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Pleural cavity type 2 innate lymphoid cells precede Th2 expansion in murine *Litomosoides sigmodontis* infection

Alexis Boyd [a, 1], Kristin Killoran [b], Edward Mitre [b], Thomas B. Nutman [a, *]

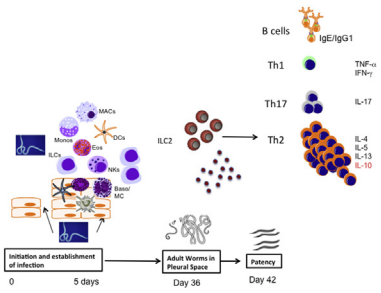
[a] *Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA*

[b] *Department of Microbiology and Immunology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd, Bethesda, MD 20892, USA*

HIGHLIGHTS

- ILC2 expansion seen in the pleural space in *Litomosoides sigmodontis* infection.
- Little to no systemic expansion of ILC2s in response to infection.
- The site-specific ILC2 expansion paralleled the localized Th2 response.

GRAPHICAL ABSTRACT



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ABSTRACT

Recently, a family of innate cells has been identified that respond to IL-25 and IL-33 in murine intestinal helminths. Termed Type 2 innate lymphoid cells (ILC2s) they facilitate the development of Th2 responses responsible for helminth clearance. We evaluated these cells in a tissue-invasive helminth model. Using *Litomosides sigmodontis* (a strong Th2 polarizing filarial infection) we observed a robust Th2 response in the pleural cavity, where adult worms reside, marked by increased levels of IL-5 and IL-13 in infected mice. In parallel, ILC2s were expanded in the pleural cavity early in the infection, peaking during the prepatent period. *L. sigmodontis* also elicits a strong systemic Th2 response, which includes significantly increased levels of IgG1, IgE and IL-5 in the plasma of infected mice. Although ILC2s were expanded locally, they were not expanded in the spleen, blood, or mediastinal lymph nodes in response to *L. sigmodontis* infection, suggesting that ILC2s function primarily at the site of infection. The increase in ILC2s in the pleural cavity and the expansion in Th2 responses indicates a probable role for these cells in initiating and maintaining the Th2 response and highlights the importance of these cells in helminth infections and their role in Th2 immunity.

1. Introduction

Recently, a family of innate cells was identified that responded to IL-25 and IL-33 in the context of murine intestinal helminth infection and facilitated the development of the Th2 immune response necessary to expel the worms. Originally termed

* Corresponding author. 4 Memorial Drive, Bethesda, MD 20892, USA.
E-mail address: tnutman@niaid.nih.gov (T.B. Nutman).

[1] Alexis Boyd is currently at the Enteric and Diarrheal Diseases Division, Bill and Melinda Gates Foundation, 440 5th Ave N, Seattle, WA 98109, USA.

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nuocytes, innate helper type 2 cells, multipotent progenitor cells and natural helper cells, 3 of the 4 cell types are now classified into a two families of cells, the multipotent progenitor cells and the rest of the cells falling into the family of innate lymphoid cells (ILCs) (Spits et al., 2013) based on analogous functions and shared surface molecule expression patterns.

ILCs are comprised of subsets termed ILC1, ILC2 and ILC3 that have specific cytokine profiles driven by discrete transcription factors (Spits et al., 2013). ILC1s have been shown to produce IL-12 primarily and rely on the transcription factor Tbet; ILC2s produce IL-13, IL-5 and some IL-4 and their differentiation is driven by GATA3; and ILC3s express Rorγt and produce IL-22 and IL-17. These ILC subsets parallel those seen among CD4+ T cells and are thought to influence Th subset differentiation (Spits et al., 2013). Based on these definitions, the ILCs identified initially in mice (Moro et al., 2010; Neill et al., 2010; Price et al., 2010) were from the ILC2 subset.

While ILC2s (Moro et al., 2010; Neill et al., 2010; Price et al., 2010) have been shown to be important for the initiation of the Th2 response to intestinal helminths, there have been no reports to date of these cells types in more systemic helminth infection nor of their presence in sites other than the skin, gastrointestinal tract or the lung proper. ILC2s have been shown to be expanded in the lung during infection with the tissue transiting helminth *Strongyloides venezuelensis*, and have been shown to produce IL-13 and IL5 in response to epithelial-cell derived IL-33 (Yasuda et al., 2012).

Here, using a model of the tissue-invasive filarial parasite *Litomosoides sigmodontis*, we investigated the presence of ILC2s within the pleural cavity, the location where adult *L. sigmodontis* worms reside.

2. Materials and methods

2.1. Parasites and mouse infection

Litomosoides sigmodontis infective stage (L3) parasites were isolated by lavage from the pleural cavity of 4 day infected *Meriones unguiculatus* jirds obtained from TRS Laboratory, Athens, GA, as previously described (Hubner et al., 2009). Wild type female BALB/c mice 6 weeks of age were infected by subcutaneous injection of 40 L3. The mice were maintained at the Uniformed Services University of the Health Sciences animal facility and all experiments were performed under a protocol approved by the Uniformed Services University Institutional Animal Care and Use Committee (Bethesda, MD). Animals were anesthetized with pentabarbitol at the end points of the experiments.

Three separate experiments were conducted. In the first experiment, 3 mice were infected and 4 were used as control mice for each time point. Spleens, whole blood, and cells from a pleural lavage were obtained at days 1, 5, 14, 42 and 60 post-infection. Whole blood and pleural lavage samples were pooled for each experimental condition at each time point, and cells were isolated from these samples as described below. For the second experiment, 5 mice were infected as above and 5 mice were used as uninfected controls for each time point; samples were collected day 5, 14, 42, and 60 post-infection. Again, spleens, whole blood, and pleural lavage fluid were collected as were mediastinal lymph nodes (MLN). Cells collected from the pleural lavage fluid and MLN were pooled for each experimental condition at each time point. Plasma was isolated from the whole blood and used for antibody and cytokine analysis (see below). The third experiment again used 5 infected and 5 uninfected mice per time point. Spleens, pleural lavage (both cells and fluid) and plasma were collected at days 36 and 42 post-infection. Pleural lavage fluid and pleural lavage cells were pooled at each time point for each experimental condition. The course of infection in the mouse and the time points taken are depicted in Fig. 1.

2.2. Cell processing

Mouse splenocytes were isolated from female wild type BALB/c mice. Briefly, spleens were taken from anesthetized mice and placed in 5 ml of RPMI (Cellgro, Manassas, VA) supplemented with 3% FBS (Valley Biomedical, Winchester, VA), 1% penicillin/streptomycin (Cellgro), 1% L-glutamine (Cellgro) and 20 mM HEPES (Cellgro). The spleens were then pushed through a 70 um filter (BD Biosciences) in a 100 mm petri dish using a syringe plunger from a 3 ml syringe (BD Biosciences) and washed with an additional 5 ml of 3% RPMI. Cells were spun at 400 rpm for 5 min at RT, resuspended in 2 ml of ACK Lysing buffer (Quality Biological, Gaithersburg, MD) and lysed for 5 min. After lysing, 5 ml of 3% RPMI was added, the cells centrifuged as before and resuspended in 10 ml of 3% RPMI for counting. Then the cells were centrifuged and stained with Violet Live/Dead (Invitrogen) stain at 1:4000, fixed for 5 min in 4% paraformaldehyde (Sigma Aldrich) and cryopreserved in 1X PBS (Quality Biological) with 10% DMSO (Sigma Aldrich). Sections of harvested mouse lungs were processed in the same manner, as were lymph nodes but without the lysing step. Additional sections of mouse lungs were fixed whole in paraformaldehyde or perfused with OTC (Sakura Finetek, Torrance, CA), frozen and stored in liquid nitrogen.

Pleural lavage cells were obtained by cutting a small window in the diaphragm and using a transfer pipette to instill 3 ml of RPMI into the cavity to collect the cells in that space. After harvesting, the pleural lavage cells were centrifuged and resuspended in 3% RPMI for counting, Live/Dead stained and cryopreserved as detailed above.

The pooled pleural lavage from uninfected or infected mice was concentrated from 15 ml to 1.5 ml each using Amicon Ultra Ultracel 3 K centrifugal filters (Millipore) and transferred to tubes for storage at −80 °C.

Whole blood was taken from each mouse by puncture of the inferior vena cava. The blood was lysed at a ratio of 1 part blood to 9 parts Immuno-lyse (1:25 dilution for 1X solution) (Beckman Coulter, Brea, CA) and vortexed for 1 min or until the solution was no longer cloudy. 2–3 volumes of 1X PBS was added and samples were centrifuged at 400 rpm for 5 min. Supernatants were aspirated, and the cells resuspended in 3% RPMI for counting, staining with Live/Dead (Invitrogen) stain and cryopreservation (First experiment). For analysis of plasma cytokines, blood was collected in Microtainer plasma separator tubes with lithium heparin (BD), centrifuged and the plasma placed in cryovials (Sarstedt) and stored at −20 °C.

2.3. Cytokine analysis

IL-4, IL-5, IL-13, IL-10, IL-17, IFN-γ and TNF-α concentrations in plasma and concentrated pleural lavage fluid samples were measured using a multiplex bead array assay per the manufacturer's instructions. The minimum detection limits for these assays were as follows: 1.1 pg/ml for IFN-γ, 3.1 pg/ml for TNF-α, 0.3 pg/ml for IL-4, 0.4 pg/ml for IL-5, 2.6 pg/ml for IL-10, 12.4 pg/ml for IL-13 and 0.7 pg/ml for IL-17.

2.4. Flow cytometry

Cryopreserved spleen, pleural lavage, MLN and whole blood cells were thawed and washed once in 1X PBS supplemented with 1% BSA. The cells were then incubated in 1% BSA for 20 min. The cells were stained with anti-mouse lineage cocktail (anti-CD3, anti-CD45R, anti-CD11b, anti-TER-119 and anti-Ly-G6) in Pacific Blue