Characterization and TCR variable region gene use of mouse resident nasal γδ T lymphocytes

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Abstract: Tissue-resident γδ T lymphocytes, such as dendritic epidermal T cells, intestinal intraepithelial lymphocytes (IEL), and resident pulmonary lymphocytes, are known to support local tissue homeostasis and host defense. Inhaled antigens, toxins, and microorganisms first interact with the immune system through contact with the nasal mucosa. Herein, we characterized two populations of resident nasal lymphocytes (RNL) that are present in the nasal mucosa: nasal IEL (nIEL) and nasal lamina propria lymphocytes (nLPL). γδ TCR$^+$ and αβ TCR$^+$ nIEL and nLPL were detected by immunofluorescent staining. Mononuclear cells (5–15%) were CD3$^+$ RNL by FACS analysis. Among the CD3$^+$ RNL, 20–30% were GL3$^+$ γδ T cells, which were double-negative for CD4 and CD8 and predominantly expressed a Vγ4/Vδ1 TCR. These results demonstrate that RNL might be crucial for the host defense and tissue homeostasis in the nasal mucosa. *J. Leukoc. Biol.* 84: 1259–1263; 2008.

Key Words: *resident nasal lymphocyte · nasal intraepithelial lymphocyte · nasal lamina propria lymphocyte*

Nasal mucosa, one of the upper respiratory tract mucosal systems, is the first site of interaction among inhaled antigens, toxins, or microorganisms and the immune system. Defense mechanisms in the nasal mucosa are regulated by well-organized humoral and cell-mediated immune responses, which are mediated by immunocompetent cells such as B and T lymphocytes. There are tissue-resident γδ T lymphocytes in the skin, intestine, and lung [1, 2]. We have shown that dendritic epidermal T cells (DETC) conduct critical functions in regulating skin homeostasis and wound repair [3, 4]. Others [5, 6] have reported that resident pulmonary lymphocytes residing in the lower airway (lung) regulate airway hyper-reactivity. However, little is known about lymphocytes residing in the nasal mucosa, resident nasal lymphocytes (RNL) [7, 8], which can be classified according to their location of residence: epithelium [nasal intraepithelial lymphocyte (nIEL)] and lamina propria [nasal lamina propria lymphocyte (nLPL)]. So far, there has been no report that characterizes nIEL and nLPL and moreover, variable region gene use of RNL. We wanted to characterize mouse RNL after isolation of lymphocytes from each compartment of the nasal mucosa, epithelium versus lamina propria. Moreover, we investigated the variable region genes that were used by RNL at the message and protein levels.

First, to localize resident T lymphocytes in the mouse (C57BL/6 mice) and human nasal mucosa, immunofluorecent staining of nasal mucosa frozen sections with a mAb to the γδ TCR (GL3) and a mAb to CD3 (2C11) was conducted. We observed γδ TCR and CD3 double-positive T lymphocytes (**Fig. 1, A** and **B**) and γδ TCR-negative, CD3-positive αβ T lymphocytes (Fig. 1, A and C) in mouse nasal mucosa. After informed consent, we harvested normal inferior turbinate mucosa from three patients who received septoplasty. Immunofluorescent staining was performed on frozen sections using a mouse mAb to the human γδ TCR (B1, BD PharMingen, San Diego, CA, USA). In human nasal turbinate mucosa, we observed the nIEL and nLPL, which expressed γδ TCR (Fig. 1D). nIEL were distributed throughout the surface epithelium but were found mainly near the basement membrane.

To characterize the mouse RNL, we harvested mononuclear cells (up to 5×10^5 cells/mouse) with 2% collagenase-D. Flow cytometry was performed with a mAb to γδ TCR and CD3 using collagenase-harvested mononuclear cells. After gating for lymphocytes using forward- and side-scatter, 5–15% of mononuclear cells were CD3$^+$ RNL. Among the CD3$^+$ RNL, 20–30% (26.4±5.9%) were GL3$^+$ γδ T lymphocytes (**Fig. 2A**). αβ T cells in nasal mucosa were 70–80% of CD3$^+$ cells. We confirmed the population of αβ T cells in nasal mucosa with flow cytometry using a mAb to the αβ TCR (H57−597; data not shown). It is well known that the proportion of γδ T cells in peripheral blood or spleen is below 3% of total CD3$^+$ T lymphocytes in a wild-type mouse under normal conditions [9]. However, like other mucosal tissues, RNL contained a higher percentage of γδ T lymphocytes than in peripheral blood, which suggests that γδ T lymphocytes may play an important role in local immune responses in the nasal mucosal system. Transcardiac perfusion was performed to remove most of the blood cells from the tissue prior to collagenase treatment to exclude the possibility of "blood contamination" in the RNL cell preparations. Previous reports about RNL used an enzymatic isolation method that harvests all lymphocytes residing

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Received January 18, 2008; revised June 23, 2008; accepted July 2, 2008.
doi: 10.1189/jlb.0108050

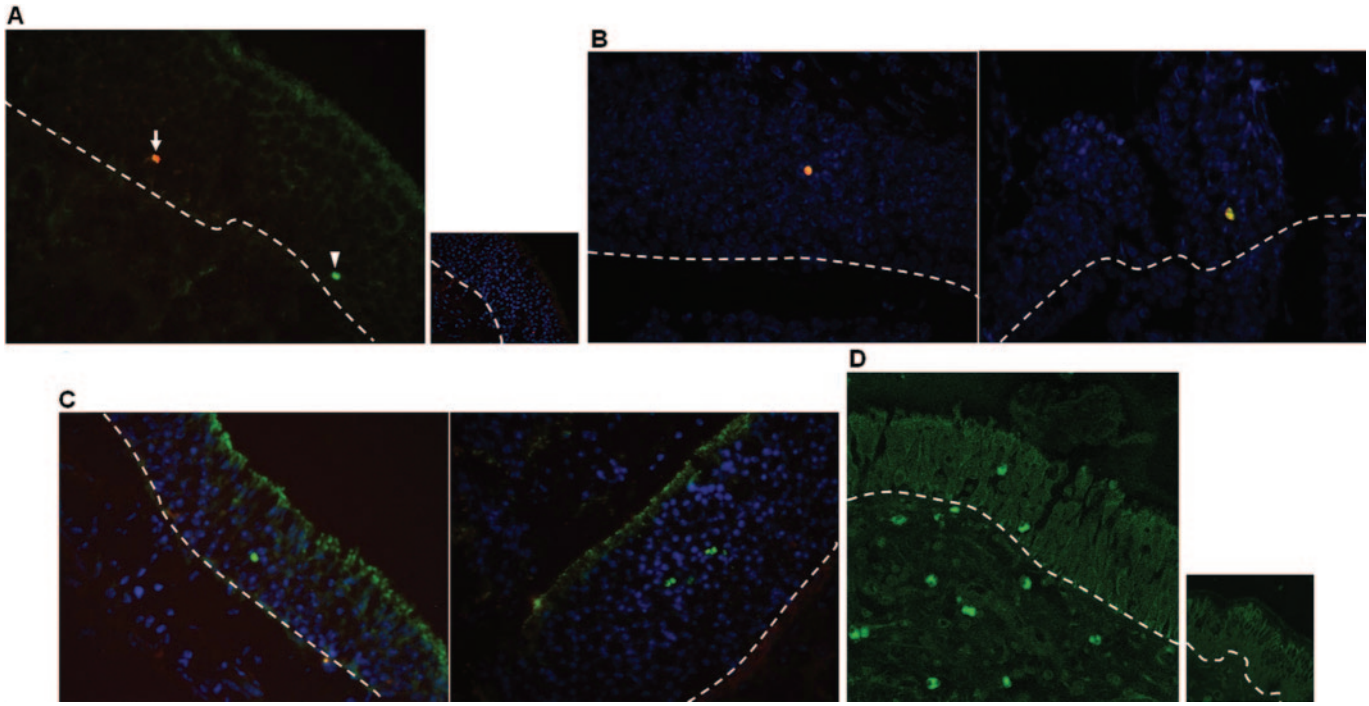

Fig. 1. Resident γδ and αβ T lymphocytes in mouse and human nasal mucosa. Frozen sections of mouse nasal mucosa (C57BL/6, female, 6–8 weeks of age) were stained with mAb to the γδ TCR (GL3; PE-labeled) and CD3 (2C11; FITC-labeled). (A) The orange-colored cell (arrow) is the γδ T lymphocyte, which is positive for CD3 and GL3, and the green-colored cell (arrowhead) is the αβ T lymphocyte, which is positive only for CD3. The small figure is for negative control. (B) Yellow-to-orange-colored cells are γδ T lymphocytes, which are positive for CD3 and GL3. (C) Green-colored cells are αβ T lymphocytes, which are positive only for CD3. (D) Frozen sections of human nasal mucosa were stained with a FITC-conjugated mAb to the γδ TCR (B1). We observed the nIEL and nLPL, which expressed γδ TCR. Dotted lines depict the basement membrane of the skin. Original magnification, ×400.

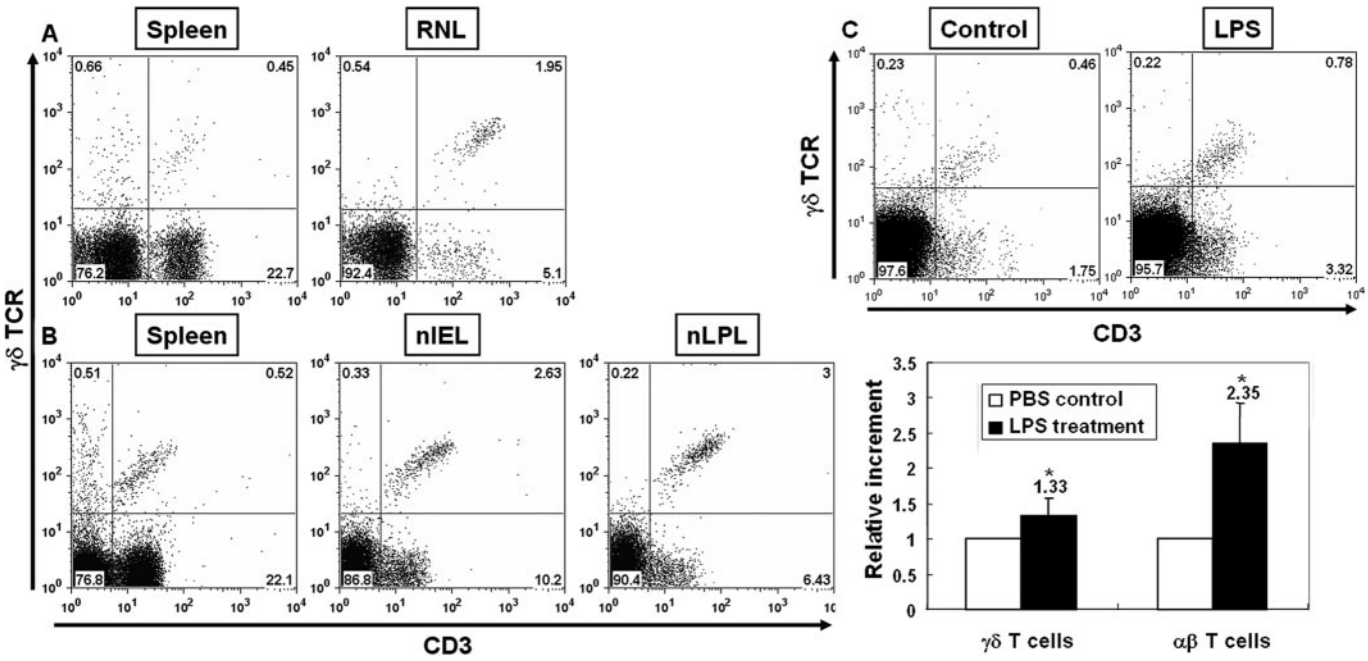

Fig. 2. γδ T lymphocyte population in RNL and nIEL versus nLPL. We harvested mononuclear cells (up to 5×10^5 cells/mouse) with collagenase. (A) Flow cytometric analysis with mAb to the γδ TCR (GL3) and CD3 (2C11) was performed on RNL. Cells were gated on live mononuclear cells (5–15%), which were $CD3^+$ RNL. Among the $CD3^+$ RNL, 20–30% (26.4±5.9%) were $GL3^+$ γδ T cells. (B) Flow cytometric analysis with mAb to the γδ TCR and CD3 was performed after isolation of nIEL and nLPL. γδ T cells were 20.7 ± 7.2% in nIEL and 25.5 ± 6.4% in nLPL. (C) Following LPS treatment (25 μg/day at a concentration of 250 μg/ml; from *Escherichia coli* serotype 0565:B5) via intranasal instillation for 3 days, γδ TCR^+ cells increased 1.33 ± 0.27 times, and αβ TCR^+ cells increased 2.35 ± 0.58 times. These differences were statistically significant (*, $P<0.05$). Data represent four to six independent experiments with four mice per experiment.

in epithelium and lamina propria of the nasal mucosa [7, 8]. To analyze nIEL and nLPL separately, we first isolated the nIEL with 0.5 M DTT/EDTA, followed by isolation of the nLPL with 2% collagenase-D. RNL were successfully isolated from each compartment. Epitope resistance was verified after DTT/EDTA and collagenase treatment (data not shown). Flow cytometric analysis was also performed after isolation of nIEL and nLPL (Fig. 2B). nLPL (25.5±6.4%) exhibited significantly ($P<0.02$) more γδ T lymphocytes than nIEL (20.7±7.2%). To study changes of the RNL population after local nasal infection, we mimicked nasal infection with intranasal LPS instillation. Mice were treated with LPS (25 μg/day at a concentration of 250 μg/ml; from *E. coli* serotype 0565:B5) via intranasal instillation for 3 days and harvested RNL on the following day. The nasal lymphocyte population of the LPS-treated group was compared with the PBS-control-treated group by FACS analysis with mAb to CD3 and γδ TCR. There was a substantial increase in γδ and αβ TCR^+ cells following LPS treatment. γδ TCR^+ cells increased 1.33 ± 0.27 times, and αβ TCR^+ cells increased 2.35 ± 0.58 times in the LPS-treated group (Fig. 2C). These differences were statistically significant ($P<0.05$). This increase in T cells in the nasal mucosa could be a result of local proliferation or recruitment of T cells to the site of infection. Both of these have been shown to occur in other mucosal tissues, such as the intestine, in response to infection [10].

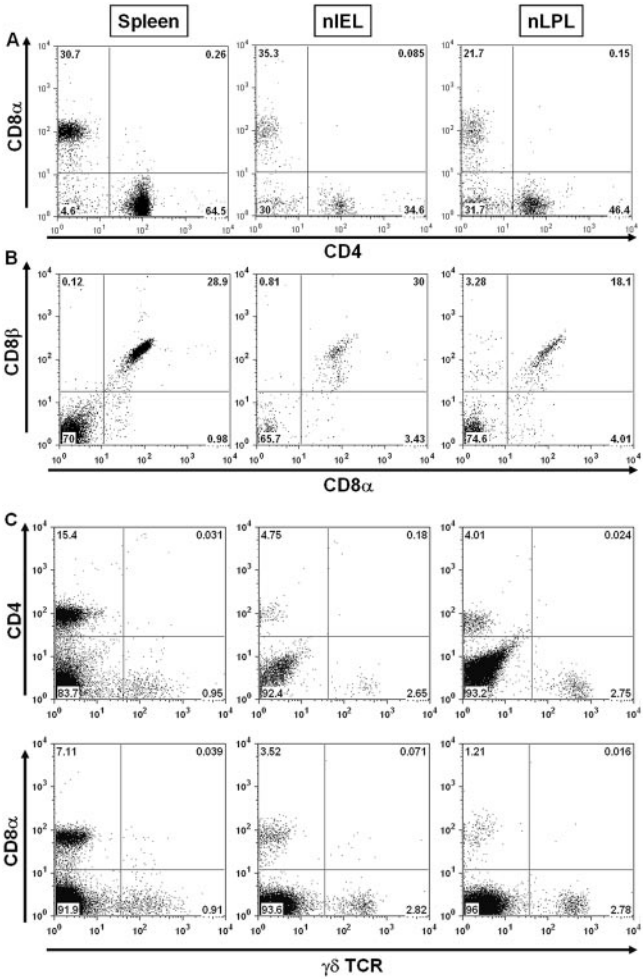

Fig. 3. Flow cytometric analysis of CD4 and CD8 expression in nIEL versus nLPL. To analyze the CD4 and CD8 coreceptor expression in nasal resident αβ T lymphocytes, we gated the mononuclear cells with TCR-β (A and B). (A) The CD4:CD8 ratio was 1.38:1 in nIEL (relatively large $CD8^+$ population) and 2.75:1 in nLPL. (B) The proportion of $CD8^+$ αβ T lymphocytes was much higher in nIEL (25.9±3.7%) than nLPL (13.3±4.2%). (C) $GL3^+$ γδ T cells were double-negative for CD4 and CD8 in nIEL and nLPL. Data represent four to six independent experiments with four mice per experiment.

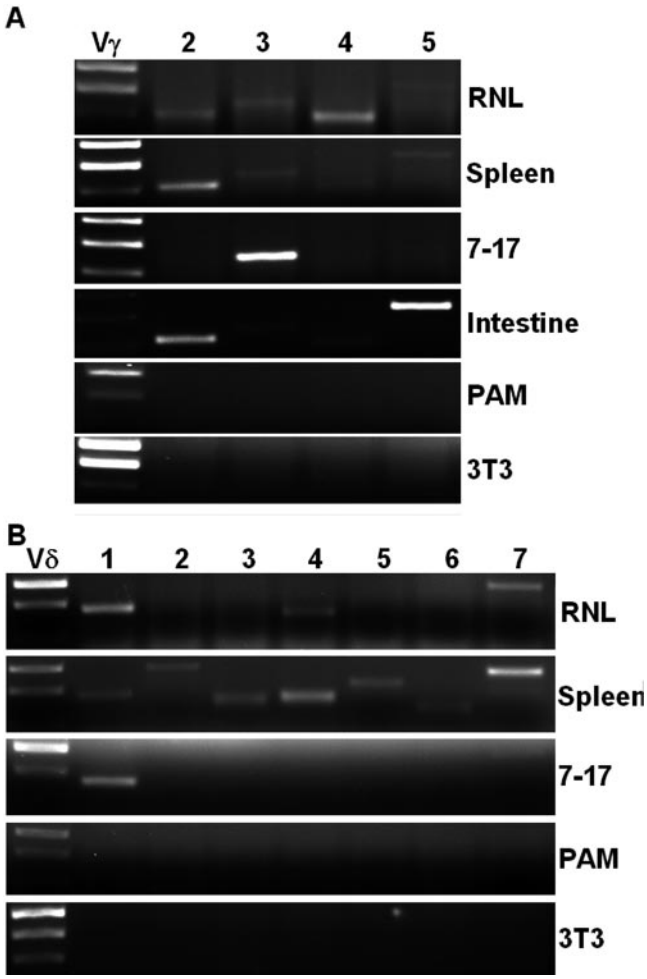

Fig. 4. Variable region gene use of RNL by RT-PCR. The sequences for primers for each of the Vγ/Jγ1 and Vδ/Jδ1 genes were used as published previously [12, 18, 19]. (A) RNL predominantly expressed Vγ4 and also expressed Vγ2 and Vγ3 TCR. (B) RNL predominantly expressed Vδ1 TCR and also expressed Vδ4 and Vδ7 TCR. Splenocytes, 7-17 cells (DETC cell line), intestinal IEL (iIEL), PAM 2-12 keratinocyte cell line, and 3T3 fibroblast cell line were used for positive and negative controls. Data represent four to six independent experiments with four mice per experiment.

To study the expression of the CD4 and CD8 coreceptors in αβ and γδ TCR^+ RNL, we performed flow cytometric analysis with mAb to CD4 (GK1.5), CD8α (53-6.7) and -β (Ly3.2), αβ TCR (H57-597), and γδ TCR (GL3) of nIEL and nLPL. In the αβ TCR^+ cells, the CD4:CD8 ratio was 1.38:1 in nIEL and 2.75:1 in nLPL as a result of a relatively large $CD8^+$ population in nIEL (**Fig. 3A**). CD8α and CD8β staining (Fig. 3B) confirmed the higher proportion of $CD8^+$ cells in nIEL (25.9±3.7% of αβ TCR^+ cells) compared with nLPL (13.3±4.2% of αβ TCR^+ cells). This low CD4:CD8 ratio in

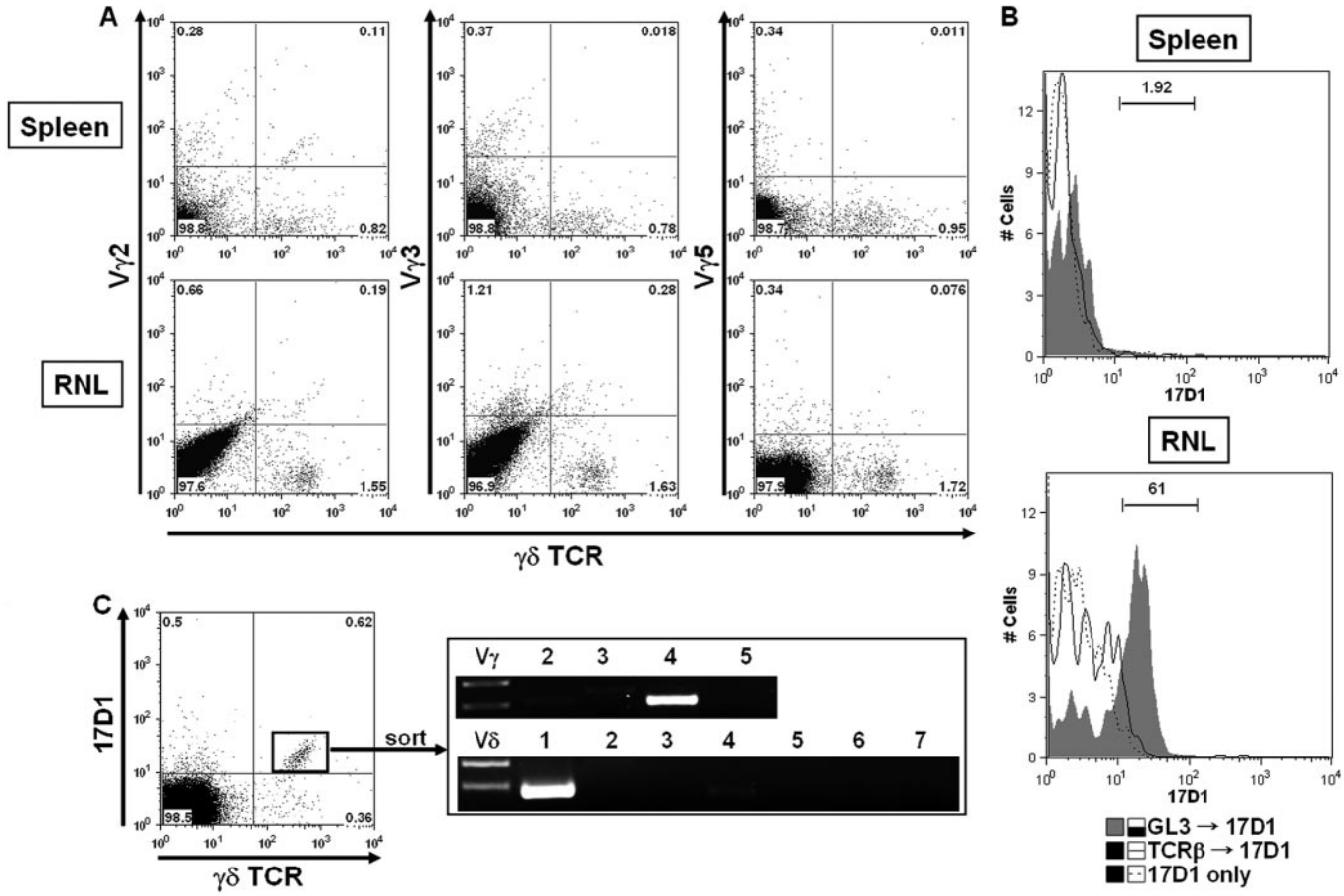

Fig. 5. Variable region gene use of RNL by flow cytometry. (A) RNL infrequently expressed variant Vγ2 and Vγ3 TCR, which is compatible with PCR data. (B) Flow cytometric analysis was performed with 17D1 antibody in GL3$^+$ RNL. 17D1 antibody is known to bind to invariant Vγ3/Vδ1 TCR and also bind to invariant Vγ4/Vδ1 TCR only after incubation with a mAb to the γδ TCR (GL3). Sixty-one percent of γδ TCR$^+$ RNL bound to 17D1 antibody only after incubation with GL3 antibody. RNL barely bound to 17D1 without any preincubation or after incubation with irrelevant antibody (TCR-β). These results indicate that 17D1-reactive cells were mostly Vγ4/Vδ1 TCR$^+$ cells, not Vγ3/Vδ1 TCR$^+$ cells. Spleen cells were used as a control. (C) To confirm the Vγ/Vδ gene use of the 17D1-positive cell population in our RNL system, RT-PCR was conducted on FACS-sorted, 17D1-positive RNL after GL3 preincubation. In this experiment, we found that this 17D1-positive population predominantly expressed Vγ4 and Vδ1 TCR. Data represent four to six independent experiments with four mice per experiment.

nIEL is compatible with a previous report that the CD4:CD8 ratio of nasal passage lymphocytes was as low as 1.7 [8]. However, nIEL and nLPL were not separated from RNL in that report. The CD4:CD8 ratio of RNL, which are in the immune effector site, was lower than those of lymphocytes in the immune inductive sites, such as spleen or Peyer's patch (CD4:CD8 ratio=3). This high proportion of CD8$^+$ lymphocytes in RNL might implicate the important role of tissue resident CD8$^+$ lymphocytes, especially nIEL. γδ T lymphocytes in nasal mucosa were negative for CD4 and CD8 in nIEL and nLP by flow cytometry (Fig. 3C). This result is in accordance with those of other tissue resident γδ T lymphocytes, such as skin DETC. There were no CD8αα$^+$ lymphocytes, which are known to be prevalent among intestinal IEL [11].

γδ T lymphocytes in specific anatomic locations use preferential TCR variable region genes for TCR-γ and -δ. Vγ2, Vγ3, and Vγ5 (nomenclature from Garman et al. [12]) are used predominantly by γδ T lymphocytes in peripheral lymphoid organs, skin, and small intestine, respectively [13–15]. Lung, tongue, and reproductive system γδ T cells preferentially express an invariant Vγ4/Vδ1 TCR [16, 17]. To determine the use of TCR variable region genes in γδ T lymphocytes of the mouse nasal mucosa, we conducted semiquantitative RT-PCR with RNL harvested by collagenase (**Fig. 4**). All positive controls exhibited expected Vγ gene use (spleen: Vγ2; 7-17 DETC cell line: Vγ3; iIEL: Vγ5 and Vγ2), and no bands were detected in the negative controls (PAM 2-12 keratinocyte cell line and 3T3 fibroblast cell line). RNL predominantly expressed Vγ4 and also expressed Vγ2 and Vγ3 TCR (Fig. 4A). Vδ1 TCR was preferentially used by RNL, but Vδ4 and Vδ7 TCR chains were also detected (Fig. 4B). These results suggest that resident γδ T lymphocytes in mouse nasal mucosa preferentially express a Vγ4 and Vδ1 TCR.

RT-PCR technique does not generate quantitative data, especially when comparing the message level of different genes that have different primers and PCR conditions in one sample. To confirm the TCR variable region gene use of RNL quantitatively at the protein level, we performed flow cytometry with mAb to mouse variable region genes for Vγ and Vδ TCR chains (**Fig. 5**). The results indicate that RNL do not use Vγ5 and infrequently use Vγ2 and Vγ3 TCR (Fig. 5A), which is compatible with the RT-PCR data. To study the use of Vγ4

TCR in RNL, we performed FACS analysis with the 17D1 antibody. There is no commercial antibody specific for mouse Vγ4. The 17D1 antibody is known to react with DETC-related invariant Vγ3/Vδ1 TCR [20], but this antibody can also detect Vγ4/Vδ1 TCR only after preincubation of cells with a mAb to Cδ (GL3) [21]. Although this sequential epitope alteration for Vγ4/Vδ1 TCR is not well understood, it suggests that there is an alternate structure for Vγ4/Vδ1 TCR, which is stabilized upon binding of the anti-Cδ antibody [21]. In this study, ~60% of γδ TCR^+ RNL bound to 17D1 only after incubation with anti-Cδ antibody (GL3; Fig. 5B). RNL barely bound to 17D1 without any preincubation or after incubation with irrelevant antibody (TCR-β). These results indicate that the 17D1-reactive cells were mostly Vγ4/Vδ1 TCR^+ cells, not Vγ3/Vδ1 TCR^+ cells. This result is also compatible with FACS data demonstrating that only a small number of γδ TCR^+ RNL bound to an antibody specific for the Vγ3 TCR (Fig. 5A). To confirm the Vγ/Vδ gene use of the 17D1-positive cell population in our RNL system, we conducted RT-PCR of FACS-sorted, 17D1-positive RNL after GL3 preincubation. In this experiment, we found that this 17D1-positive population predominantly expressed Vγ4 and Vδ1 TCR (Fig. 5C). Therefore, we conclude that nasal γδ T lymphocytes preferentially express a Vγ4/Vδ1 TCR.

The resident nasal lymphocytes have not been well characterized, in large part because of the limited tissue size and cell number [7, 8, 22]. In these published reports, nasal γδ T cells were increased significantly after infection or nasal allergy. However, TCR use of those γδ T cells and subset localization were not well documented. In this report, we isolated nIEL and nLPL separately and explored the TCR use of γδ T cells in nasal mucosa at the message and protein levels.

During mouse fetal thymocyte development, the second wave of TCR-expressing thymocytes expresses a canonical Vγ4 TCR paring with a canonical Vδ1 TCR [23, 24]. These invariant Vγ4/Vδ1 cells were known to migrate to the mucosa of the reproductive system, tongue, and lung, but the roles of these cells in local tissue are not well understood. This is the first report that resident nasal γδ T lymphocytes also express a Vγ4/Vδ1 TCR, and these Vγ4/Vδ1 cells comprise the majority of mouse resident nasal γδ T lymphocytes. The role of γδ T cells in the host defense is linked to the location of residence and TCR use of γδ T cells and also to external factors such as types and stages of infections. Future studies will focus on identification of the functional role of resident nasal γδ T lymphocytes, especially the Vγ4/Vδ1 subset, during host defense and innate immune responses of the nasal mucosa.

ACKNOWLEDGMENTS

This manuscript was supported by National Institutes of Health (AI-52257 to W. L. H.). This is manuscript #IMM-19139 from The Scripps Research Institute.

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