A novel IL-10-producing innate lymphoid cells (ILC10) in a contact hypersensitivity mouse model

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The immunoregulatory cytokine Interleukin 10 (IL-10) protein is produced by various cells during the course of inflammatory disorders. Mainly, it downregulates pro-inflammatory cytokines, antigen presentation, and helper T cell activation. In this study, we show that the ratio of IL-10-producing cells was significantly increased in lineage negative (i.e., not T, B, or leukocyte cell lineages) cells than in lineage positive cells in lymphoid and peripheral tissues. We further observed that IL-10-producing innate lymphoid cells (ILCs), here called firstly ILC10, were increased in number in oxazolone-induced contact hypersensitivity (CHS) mice. In detail, IL-10-producing lineage negative cells were elevated in the axillary, inguinal lymph node, and ear tissues of CHS mice. Notably, the cells expressed classical ILC marker proteins such as CD45, CD127, Il10, and Sca-1. Altogether, our findings suggest for the first time that ILC10s are present in various physiological settings and could be involved in numerous immune responses as regulatory cells. [BMB Reports 2016; 49(5): 293-296]

INTRODUCTION

Recent studies have shown that innate lymphoid cell (ILC) subsets participate in intestinal homeostasis, lymphoid organogenesis, cytotoxicity, tissue remodeling during wound healing, and early immune responses against foreign antigens, including various pathogens. Although the cells were initially identified as potent effector immune cells in the process of various immune diseases, other subsets were additionally recognized as regulatory cells (10, 12). In particular, some regulatory roles are dependent on IL-10 in Th1, regulatory T cells (Tr1), regulatory B10 cells, myeloid-derived suppressor cells, or type 2 macrophages (M2) (12-23). However, to the best of our knowledge, there is no previously published report on the presence of regulatory subsets of ILCs.

RESULTS AND DISCUSSION

In general, it is well established that T cells, B cells, and other leukocytes act as effector cells to protect the host from foreign antigens, including various pathogens. Although the cells were initially identified as potent effector immune cells in the process of various immune diseases, other subsets were additionally recognized as regulatory cells (10, 12). In particular, some regulatory roles are dependent on IL-10 in Th1, regulatory B10 cells, myeloid-derived suppressor cells, or type 2 macrophages (M2) (12-23). However, to the best of our knowledge, there is no previously published report on the presence of regulatory subsets of ILCs.

Keywords: Contact hypersensitivity, ILC10, Innate lymphoid cells (ILCs), Interleukin-10, regulatory ILCs
We hypothesized that IL-10-producing ILC10s may exist in various immune tissues. In this study, we tested first whether or not IL-10-producing cells would exist in lineage negative cells, which excludes T cells (CD3\(^+\)), B cells (CD45R\(^+\)), monocytes (CD11b\(^+\)), erythrocytes (TER-119\(^+\)), and neutrophils (Gr-1\(^+\)), in control or CHS mice. Interestingly enough, we observed that an IL-10\(^+\)Lin\(^-\) subset was definitely present, albeit rarely, in the spleen, axillary lymph node (aLN), inguinal lymph node (iLN), and ear (Fig. 1A). Furthermore, the ratio of IL-10\(^-\)IL-10\(^+\) cells was significantly higher in Lin\(^-\) than in Lin\(^+\) cells from second lymphoid tissues such as spleen, aLN, and iLN, but not ear tissue in control (ACE) mice. We also found that the ratio of IL-10\(^-\)IL-10\(^+\) Lin\(^-\) cells in aLN and ear tissues significantly increased in CHS mice (Figs. 1A and 1B). Accordingly, the frequency and number of IL-10\(^+\)Lin\(^-\) cells were substantially increased in the aLN, iLN, and ear, whereas the spleen was unaffected (Fig. 2), suggesting that mostly local drained LN IL-10\(^-\) Lin\(^-\) cells, but not those from the spleen, are associated with inflammation in CHS mice.

The above results prompted us to investigate whether phenotypically IL10\(^+\)Lin\(^-\) cells contain a new subset of ILCs (24). Notably, some subsets of IL-10\(^+\)Lin\(^-\) cells expressed several typical ILC markers such as CD45, CD127, and Sca-1, but CD25 was expressed in a low amount. We also observed that the expression of Sca-1 was increased more in IL-10\(^-\)Lin\(^-\) cells than in IL-10\(^+\)Lin\(^-\) cells, but did not detect significant differences in CD45 and CD127 expression between IL-10\(^-\) and IL-10\(^+\)Lin\(^-\) cells (Fig. 2A). We also observed no phenotypical difference in CD25 expression on the spleen, aLN, and iLN-derived IL-10\(^+\) or IL-10\(^-\) Lin\(^-\) cells. However, compared to IL-10\(^-\) Lin\(^-\) cells, the expression of CD25 was markedly increased in IL-10\(^-\)Lin\(^-\) cells in CHS-induced ear tissues (Fig. 3). Finally, we found that the number of IL-10\(^-\) ILCs (ILC10, Lin CD45\(^+\)CD127\(^+\)Sca-1\(^-\)) was significantly increased in the spleen, aLN, iLN, and ear in CHS mice (Fig. 4), suggesting that IL-10-producing ILC10s are associated with regulation of CHS inflammation. Given, particularly, the critical role of T cells in the progress of CHS inflammation (25), it would be of interest to determine whether ILC10s could regulate T cells under pathological circumstances.

In summary, for the first time we identified the presence of a IL-10\(^+\) ILC (Lin CD45\(^+\)CD127\(^+\)Sca-1\(^-\)) subset in lymphoid

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**Fig. 1.** IL-10 expression in lineage-negative cells in control or contact hypersensitivity mice. (A) Two days after being challenged with oxazolone, the frequency of lineage (Lin\(^+\)) or IL-10\(^+\) or cells in spleen, aLN, iLN, and ear tissues was measured as described in "Materials and Methods." (B) The ratio of IL-10\(^+\)IL-10\(^+\) in Lin\(^+\) or cells from contact hypersensitivity mice. Plots in (A) are representative images and data in (B) are the mean ± SEM from three independent experiments. *P < 0.05; **P < 0.01; n.s., not significant. Oxz, oxazolone; ace, acetone; aLN, axillary lymph node; iLN, inguinal lymph node.

**Fig. 2.** IL-10 expression in lineage-negative cells from various tissues in the contact hypersensitivity mouse model. (A) Shown are the representative gating strategies for flow cytometry analysis of splenic IL-10\(^+\)Lin\(^-\) cells from contact hypersensitivity mice. (B) The frequency and count of IL-10\(^+\)Lin\(^-\) cells were determined by flow cytometric analysis as described in "Materials and Methods." Data are the mean ± SEM from three independent experiments. *P < 0.05; **P < 0.01; n.s., not significant. Oxz, oxazolone; ace, acetone; aLN, axillary lymph node; iLN, inguinal lymph node.
tissues, spleen, LN, and ear tissue, which we call ILC10. We further suggest that ILC10s could regulate some inflammatory immune responses in contact hypersensitivity and possibly other inflammatory diseases. Further studies are necessary to evaluate the physiology of ILC10s in various immune processes.

MATERIALS AND METHODS

Mice
C57BL/6 mice were purchased from Orientbio (Seongnam, Korea) and housed in a specific pathogen-free animal facility at Konkuk University (Seoul, Korea) with a sterilized diet and autoclaved water, unless indicated otherwise. The mice used were between 6 and 8 weeks of age. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

Induction of contact hypersensitivity in mice
Contact Hypersensitivity (CHS) was induced according to a previously reported method (18). Briefly, mice were sensitized with 25 μl of Oxazolone (100 mg/ml, Sigma, St. Louis, MO) in Acetone/Olive Oil (4:1 v/v) on the shaved hind flank for 2 consecutive days. Five days later, mice were challenged by the application of 10 μl of oxazolone (10 mg/ml) on each ear. Two days after they were challenged with Oxazolone, single cells were isolated for flow cytometric analysis.

Flow cytometric analysis
Single-cell suspensions were prepared from the spleen, axillary, inguinal lymph node, and ear tissues. To detect intracellular IL-10 in lineage negative (Lin-1) or Lin-2CD45+ cells from each tissue type, isolated cells were re-suspended and incubated with Phorbol 12-Myristate 13-Acetate (PMA, 50 ng/ml; Sigma), Ionomycin (500 ng/ml; Sigma), and Brefeldin A (3 mg/ml; eBioscience, San Diego, CA, USA). Before cell surface markers were stained, Fc receptors were blocked with Anti-CD16 and Anti-CD32 monoclonal antibodies (2.4G2, BD Biosciences). The antibodies against cell surface proteins were as follows: mouse hematopoietic lineage (anti-CD3 (17A2), anti-CD45R (B220, RA3-6B2), anti-CD11b (M1/70), anti-Ter-119 (TER-119), and anti-Ly-G6 (Gr-1, RB6-8C5)), anti-CD127 (IL-7Rα, A7R34), Sca-1 (Ly-6A/E, Ly-6C/E, or Ly-6E/C), and Anti-IL-10 (JES5-315, eBioscience).
D7), and anti-CD25 (PC61.5), which were purchased from eBioscience, and anti-CD45 (30-F11), which was purchased from BD Biosciences. Cells were fixed and permeabilized with a Cytofix/Cytoperm kit (eBioscience) and then were incubated with anti-IL-10 monoclonal antibody (JES5-16E3, eBioscience) at 4°C for 30 min. Cells were analyzed with FACSaria or FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and FlowJo Version 10 software (TreeStar).

Statistical analysis

Data are expressed as the mean ± SEM from at least three independent experiments. Statistical analysis was performed using one-way ANOVA and the Student’s t-test for unpaired values unless stated otherwise. Statistical significance (*P < 0.05 and **P < 0.01) was performed using the software SigmaStat (Systat Software, Inc., San Jose, CA, USA).

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