Journal of Bacteriology and Virology 2015. Vol. 45, No. 4 p.354 – 363
http://dx.doi.org/10.4167/jbv.2015.45.4.354

Original Article

Eosinophils are Required for Immune Responses Induced by Oral Immunization

YunJae Jung*

Department of Microbiology, School of Medicine, Gachon University, Incheon Korea

Eosinophils are multifunctional leukocytes that reside in several tissues, most abundantly in the small intestinal lamina propria under the steady state. To date, the phenotypic and functional characteristics of small intestinal eosinophils have remained poorly understood. In this study, we found that proliferation of ovalbumin (OVA)-specific $CD4^+$ T cells isolated from the mesenteric lymph nodes of eosinophil-deficient ΔdblGATA mice were decreased relative to wild-type mice after oral immunization with OVA and cholera toxin (CT), the typical mucosal adjuvant that induces $CD4^+$ T cell-dependent responses. ΔdblGATA mice showed reduced mucosal secretion of OVA-specific IgA and IgG1 while maintaining a systemic level of anti-OVA IgG1 upon oral immunization with OVA and CT. These findings suggest that eosinophils might have a role in the modulation of T cell-mediated immune responses including mucosal antibody responses in the gastrointestinal tract following oral immunization.

Key Words: Eosinophils, Small intestine, Mucosal immunity, Oral immunization, Antibody responses

INTRODUCTION

Eosinophils are multifunctional proinflammatory leukocytes that play an important role in parasitic infections and allergic inflammations (1, 2). The cytoplasm of mature eosinophils contains secondary granules such as eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin, and major basic protein the exocytotic release of which contributes to host protection (1, 3). However, accumulating evidence indicates that eosinophils are involved in biologic processes such as modulation of T cell-mediated immune responses (2, 4).

Eosinophils develop in the bone marrow under regulation of the transcription factors GATA-1, GATA-2, and c/EBP (5); accordingly, deletion of a high-affinity GATA-binding site in the GATA-1 promoter (ΔdblGATA) generates eosinophil-deficient mice (6). Most eosinophils, developed in the bone marrow, migrate to the lamina propria (LP) of the gastrointestinal (GI) tract, but not to the esophagus, under homeostatic conditions (7). Although eosinophils in the steady state are present in the thymus, mammary glands, and uterus (1), they are most abundant in the LP of the GI tract. Intestinal eosinophils have characteristics associated with prolonged

Received: August 10, 2015/ Revised: August 28, 2015/ Accepted: September 23, 2015
*Corresponding author: YunJae Jung, MD, PhD. Department of Microbiology, School of Medicine, Gachon University, 191 Hambakmoe-ro, Yeonsu-gu, Incheon 21936, Korea.
Phone: +82-32-820-4753, Fax: +82-32-820-4744, e-mail: yjjung@gachon.ac.kr
**This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2013R1A1A2004820). The author thanks Dr. So-Youn Woo (Ewha Woman's University, Korea) for supporting works with ΔdblGATA mice. The author has no financial conflicts of interest.

survival due to common γ-chain-dependent signaling (8) that contributes to their accumulation in the GI tract. Additionally, intestinal eosinophils constitutively express signal-regulatory protein α/CD172a, the interaction of which with CD47 also leads, through inhibition of eosinophil degranulation, to prolonged eosinophil survival (9). Although eosinophils are much more abundant in the GI tract than in other tissues, their functional characteristics in the intestinal immune system have yet to be elucidated.

The intestinal immune system is a unique environment that invokes strong protective immunity against pathogens while maintaining tolerance to dietary proteins or commensal bacteria (10). A prominent feature of the intestinal immune system's blocking of harmful pathogens is the production of immunoglobulin (Ig) A, the most abundant antibody isotype in the human body (11). It is well established that oral administration of cholera toxin (CT) induces mucosal IgA, IgG1, and IgE responses to co-administered protein (12, 13). Production of antibodies upon oral CT-protein immunization is a T cell-dependent response that depends on CD40 signals of $CD4^+$ T cells activated by dendritic cells (DC) (14). The roles of other molecules and cells in the induction of immune responses following CT oral immunization have been extensively investigated. We recently reported that eosinophils in the GI tract regulate IgA production under the steady state through secretion of IL-1β (15). Additionally, CD47, a membrane protein that contributes to prolonged survival of eosinophils, is critical to the induction of secretory IgA synthesis upon oral immunization (16).

In the present study, I investigated the roles of eosinophils in immune responses following oral immunization of eosinophil-deficient ΔdblGATA and wild-type (WT) mice with ovalbumin (OVA) and CT. ΔdblGATA mice showed decreased proliferation of $CD4^+$ T cells following oral immunization and intestinal secretion of OVA-specific antibodies was also impaired in this eosinophil-deficient mice. The frequency of IgA^+ cells in the LP of ΔdblGATA mice was decreased compared to WT mice following oral immunization. Therefore, the defective immune responses of ΔdblGATA mice upon oral immunization are likely to be linked to the absence of eosinophils in the GI tract.

MATERIALS AND METHODS

Mice

Female BALB/c WT mice (Orientbio, Gapyeong, Korea) and ΔdblGATA mice (Jackson Laboratory, Bar Harbor, ME, USA), aged 6 to 8 weeks were housed under standard laboratory conditions of temperature and humidity at Gachon University. All experiments complied with the institutional guidelines for animal welfare.

Preparation of cells

Segments of the small intestine were incubated with FACS buffer (phosphate-buffered saline (PBS) containing 10% FCS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM EDTA, and 10 μg/ml polymyxin B) for 30 min at 37℃ to remove epithelial cells, then washed extensively with PBS. Small intestinal segments and Peyer's Patch (PP) were digested with 400 Mandl U/ml collagenase D (Roche, Mannheim, Germany) and 10 μg/ml DNase I (Roche) in RPMI 1640/ 10% FCS with continuous stirring at 37℃ for 30 min. EDTA was added (10 mM final), and the cell suspension was incubated for an additional 5 min at 37℃. After washing, the cells were subjected to density-gradient centrifugation in 40%/75% Percoll. The cells harvested from the interface were washed and used as LP leukocytes in assays. Blood and bone marrow cells were collected from WT and ΔdblGATA mice and resuspended in ACK lysis buffer to lyse erythrocytes.

Flow cytometric analysis

To characterize the surface phenotype, cells were isolated and resuspended in FACS buffer. After Fc receptor blocking with anti-mouse CD16/CD32 (2.4G2, BD Biosciences, San Diego, CA, USA) for 15 min at 4℃, the cells were stained with the antibodies identified below for 30 min at 4℃. Monoclonal antibodies (mAb) against Siglec F (E50-2440), CD11b (M1/70), CCR3 (83103), IL-5Rα (T21), CD11c (HL3), and CD4 (RM4-5) were purchased from BD Bio-

sciences. Anti-CD172a (P84), anti-CD47 (miap301), anti-CD8a (53-6.7), and anti-B220 (RM3-6B2) mAb were purchased from eBioscience (San Diego, CA, USA) and anti-Gr-1 (RB6-8C5) mAb were obtained from BioLegend (San Diego, CA, USA). Unbound antibodies were washed with FACS buffer, and cells showing the light scatter pattern unique to eosinophils were electronically gated. To examine the intracellular IgA, cells were fixed and permeabilized using a Cytofix/Cytoperm Kit (BD Biosciences), then stained with anti-mouse IgA (C10-3, BD Biosciences). Each sample was acquired with a FACSCalibur (BD Biosciences), and the data were processed with the FlowJo software (Tree Star, Ashland, OR, USA).

Oral immunization

Mice were deprived of food for two hours and then given 0.5 ml of sodium bicarbonate to neutralize stomach acidity (17). After 30 min, mice were orally immunized by gastric intubation with 1 mg OVA (Grade II, Sigma-Aldrich, St. Louis, MO, USA) in the presence of 10 μg of CT (List Biological Laboratories, Campbell, CA, USA) as the mucosal adjuvant. The oral immunization procedure was conducted at seven-day intervals three times. One week after the final immunization, mice were sacrificed and blood was collected. For intestinal lavage, the small intestine was removed and 2 ml of PBS containing 0.1% bovine serum albumin (BSA), 50 mM EDTA, and 0.1 mg of soybean trypsin inhibitor (Sigma-Aldrich) per ml was passed through and collected (18). Subsequently, phenylmethylsulfonyl fluoride (1 mM, Sigma-Aldrich) was added to the intestinal wash. All of the intestinal washes were vigorously vortexed and centrifuged at 1,000 × g for 20 min to remove debris, after which the supernatants were collected and 0.1% NaN_3 was added.

T cell proliferation assay

$CD4^+$ T cells were enriched from the mesenteric lymph nodes (MLN) of mice by positive selection magnetic separation using a MACS LS column (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD4^-$ fraction was used for antigen presenting cells (APC) after treatment with mitomycin C (Sigma-Aldrich). Isolated $CD4^+$ T cells were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) for 10 min at 37℃, then washed three times. CFSE-labeled $CD4^+$ T cells (1×10^4) were incubated with OVA-loaded APC (1×10^5) or anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies for 72 h. T cell proliferation was then assessed by flow cytometric analysis of the CFSE dilutions.

Detection of antibodies by ELISA

To measure the OVA-specific antibody level, 96-well plates were coated with OVA (50 μg/ml) and blocked with PBS/BSA. Diluted serum and intestinal washes were incubated, and biotinylated anti-mouse IgA (RMA-1, BioLegend) or IgG1 (A85-1, BD Biosciences) was added. After reacting with streptavidin-HRP, the plates were developed with TMB substrate and the absorbance was read at 450 nm.

Statistical analysis

The data are presented as mean ± s.e.m. All experiments were performed in triplicate. When necessary, a two-group comparison was performed using the Student's *t*-test. A p value < 0.05 was considered statistically significant.

RESULTS

Phenotypic characterization of eosinophils in the LP of the small intestine

A forward light scatter/side scatter (SSC)-based analysis of the small intestinal LP cells of WT mice by flow cytometry indicated the presence of two main cell subsets: a granulocyte population with medium to high SSC (R1; 30 ± 1%), and a mononuclear cell population with low SSC (R2; 48 ± 4%), which is consistent with the relevant previous findings (Fig. 1A) (9, 15). Staining with mAb against molecules expressed by intestinal eosinophils, such as CD11b, Siglec F, CCR3, and IL-5Rα, confirmed that almost 95% of the R1 cells were eosinophils (Fig. 1A). Compared to the intestinal LP, blood and bone marrow harbored only limited numbers of $CCR3^+SiglecF^+$ eosinophils (4.4 ± 0.5% and 1.9 ± 0.7%, respectively, Fig. 1). The expression of CD11c and Gr-1 was observed in the R1 eosinophils, but none of

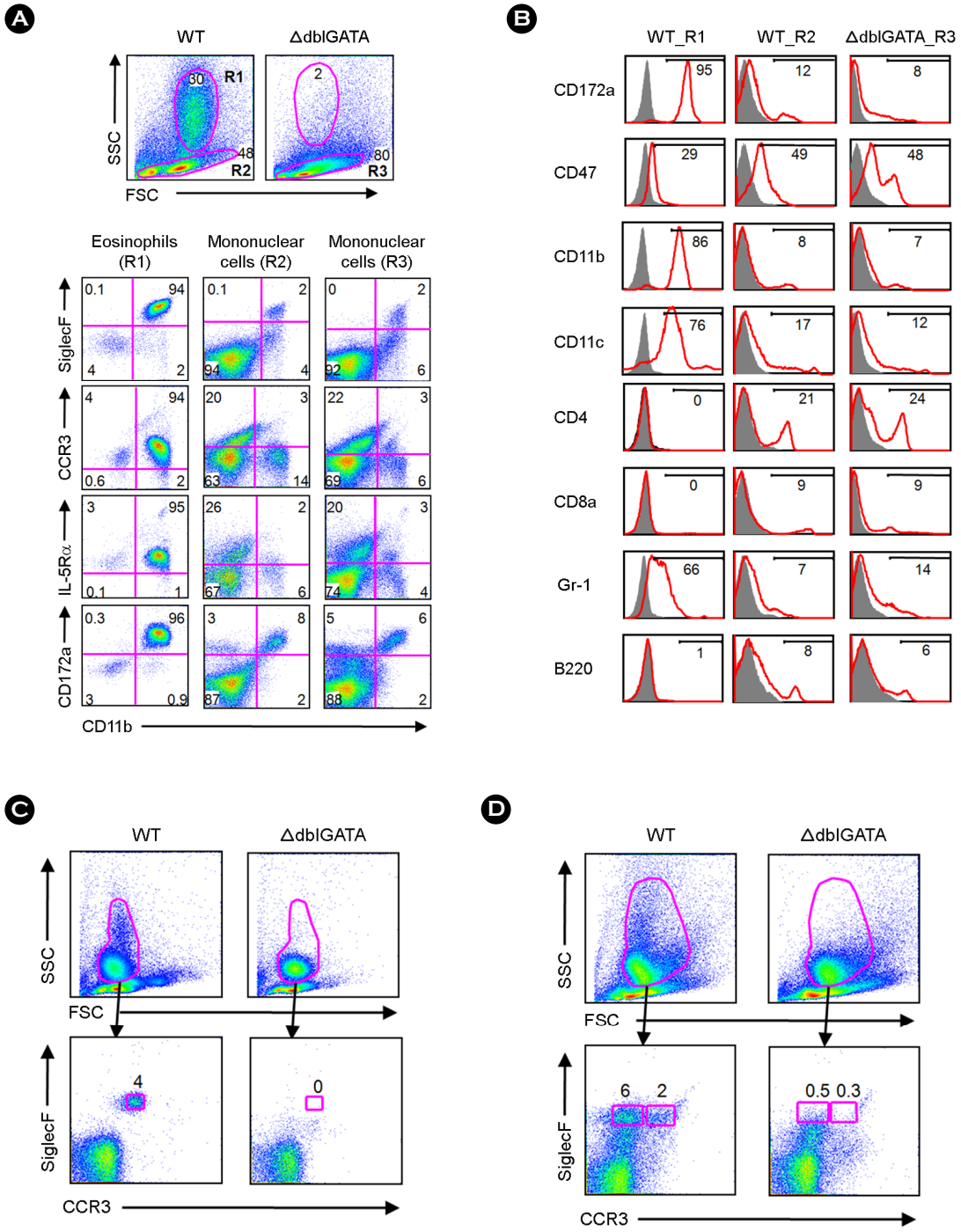

Figure 1. Phenotypic characteristics of leukocytes isolated from the small intestinal lamina propria (LP) of wild-type (WT) and ΔdblGATA mice. (A) Small intestinal LP cells of WT and ΔdblGATA mice were stained with anti-CD11b and anti-CD172a, anti-SiglecF, anti-CCR3, or anti-IL-5Rα, then analyzed by flow cytometry. The R1 gate represents the medium to high side scatter (SSC) subset, and the R2 and R3 gates correspond to the mononuclear cell fractions of WT and ΔdblGATA mice, respectively. FSC indicates a forward light scatter. (B) The R1 and R2 of the LP cells of WT mice and the R3 of ΔdblGATA intestinal LP cells were analyzed for the expression of various cell-surface molecules. (C and D) Isolated blood (C) and bone marrow cells (D) were stained with anti-CCR3 and anti-SiglecF and analyzed by flow cytometry. A granulocyte population with medium to high SSC was gated and analyzed for their expression of CCR3 and SiglecF. Eosinophil populations ($CCR3^+SiglecF^+$) were not observed in the blood or bone marrow of ΔdblGATA mice. The $CCR3^-SiglecF^+$ subset, observed only in the bone marrow of WT mice, might represent an immature type of eosinophil. The results shown are representative of three independent experiments.

the T or B cell markers (i.e. CD4, CD8a, and B220) were detected (Fig. 1B). Additionally, small intestinal eosinophils expressed CD172a at a high level (Fig. 1A), as previously described (9). Compared with the R1 subset, cells in the mononuclear R2 fraction showed only minor expression of CD172a, CD11b, SiglecF, CCR3, and IL-5Rα (Fig. 1A). In the small intestinal LP of ΔdblGATA mice, the medium to high SSC subset representing intestinal eosinophils was completely depleted (Fig. 1A), and the mononuclear cells (R3) of those mice shared characteristics with R2 of WT mice (Fig. 1).

Reduced proliferation of $CD4^+$ T cells isolated from the MLN of ΔdblGATA mice after oral immunization

Impaired production of IgA and IgG has been reported in CD47-deficient mice following oral immunization with OVA and CT (16). CD172a transmits negative signals upon ligation by CD47, a ubiquitously expressed plasma membrane protein (19). As CD172a signaling of small intestinal eosinophils contributes to the prolonged survival of murine intestinal eosinophils (9), decreased antibody responses observed in CD47-deficient mice might be attributable to the reduced viability of LP eosinophils. Given that CT acts as a mucosal adjuvant through induction of Th2-type responses (20), we investigated $CD4^+$ T cell proliferation of WT and ΔdblGATA mice after oral immunization. CFSE-labeled $CD4^+$ T cells purified from the MLN of oral-immunized mice were co-cultured with OVA-loaded APC and a CFSE dilution of T cells was measured after 72 h. As shown in Fig. 2, the fraction of T cells that had entered division was markedly reduced in $CD4^+$ T cells isolated from the MLN of ΔdblGATA mice following oral immunization with OVA-plus-CT. Proliferation of T cells isolated from WT mice fed OVA and CT showed a significant increase ($p = 0.0006$) relative to the same group of ΔdblGATA mice, though no significant differences were observed between WT and ΔdblGATA mice fed PBS ($p = 0.3896$) or OVA ($p = 0.1557$). There was no significant difference between ΔdblGATA and WT mice after oral immunization with OVA and CT in terms of proliferation of $CD4^+$ T cells stimulated by anti-CD3 and anti-CD28 antibodies (Fig. 2, C and D). These results indicate that ΔdblGATA mice might have a reduced ability to induce proliferation of $CD4^+$ T cells after feeding with OVA in the presence of mucosal adjuvant.

Antigen-specific intestinal antibody titers are significantly reduced in ΔdblGATA mice

I next assessed the OVA-specific antibody responses in WT and ΔdblGATA mice upon oral immunization with OVA and CT. As previously reported (15), ΔdblGATA mice showed decreased IgA^+B220^+ cells (post-class switch recombination IgA^+ B cells) in the PP and IgA^+B220^- plasma cells in the LP under the steady state (Fig. 3A). The frequency of IgA^+ cells in the PP and LP of ΔdblGATA was also reduced than WT mice after oral immunization with OVA and CT (Fig. 3A). In support of these findings, ΔdblGATA mice orally immunized with OVA and CT showed significantly reduced mucosal production of anti-OVA IgA and IgG1 and systemic anti-OVA IgA relative to WT mice (Fig. 3B). However, compared with PBS fed ΔdblGATA mice, OVA-plus-CT oral immunization induced significant levels of anti-OVA-specific IgA in ΔdblGATA mice (Fig. 3B). As for OVA-specific serum IgG1, OVA-plus-CT oral immunized ΔdblGATA showed a significant increase ($p < 0.0001$) relative to the same group of WT mice (Fig. 3B). On the basis of a recent study reporting increased mast cell activation in ΔdblGATA mice by OVA challenge (21), the upregulated serum OVA-specific IgG1 upon OVA plus CT oral immunization in ΔdblGATA mice might originate from enhanced systemic IgG1 responses in the absence of eosinophils.

DISCUSSION

Eosinophils are generally thought of as circulating pro-inflammatory cells involved in the pathogenesis of allergic diseases and protection against helminth infection (1, 2). However, eosinophils are much more abundant in the LP of the intestine than in other tissues under the steady state (1~3), and previous studies have shown that eosinophils are required for mucosal production of IgA and maintenance of PP cellularity (15, 22). Recently, isolation of intestinal

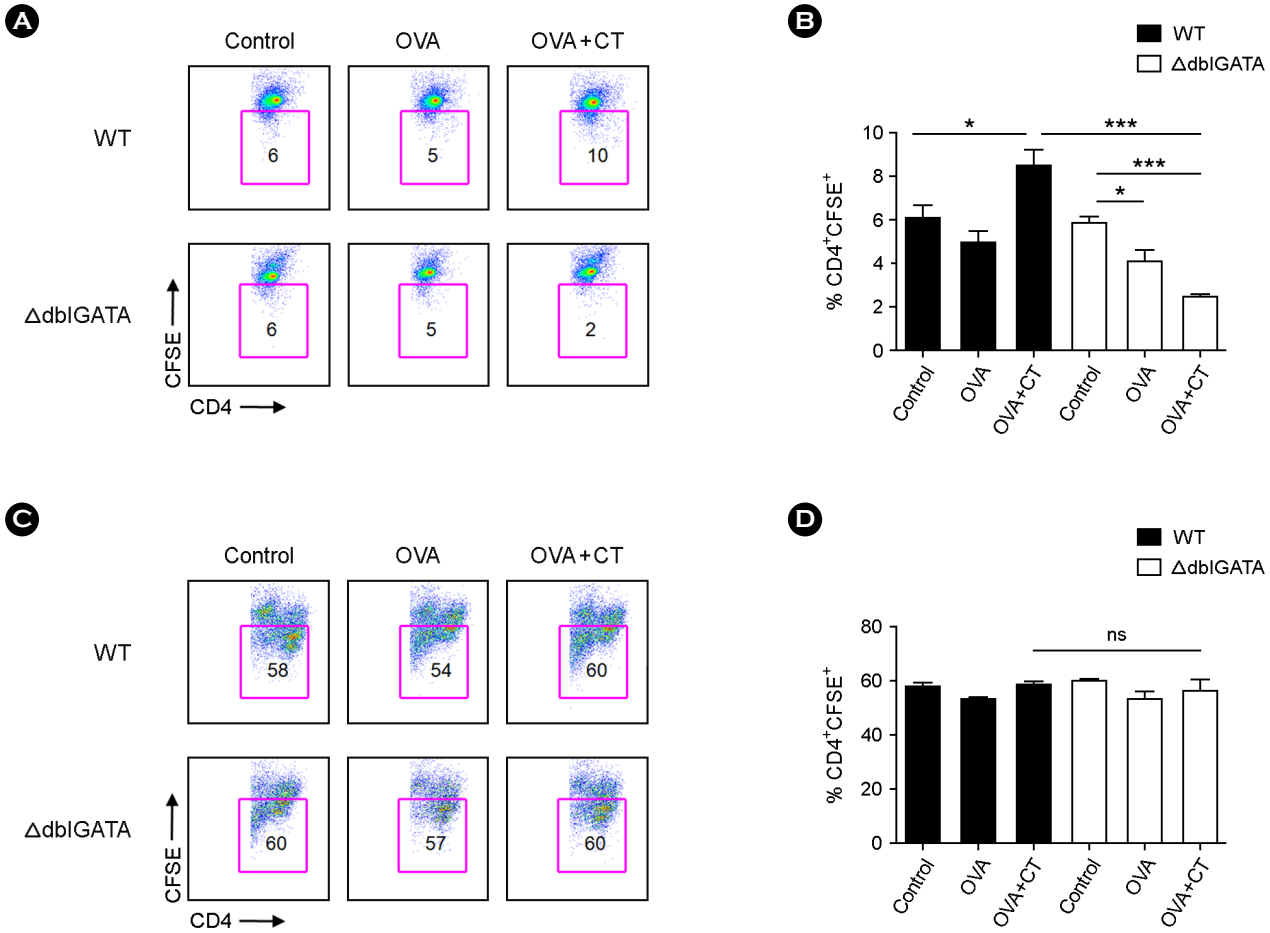

Figure 2. Reduced proliferation of CD4$^+$ T cells isolated from the mesenteric lymph node (MLN) of ΔdblGATA mice after oral immunization with ovalbumin (OVA) and cholera toxin (CT). (A and B) CD4$^+$ T cells isolated from the MLN of WT or ΔdblGATA mice after oral immunization with PBS (control), OVA or OVA+CT were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and co-cultured with OVA-loaded APC for 72 h. Representative flow cytometry analysis of CD4$^+$CFSE$^+$ cells (A). (B) CD4$^+$ T cell proliferation presented as percentage of CD4$^+$CFSE$^+$ cells. (C and D) CD4$^+$ T cells isolated from the MLN of WT or ΔdblGATA mice after oral immunization with PBS, OVA or OVA+CT were labeled with CFSE and cultured for 72 h with anti-CD3 and anti-CD28 antibodies. Representative flow cytometry analysis of CD4$^+$CFSE$^+$ cells (C). (D) CD4$^+$ T cell proliferation presented as a percentage of CD4$^+$CFSE$^+$ cells. Data are mean ± s.e.m. values. *$p < 0.05$, ***$p < 0.001$ (Student's *t*-test).

eosinophils and their definitive phenotypes have been reported (8, 9, 23), enabling us to examine functional properties of eosinophils in the GI tract. In the present study, we demonstrated that eosinophils are required for mucosal production of antigen-specific IgA and IgG1 following oral immunization of protein antigen with CT adjuvant. The proliferation capacity of CD4$^+$ T cells purified from the MLN of eosinophil-deficient ΔdblGATA mice was reduced compared to that of WT mice, which could contribute to the reduced levels of antigen-specific antibodies in ΔdblGATA mice upon oral immunization.

The intestinal immune system protects against microbial pathogens and maintains a homeostatic interaction with the dense community of commensal bacteria. In this environment, intestinal IgA neutralizes pathogenic toxins and microbes in a non-inflammatory manner owing to its inability to activate the complement cascade, thereby promoting both immune protection and intestinal homeostasis (24). Intestinal

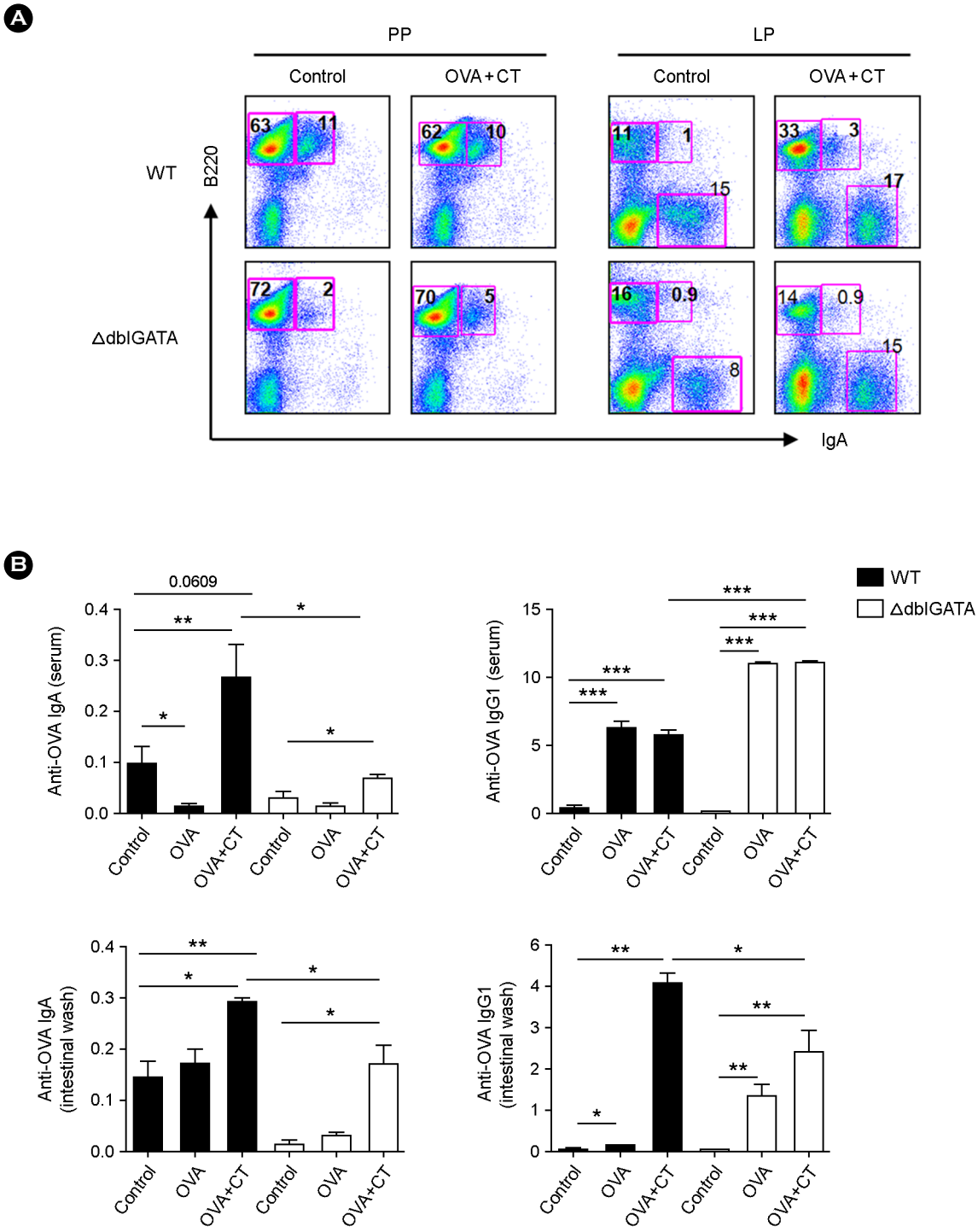

Figure 3. OVA-specific intestinal IgA and IgG1 are reduced in ΔdblGATA mice orally immunized with OVA plus CT. (A) The frequencies of IgA^+ cells in the Peyer's patch (PP) and small intestinal LP of WT and ΔdblGATA mice after oral immunization with PBS (control) or OVA+CT. The results shown are representative of three independent experiments. (B) OVA-specific IgA and IgG1 titers in serum and intestinal wash of WT and ΔdblGATA mice after oral immunization with PBS (control), OVA or OVA+CT. Each group consists of at least three individual mice. The absorbance at 450 nm represents antibody titers. Data are mean ± s.e.m. values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test).

IgA is synthesized through several mechanisms including natural, T cell-dependent, and T cell-independent pathways (11, 25). T cell-dependent IgA production is initiated in the organized tissue of PP, MLN, and isolated lymphoid follicles as antigen-loaded DC activate $CD4^+$ T cells (11). B cells are switched from the IgM to the IgA isotype under the influence of activated T cells, and cytokines (14, 26). Production of IgA upon oral immunization with CT adjuvant is a typical T cell-dependent response to the dependence on the CD40 signals of $CD4^+$ T cells. Accordingly, these results constitute support for the necessary role of eosinophils in T cell-dependent antibody synthesis induced by mucosal immunization. Although few eosinophils are present in PP under the healthy state, Th2-associated conditions induce accumulation of a considerable number of eosinophils in the outer cortex of PP (27). Therefore, it is plausible that eosinophils are traffic to the PP and contribute to IgA switching upon oral immunization with OVA and CT, since CT acts as a mucosal adjuvant inducing antigen-specific Th2-type response in the PP. In T cell-dependent IgA synthesis, eosinophils are unlikely to function as APC, since LP eosinophils express only negligible levels of CD86 and MHC class II (28). Instead, we speculate that eosinophils contribute to IgA synthesis by optimizing proliferation of IgA-secreting plasmablasts in the small intestinal LP. IgA-producing precursors induced in the PP migrate to the LP of the small intestine through DC-mediated up-regulation of gut-homing receptors (29). It is generally accepted that, after antigenic stimulation in the PP, IgA^+B220^+ plasmablasts home to the LP of the small intestine (30). Migrated IgA-producing precursors repopulate in the LP, where they fully differentiate into IgA-producing plasma cells (31). In fact, IgA-producing plasma cells can be generated in the LP independent of T cells under the influence of several factors and cytokines, such as transforming growth factor (TGF)-β, tumor-necrosis factor family (BAFF), proliferation-inducing ligand (APRIL), and IL-6 produced by DC and intestinal epithelial cells (32~34). Therefore, expansion of IgA-committed plasma cell precursors in the small intestinal LP following T cell-dependent class switching in the PP has been suggested as the T cell-independent second phase of IgA production (31). In line with this idea, IgA^+B220^+ cells induced in the LP of WT mice upon oral immunization with OVA and CT were markedly decreased in ΔdblGATA mice (Fig. 3A). As previously demonstrated, eosinophils in the bone marrow synthesize APRIL and IL-6, which supports survival of plasma cells and the long-term maintenance of protective antibody titers (35). Furthermore, eosinophils are constitutively located in the intestinal LP for almost 30% of isolated cells there, and small intestinal eosinophils produce IL-1β, which enables the intestinal microenvironment to be more favorable for IgA class switching (15). Thus, eosinophils might play a role in the secondary expansion and differentiation of IgA-producing cells that have undergone class switching in organized lymphoid organs in response to antigen challenge, which would explain why OVA-plus-CT oral immunization still generated certain levels of mucosal anti-OVA-specific IgA and IgG1 in ΔdblGATA mice, albeit at lower levels than in WT mice.

MLN $CD4^+$ T cells isolated from ΔdblGATA mice, orally immunized with OVA and CT, showed defective proliferation against *in vitro* OVA stimulation. The preferential expansion of antigen-specific Th2 cells has been suggested for the induction of mucosal antibody synthesis following oral protein administration with adjuvant (12). However, MLN T cells taken soon after oral immunization harbor undifferentiated precursors of helper T cells (36). Considering that the cytokine microenvironment during T cell differentiation determines the functional fate of T cells and IL-4 preferentially induces differentiation of T cells into Th2 (37, 38), it can be posited that eosinophils play a supportive role in the differentiation of functionally matured Th2 cells upon antigenic stimulation. $CD4^+$ T cell proliferation was not significantly different between WT and ΔdblGATA mice after oral immunization with OVA plus CT when cells were stimulated by anti-CD3 and anti-CD28 antibodies. As ligation of CD3/CD28 provides generalized activation signals to T cells (39), it seems plausible that $CD4^+$ T cells in ΔdblGATA mice have a diminished ability for Th2 differentiation upon oral immunization without primary defects in proliferation.

In summary, this study shows that eosinophils are required

for mucosal production of antigen-specific immune responses following oral immunization. The increasing number of recent experimental observations indicates that eosinophils are multifunctional leukocytes involved in modulation of the intestinal immune system. Further studies concerning antigen-presenting capabilities and cytokine production of stimulated eosinophils should be central to identify the role of eosinophils in mucosal immune responses stimulated by allergen challenge.

REFERENCES

1) Rothenberg ME, Hogan SP. The eosinophil. Annu Rev Immunol 2006;24:147-74.
2) Jung Y, Rothenberg ME. Roles and regulation of gastrointestinal eosinophils in immunity and disease. J Immunol 2014;193:999-1005.
3) Rothenberg ME. Eosinophilic gastrointestinal disorders (EGID). J Allergy Clin Immunol 2004;113:11-28.
4) Yang D, Chen Q, Su SB, Zhang P, Kurosaka K, Caspi RR, *et al*. Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances Th2 immune responses. J Exp Med 2008;205:79-90.
5) Hirasawa R, Shimizu R, Takahashi S, Osawa M, Takayanagi S, Kato Y, *et al*. Essential and instructive roles of GATA factors in eosinophil development. J Exp Med 2002;195:1379-86.
6) Yu C, Cantor AB, Yang H, Browne C, Wells RA, Fujiwara Y, *et al*. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage *in vivo*. J Exp Med 2002;195:1387-95.
7) Mishra A, Hogan SP, Lee JJ, Foster PS, Rothenberg ME. Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. J Clin Invest 1999;103:1719-27.
8) Carlens J, Wahl B, Ballmaier M, Bulfone-Paus S, Förster R, Pabst O. Common gamma-chain-dependent signals confer selective survival of eosinophils in the murine small intestine. J Immunol 2009;183:5600-7.
9) Verjan Garcia N, Umemoto E, Saito Y, Yamasaki M, Hata E, Matozaki T, *et al*. SIRPalpha/CD172a Regulates Eosinophil Homeostasis. J Immunol 2011;187:2268-77.
10) Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol 2003;3:331-41.
11) Fagarasan S, Kawamoto S, Kanagawa O, Suzuki K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu Rev Immunol 2010;28:243-73.
12) Elson CO, Ealding W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J Immunol 1984;132:2736-41.
13) Lycke N, Holmgren J. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. Immunology 1986;59:301-8.
14) Bergqvist P, Gärdby E, Stensson A, Bemark M, Lycke NY. Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. J Immunol 2006;177:7772-83.
15) Jung Y, Wen T, Mingler MK, Caldwell JM, Wang YH, Chaplin DD, *et al*. IL-1beta in eosinophil-mediated small intestinal homeostasis and IgA production. Mucosal Immunol 2015;8:930-42.
16) Westlund J, Livingston M, Fahlén-Yrlid L, Oldenborg PA, Yrlid U. CD47-deficient mice have decreased production of intestinal IgA following oral immunization but a maintained capacity to induce oral tolerance. Immunology 2012;135:236-44.
17) Yamamoto M, Rennert P, McGhee JR, Kweon MN, Yamamoto S, Dohi T, *et al*. Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. J Immunol 2000;164:5184-91.
18) Orr N, Robin G, Cohen D, Arnon R, Lowell GH. Immunogenicity and efficacy of oral or intranasal Shigella flexneri 2a and Shigella sonnei proteosome-lipopolysaccharide vaccines in animal models. Infect Immun 1993;61:2390-5.
19) Seiffert M, Cant C, Chen Z, Rappold I, Brugger W, Kanz L, *et al*. Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counter-receptor CD47. Blood 1999;94:3633-43.
20) Xu-Amano J, Kiyono H, Jackson RJ, Staats HF, Fujihashi K, Burrows PD, *et al*. Helper T cell subsets for immuno-

globulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. J Exp Med 1993; 178:1309-20.
21) Cho KA, Suh JW, Sohn JH, Park JW, Lee H, Kang JL, *et al*. IL-33 induces Th17-mediated airway inflammation via mast cells in ovalbumin-challenged mice. Am J Physiol Lung Cell Mol Physiol 2012;302:L429-40.
22) Chu VT, Beller A, Rausch S, Strandmark J, Zänker M, Arbach O, *et al*. Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and contribute to gut immune homeostasis. Immunity 2014;40: 582-93.
23) Wen T, Mingler MK, Blanchard C, Wahl B, Pabst O, Rothenberg ME. The pan-B cell marker CD22 is expressed on gastrointestinal eosinophils and negatively regulates tissue eosinophilia. J Immunol 2012;188:1075 -82.
24) Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. The immune geography of IgA induction and function. Mucosal Immunol 2008;1:11-22.
25) Cerutti A, Rescigno M. The biology of intestinal immunoglobulin A responses. Immunity 2008;28:740-50.
26) Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA *in vivo*. Immunity 2000;13:443-51.
27) Mishra A, Hogan SP, Brandt EB, Rothenberg ME. Peyer's patch eosinophils: identification, characterization, and regulation by mucosal allergen exposure, interleukin-5, and eotaxin. Blood 2000;96:1538-44.
28) Uematsu S, Fujimoto K, Jang MH, Yang BG, Jung YJ, Nishiyama M, *et al*. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol 2008;9: 769-76.
29) Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, *et al*. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 2006;314:1157 -60.
30) Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. Nature 2001;413:639-43.
31) Yuvaraj S, Dijkstra G, Burgerhof JG, Dammers PM, Stoel M, Visser A, *et al*. Evidence for local expansion of IgA plasma cell precursors in human ileum. J Immunol 2009;183:4871-8.
32) He B, Raab-Traub N, Casali P, Cerutti A. EBV-encoded latent membrane protein 1 cooperates with BAFF/BLyS and APRIL to induce T cell-independent Ig heavy chain class switching. J Immunol 2003;171:5215-24.
33) Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, *et al*. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol 2002;3:822-9.
34) Xu W, He B, Chiu A, Chadburn A, Shan M, Buldys M, *et al*. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. Nat Immunol 2007;8:294-303.
35) Chu VT, Fröhlich A, Steinhauser G, Scheel T, Roch T, Fillatreau S, *et al*. Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nat Immunol 2011;12:151-9.
36) Schaffeler MP, Brokenshire JS, Snider DP. Detection of precursor Th cells in mesenteric lymph nodes after oral immunization with protein antigen and cholera toxin. Int Immunol 1997;9:1555-62.
37) Demeure CE, Yang LP, Byun DG, Ishihara H, Vezzio N, Delespesse G. Human naive CD4 T cells produce interleukin-4 at priming and acquire a Th2 phenotype upon repetitive stimulations in neutral conditions. Eur J Immunol 1995;25:2722-5.
38) Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc Natl Acad Sci U S A 1993;90:10188-92.
39) Thompson CB, Lindsten T, Ledbetter JA, Kunkel SL, Young HA, Emerson SG, *et al*. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. Proc Natl Acad Sci U S A 1989; 86:1333-7.