ T cells isolated from OT-II mice were co-cultured with $OVA_{323–339}$-loaded T cell-depleted splenocytes as APCs. We found that LF and TGF-β1 increased Foxp3 expression by $OVA_{323–339}$-stimulated OT-II CD4⁺ T cells (**Fig. 2A**). Subsequent kinetic analyses revealed that TGF-β1–induced Foxp3 expression was increased at lower $OVA_{323–339}$ concentrations (**Fig. 2B**). Further, the combined effect of TGF-β1 and LF on Foxp3 expression was evident at high doses of loaded peptides (≥0.5 μg/ml) but not at low doses (0.125 μg/ml). We then examined the effect of CD28 intensity on Foxp3 expression under high TCR signaling intensity at the sufficient $OVA_{323–339}$ concentration (**Fig. 2C**). Results show that stimulation of CD28 with anti-CD28

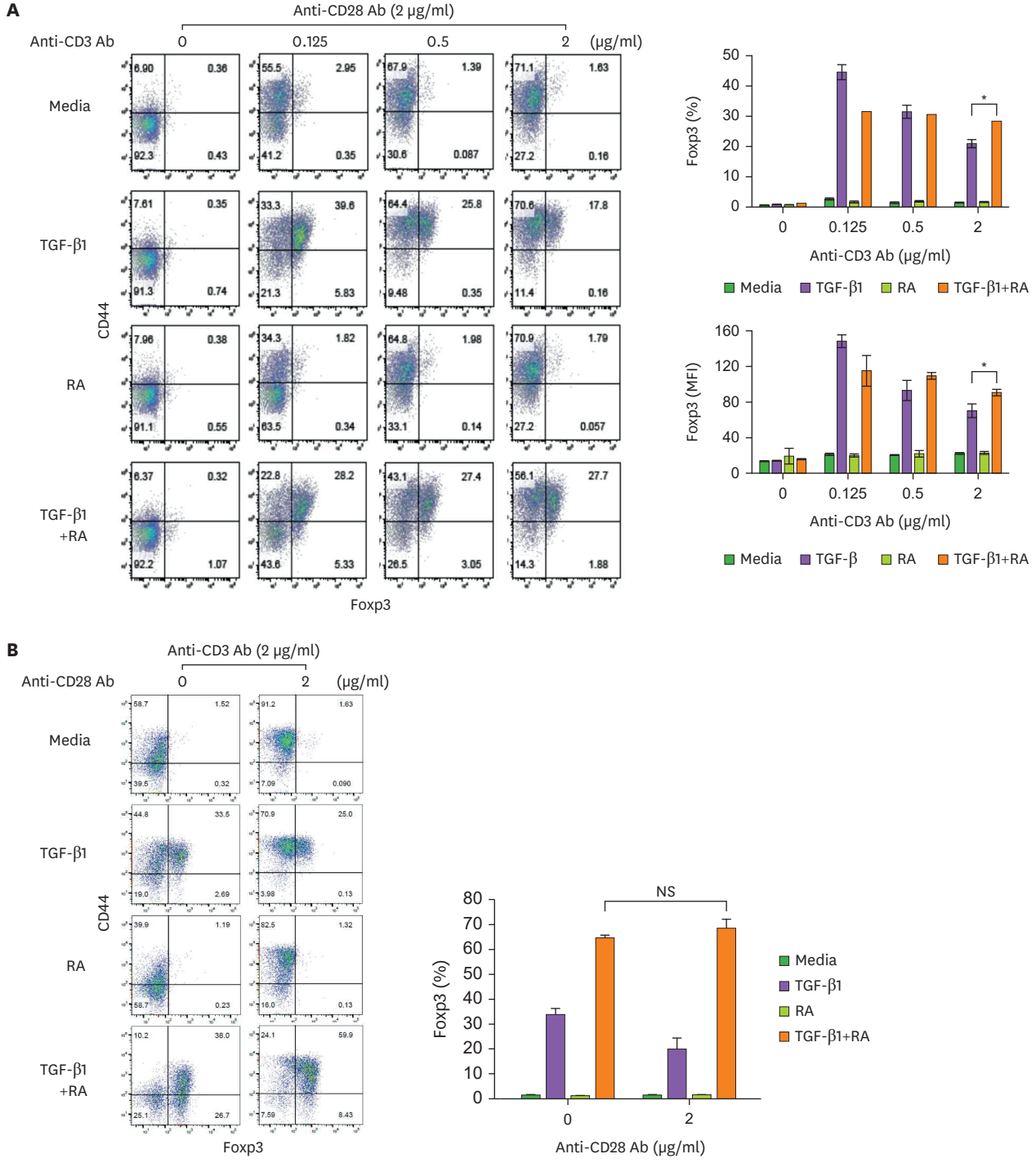

Figure 1. Effect of TCR/CD28 signaling intensity on TGF-β1/RA–mediated Foxp3 expression. (A) Naïve CD4⁺ T cells were activated with anti-CD3e Ab (2, 0.5, 0.125, 0 μg/ml), anti-CD28 Ab (2 μg/ml), and IL-2 (100 IU/ml) and then treated with TGF-β1 (0.5 ng/ml) and RA (50 nM) for 3 days. (B) Naïve CD4⁺ T cells were activated with anti-CD3 Ab (2 μg/ml), anti-CD28 Ab (0 and 2 μg/ml), and IL-2 (100 IU/ml) in the presence of TGF-β1 (0.5 ng/ml) and RA (50 nM) for 3 days. Intracellular Foxp3 expression was determined by FACS. The data represent the average percent of three independent experiments with the SEM indicated by the bars. The significance was determined by Student's *t*-test.
NS, not significant.
*p<0.05.

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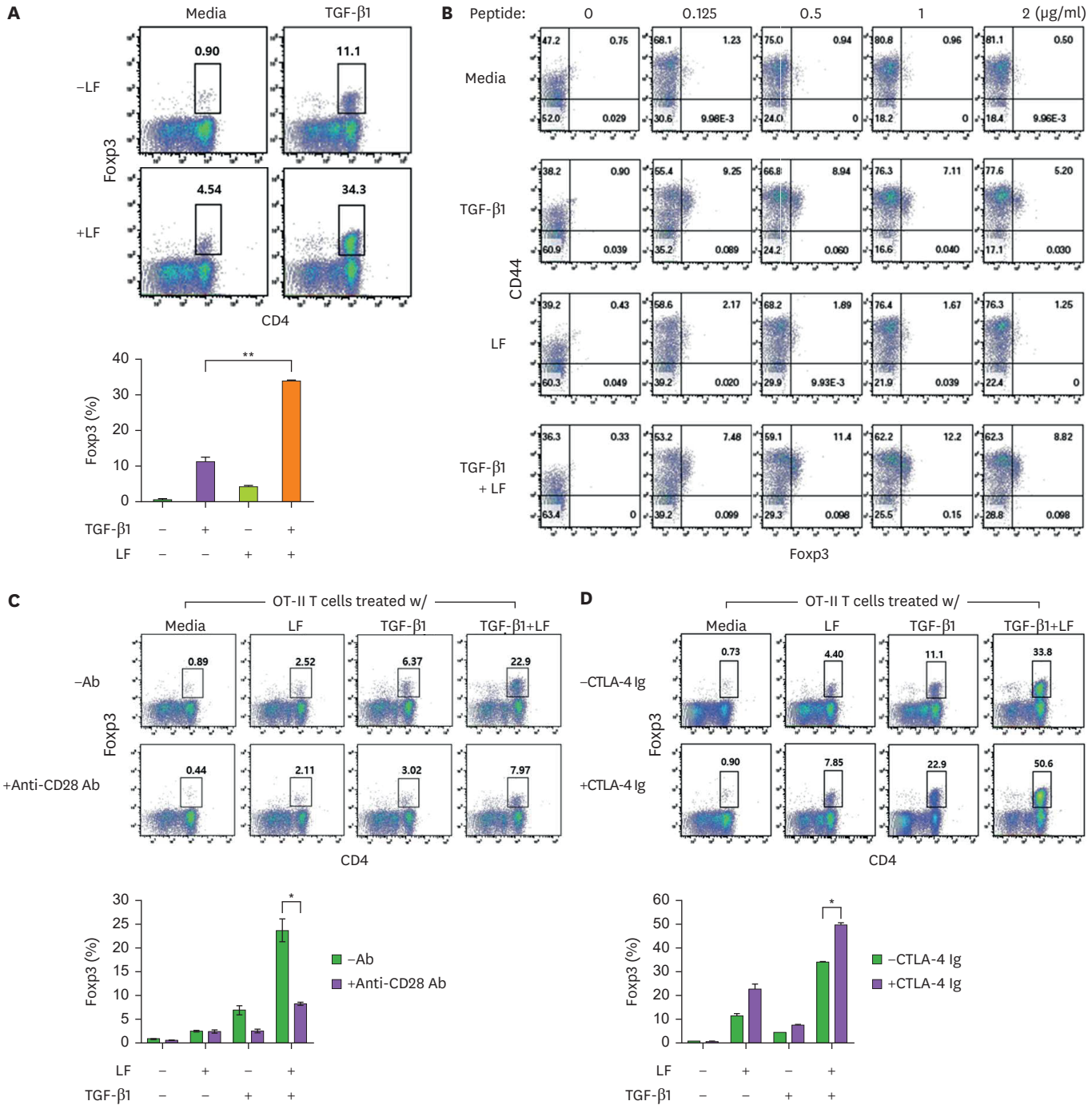

Figure 2. Effect of TGF-β1 and LF on OVA-specific Foxp3⁺ T cell differentiation. (A) OT-II naïve CD4⁺ T cells were cocultured with $OVA_{323-339}$-pulsed splenoblasts in the presence of TGF-β1 (1 ng/ml) and LF (200 μg/ml). After 3 days of culture, cells were analyzed for surface CD4 and intracellular Foxp3 expression using flow cytometry. (B) Naïve CD4⁺ T cells were cultured as described in (A) with the indicated dose of $OVA_{323-339}$. After 3 days of culture, cells were analyzed for CD4 and intracellular Foxp3 expression using flow cytometry. (C, D) Naïve CD4⁺ T cells were cultured as described in (A) with anti-CD28 Ab (2 μg/ml, C) or CTLA-4 Ig (4 μg/ml, D). After 3 days of culture, cells were analyzed for CD4 and intracellular Foxp3 expression using flow cytometry. The data represent the average percent of three independent experiments with SEM (bars). The significance was determined by Student's *t*-test.
*p<0.05; **p<0.01.

Ab substantially decreased TGF-β1/LF–mediated Foxp3 expression. Consistent with these observations, blocking the B7 costimulatory molecule by CTLA-4 Ig markedly increased TGF-β1/LF–mediated Foxp3 expression (**Fig. 2D**). These results reveal that, like RA, LF is

required for the strong generation of a Foxp3$^+$ T cell population induced by TGF-β1 at high TCR signaling intensity. Further, under these conditions, lower levels of CD28 stimulation promote enhanced Foxp3 expression.

Combinatorial effects of TGF-β1, RA, and LF on iTreg differentiation

Thus far, we found that both RA and LF substantially increase TGF-β1–mediated Foxp3 expression at high TCR signaling intensity. Moreover, LF alone increased Ag-specific Foxp3 expression (**Fig. 2A**), whereas RA alone had little effect on Ag-nonspecific Foxp3 expression (**Fig. 1A**). Thus, it was necessary to characterize the combinatorial effects of the three molecules (i.e., TGF-β1, RA, and LF) on Ag-specific iTreg differentiation at high TCR signaling intensity. As observed above for Ag-nonspecific T cell responses (**Fig. 1A**), we found that RA alone had little effect on Foxp3 expression by $OVA_{323–339}$-stimulated OT-II CD4$^+$ T cells (**Fig. 3**). In contrast, like LF, RA markedly enhanced TGF-β1–mediated Foxp3 expression. Interestingly, LF and RA in combination significantly increased Foxp3 expression, suggesting that the mechanisms by which these molecules enhance TGF-β1–induced Foxp3 expression are distinct. Finally, we tested the combination of TGF-β1, RA, and LF and found that this promotes robust expression of Foxp3.

Given that the effect of the triple combination on Foxp3 expression was greater than any dual combination, we further investigated the effects of TGF-β1, RA, and LF on other phenotypes of this T cell population. To this end, we measured the expression of CD25, CD44, and CD62L. The naïve T cell marker CD62L is known to be rapidly shed upon CD4$^+$ T cell activation (14). Here, we found that TGF-β1 and RA induced significant CD62L shedding, and LF similarly augmented CD62L shedding in response to TGF-β1 (**Fig. 4A**). However, unexpectedly, we found that LF inhibited CD62L shedding induced by TGF-β1 and RA in combination. In contrast, CD25 and CD44 (T cell activation markers) were upregulated by TGF-β1 but downregulated by LF and RA (**Fig. 4B and C**). This observation suggests that both LF and RA inhibit T cell activation, possibly contributing to Treg differentiation. We summarize these phenotypes and the effects on Foxp3 expression in **Fig. 5**, which illustrates that Foxp3 expression increases as RA and LF are added sequentially. Further, the expression levels of other proteins (CD62L, CD25, and CD44) differ among the three Foxp3$^+$ T cell populations, indicating that their differentiation stages may be distinct. These

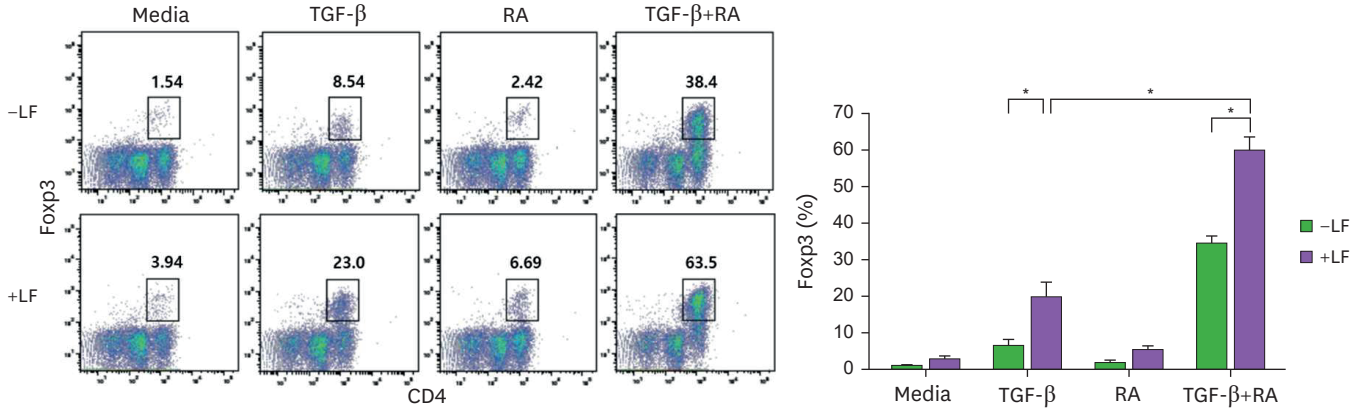

Figure 3. Effect of LF, TGF-β1, and RA on OVA-specific Foxp3$^+$ T cell differentiation. OT-II naïve CD4$^+$ T cells were cocultured with $OVA_{323–339}$-pulsed splenoblasts in the presence of TGF-β1 (1 ng/ml), RA (100 nM), and LF (200 μg/ml). After 3 days of culture, cells were analyzed for surface CD4 and intracellular Foxp3 expression using flow cytometry. The data represent the average percent of three independent experiments with SEM (bars). The significance was determined by Student's *t*-test.
*$p<0.05$.

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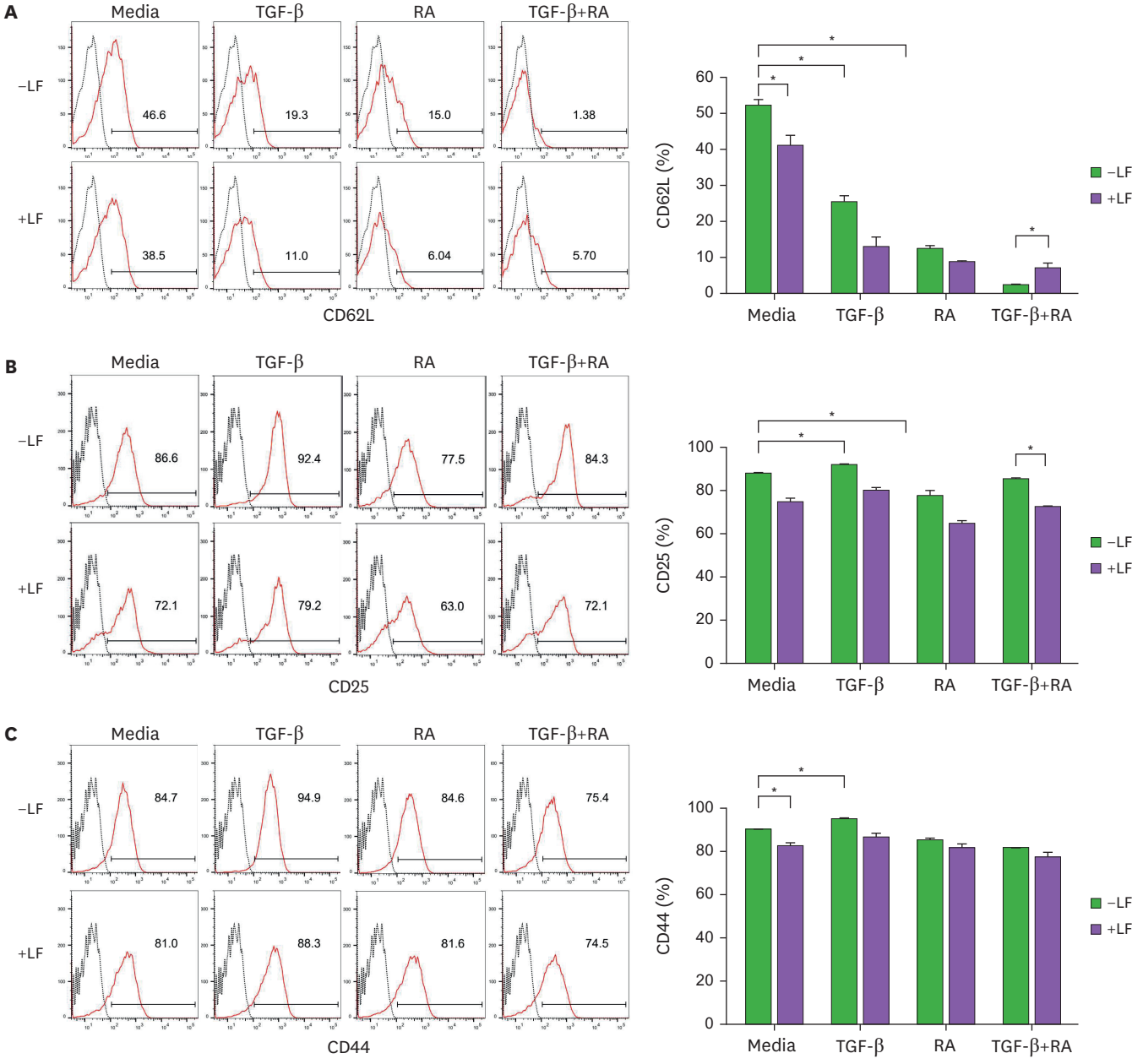

Figure 4. Effect of LF, TGF-β1, and RA on phenotypes of OVA-specific Foxp3$^+$ T cells. $OVA_{323-339}$-activated OT-II naïve CD4$^+$ T cells were cultured with TGF-β1 (1 ng/ml), RA (100 nM), and LF (200 μg/ml). After 3 days of culture, expression of CD62L (A), CD25 (B), and CD44 (C) were analyzed by flow cytometry. The data represent the average percent of three independent experiments with SEM (bars). The significance was determined by Student's *t*-test.
*p<0.05.

results prompted us to assess the suppressor activity of the three Foxp3$^+$ T cell populations. Consistent with the observed levels of Foxp3 induction, the three Foxp3$^+$ T cell populations displayed corresponding levels of suppressive activity against responder CD4$^+$ T cell proliferation (**Fig. 6**). These results indicate that TGF-β1, RA, and LF in combination can induce functionally optimized Ag-specific Treg differentiation.

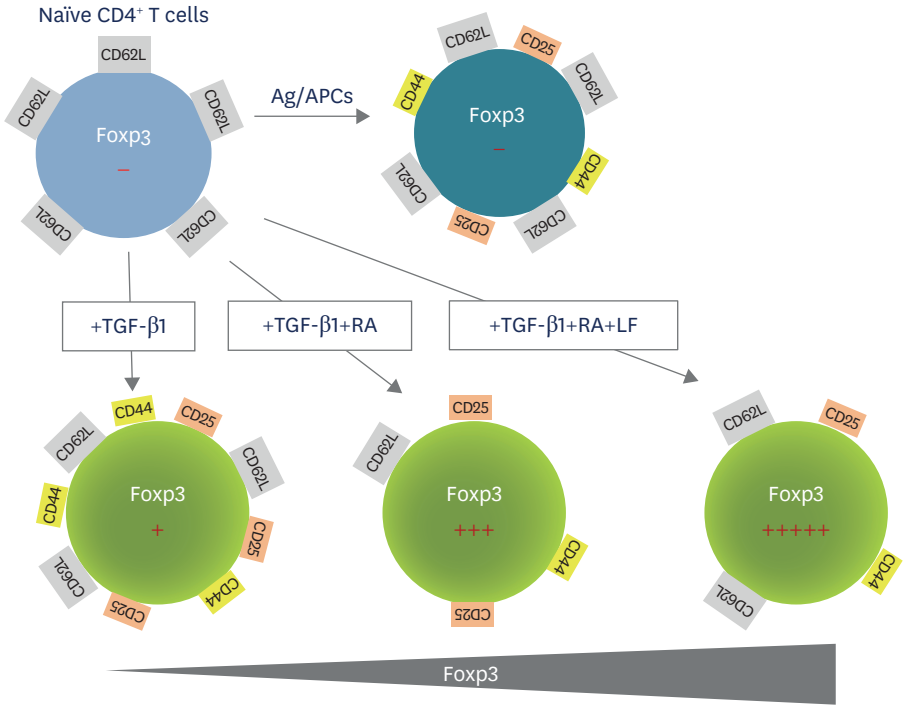

Figure 5. Schematic diagram illustrating the phenotypes of TGF-β1/RA/LF–induced Ag-specific Foxp3⁺ T cells.

DISCUSSION

In the present study, we demonstrate that TGF-β1–mediated iTreg generation is powerfully enhanced by the addition of RA and LF. However, the mechanism by which this combination might cause such synergistic iTreg generation remains unknown. It is well established that TGF-β/Smad3–dependent signaling induces Foxp3⁺ Treg differentiation (3,4), and TGF-β1–mediated iTreg generation is further enhanced by LF and its peptides (7,11). These studies reveal that LF promotes Foxp3 upregulation through two mechanisms: by phosphorylating Smad3 through activation of membrane-bound TGF-β and attenuating TCR/CD28 signaling, both of which occur via TGF-β type III receptor. We further note that the present study does not address the specific mechanisms by which RA enhances TGF-β1–mediated iTreg generation. However, prior studies have demonstrated that Foxp3 expression is induced by either increasing the levels of total Smad3/phosphorylated Smad3 (pSmad3) or enhancing pSmad3 binding to a conserved enhancer region of the *Foxp3* gene in TGF-β/RA–stimulated T cell cultures (5,6). These findings suggest that RA may promote TGF-β signaling toward Foxp3 expression. Additionally, results from the present study reveal that RA, like LF, inhibits T cell activation (**Fig. 4A**). This action of RA must contribute to Foxp3⁺ Treg differentiation, given that, like LF, RA is expected to attenuate TCR/CD28 signaling.

When considering the physiological implications of synergistic TGF-β1/RA/LF–mediated Ag-specific Foxp3⁺ Treg generation, it is worthwhile to view this phenomenon in the context of intestinal immune tolerance. The gastrointestinal tract is in constant contact with food Ags, commensal microorganisms, and potential pathogens. Foxp3⁺ Tregs are known to be abundant in the gut, where they play a central role in maintaining intestinal immune homeostasis (15). Moreover, TGF-β, RA, and LF are ubiquitous in mucosal tissues.

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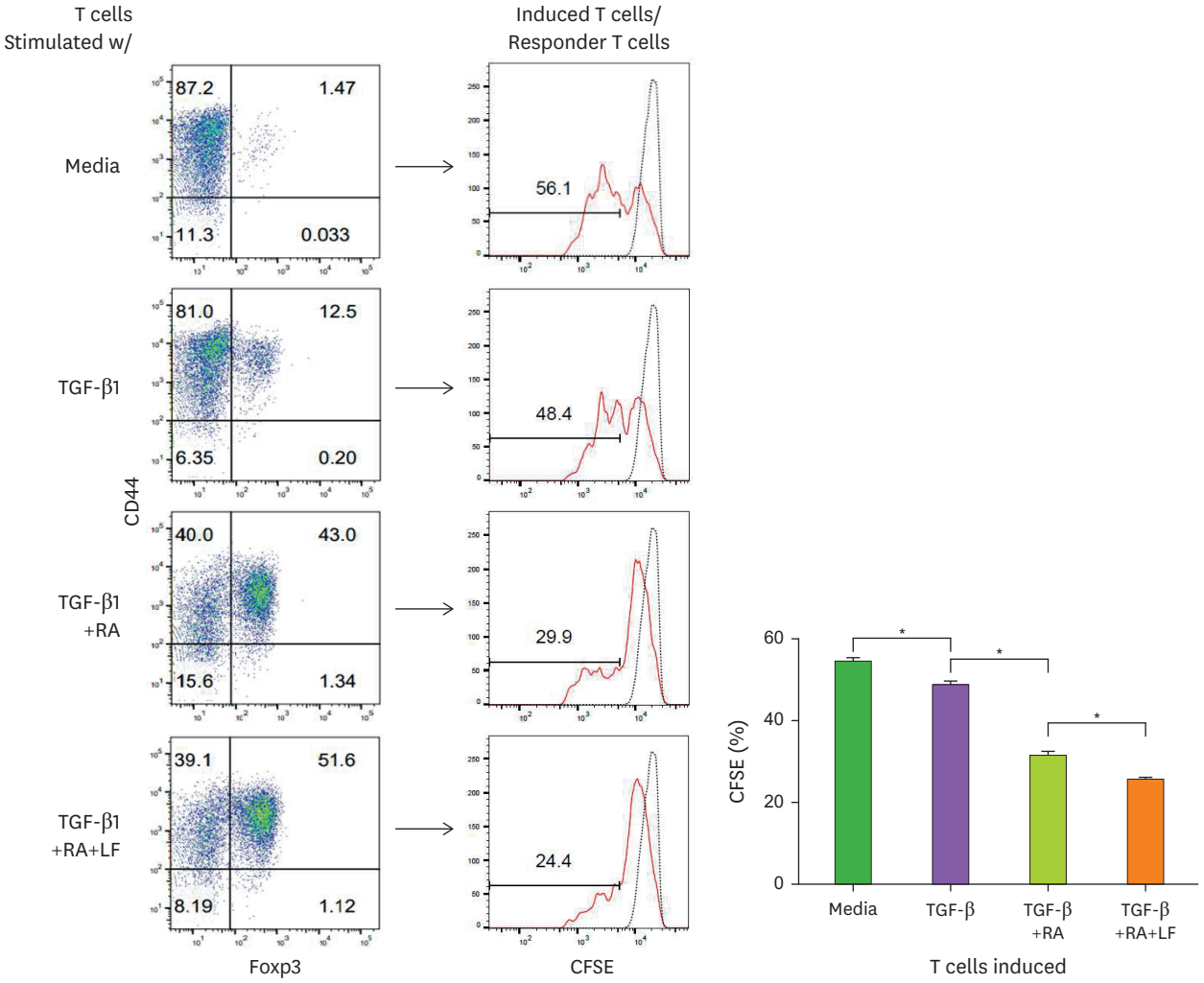

Figure 6. LF/TGF-β1/RA–induced OVA-specific Foxp3$^+$ T cells suppress responder T cell activation. OVA-specific Foxp3$^+$ T cells were obtained by culturing as described in **Fig. 3**. Responder T cells were prepared by labeling OT-II naïve CD4$^+$ T cells with CFSE and then stimulating them with $OVA_{323-339}$-pulsed splenoblasts. The two cell populations were cultured for 3 days, and proliferation was determined by analyzing CFSE dilution using flow cytometry. The data represent the average percent of three independent experiments with SEM (bars). The significance was determined by Student's *t*-test.
*p<0.05.

Thus, the gut is a TGF-β–rich environment in which most cell types can both produce and respond to this cytokine (16). The small intestinal microenvironment is particularly high in RA, containing enterocytes that metabolize retinol (vitamin A) to RA via retinal aldehyde dehydrogenases (17). Notably, it was previously shown that lamina propria dendritic cells promote *de novo* generation of Foxp3$^+$ Tregs via RA (18). LF is synthesized by epithelial cells and is also found primarily in mucosal secretions (19). As noted above, we found that the highest levels of Foxp3 expression induced by TGF-β1/RA/LF in combination are present in parallel with the most powerful regulatory function (**Fig. 6**). Moreover, this combined effect occurs at high TCR signaling intensity. In fact, both RA and LF inhibit TGF-β1–mediated Foxp3 expression at lower TCR signaling intensity (**Figs. 1** and **2**). Thus, our *in vitro* studies, combined with published reports (16-19), raise the possibility that RA, LF, and TGF-β1 may modulate intestinal iTreg generation, especially during strong stimulation of TCR by foreign Ags (e.g., unwanted food Ags). Thus, it is highly plausible that the three molecules have pivotal roles in maintaining intestinal immune homeostasis.

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It is now extensively studied that iTregs are potentially important substitutes for anti-inflammatory agents to treat various autoimmune diseases and allogenic transplantation (8,20). In this regard, our findings that Ag-specific iTreg populations strongly generated *ex vivo* by TGF-β1/RA/LF would be useful for the treatment of patient-specific hypersensitive immune disorders as mentioned above.

ACKNOWLEDGEMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant (NRF-2022R1I1A3060443).

REFERENCES

1. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003;198:1875-1886.
PUBMED | CROSSREF
2. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, et al. Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol* 2013;14:307-308.
PUBMED | CROSSREF
3. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-1061.
PUBMED | CROSSREF
4. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* 2008;9:194-202.
PUBMED | CROSSREF
5. Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol* 2008;181:2277-2284.
PUBMED | CROSSREF
6. Xu L, Kitani A, Stuelten C, McGrady G, Fuss I, Strober W. Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I. *Immunity* 2010;33:313-325.
PUBMED | CROSSREF
7. Jang YS, Song HE, Seo GY, Jo HJ, Park S, Park HW, Kim TG, Kang SG, Yoon SI, Ko HJ, et al. Lactoferrin potentiates inducible regulatory t cell differentiation through TGF-β receptor III binding and activation of membrane-bound TGF-β. *J Immunol* 2021;207:2456-2464.
PUBMED | CROSSREF
8. Dong Y, Yang C, Pan F. Post-translational regulations of foxp3 in Treg cells and their therapeutic applications. *Front Immunol* 2021;12:626172.
PUBMED | CROSSREF
9. Turner MS, Kane LP, Morel PA. Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion. *J Immunol* 2009;183:4895-4903.
PUBMED | CROSSREF
10. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, Knight ZA, Cobb BS, Cantrell D, O'Connor E, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A* 2008;105:7797-7802.
PUBMED | CROSSREF
11. Jang YS, Yang SW, Kim TG, Song HE, Park S, Lee EH, Kang SG, Yoon SI, Ko HJ, Lee GS, et al. Lactoferrin-derived chimeric peptide (LFch) strongly boosts TGFβ1-mediated inducible Treg differentiation possibly through downregulating TCR/CD28 signalling. *Immunology* 2023;168:110-119.
PUBMED | CROSSREF

12. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* 2007;204:1765-1774.
PUBMED | CROSSREF

13. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, Noelle RJ, Cheroutre H. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* 2009;30:471-472.
PUBMED | CROSSREF

14. Chen A, Engel P, Tedder TF. Structural requirements regulate endoproteolytic release of the L-selectin (CD62L) adhesion receptor from the cell surface of leukocytes. *J Exp Med* 1995;182:519-530.
PUBMED | CROSSREF

15. Jacobse J, Li J, Rings EH, Samsom JN, Goettel JA. Intestinal regulatory t cells as specialized tissue-restricted immune cells in intestinal immune homeostasis and disease. *Front Immunol* 2021;12:716499.
PUBMED | CROSSREF

16. Konkel JE, Chen W. Balancing acts: the role of TGF-β in the mucosal immune system. *Trends Mol Med* 2011;17:668-676.
PUBMED | CROSSREF

17. Manicassamy S, Pulendran B. Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. *Semin Immunol* 2009;21:22-27.
PUBMED | CROSSREF

18. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 2007;204:1775-1785.
PUBMED | CROSSREF

19. Mason DY, Taylor CR. Distribution of transferrin, ferritin, and lactoferrin in human tissues. *J Clin Pathol* 1978;31:316-327.
PUBMED | CROSSREF

20. Sánchez-Fueyo A, Whitehouse G, Grageda N, Cramp ME, Lim TY, Romano M, Thirkell S, Lowe K, Fry L, Heward J, et al. Applicability, safety, and biological activity of regulatory T cell therapy in liver transplantation. *Am J Transplant* 2020;20:1125-1136.
PUBMED | CROSSREF