Hematopoietic LTβR deficiency results in skewed T cell cytokine profiles during a mucosal viral infection

Tian Sun, Olga L. Rojas, Conglei Li, Dana J. Philpott, and Jennifer L. Gommerman1

Department of Immunology, University of Toronto, Toronto, Ontario, Canada

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ABSTRACT

The lymphotoxin signaling pathway plays an important role in the homeostasis and function of peripheral and mucosal dendritic cells, and dendritic cell-intrinsic lymphotoxin β receptor expression is required for optimal responses to opportunistic intestinal bacteria. However, it is unknown whether dendritic cell-intrinsic lymphotoxin β receptor signaling is required for responses to intestinal viral infections. We explored this question by orally administrating murine rotavirus to chimeric mice that lack lymphotoxin β receptor signaling in the myeloid compartment but retain lymphoid tissues. We found that although clearance of rotavirus was unimpaired in the lymphotoxin β receptor−/− wild-type chimeric mice compared with wild-type → wild-type chimeric mice, IFN-γ-producing CD8+ and CD4+ T cells were significantly increased in the small intestinal lamina propria of lymphotoxin β receptor−/− → wild-type chimeric mice. In contrast, IL-17-producing CD4+ T cells were reduced in lymphotoxin β receptor−/− → wild-type chimeric mice in the steady state, and this reduction persisted after rotavirus inoculation. In spite of this altered cytokine profile in the small intestinal lamina propria of lymphotoxin β receptor−/− → wild-type chimeric mice, the local production of rotavirus-specific IgA was unperturbed. Collectively, our results demonstrate that lymphotoxin β receptor signaling in radio-sensitive myeloid cells regulates the balance of IFN-γ and IL-17 cytokine production within the small intestinal lamina propria; however, these perturbations do not affect mucosal antiviral IgA responses. J. Leukoc. Biol. 100: 000–000; 2016.

Introduction

LT is a TNF family cytokine that can exist as a membrane-bound LTαβ2 heterotrimer or a soluble LTα3 trimer. Whereas LTα3 binds to TNFR I and II, LTαβ2 signals exclusively through the LTβR. Another LTβR ligand, TNF superfamily member (LIGHT) can also deliver signals through HVEM. Both LTαβ2 and LIGHT are expressed primarily on lymphocytes, whereas LTβR is expressed on radio-resistant epithelial and stromal cells, as well as radio-sensitive myeloid cells [1, 2]. LTαβ2/LTβR signaling is critically required for lymphoid tissue organogenesis and the maintenance of secondary lymphoid structures [3, 4]. In addition, LTβR signaling is involved in host responses to infections in mice, including responses to lymphohytic choriomeningitis virus [5], Listeria monocytogenes, and Mycobacteria tuberculosis [6]. Moreover, LTβR signaling has been found to regulate acute inflammatory reactions, such as dextran sulfate sodium-induced colitis [7, 8], and to mediate tumor cell apoptosis [9]. Hence, LTβR signaling is involved in innate and adaptive immune responses.

Recently, LTβR signaling was shown to play a protective role in the immune response to a mucosal bacterial infection, specifically in the clearance of the attaching and effacing bacterium Citrobacter rodentium [10–13]. A mouse model used to understand the consequences of enteropathogenic and enterohemorrhagic Escherichia coli in humans. Within the radio-resistant compartment, LTβR signaling in IECs is required for the recruitment of neutrophils to the infection site via production of CXCL1 and CXCL2 chemokines [11] and for protection against epithelial injury via a mechanism that depends on IL-23 [14]. Within the radio-sensitive compartment, LTβR signaling in LP DCs drives the production of IL-22 from RORγt+ ILCs to maintain barrier integrity, thus providing protection against C. rodentium [12]. Furthermore, Notch2-dependent CD103+CD11b+ cDCs, which play a critical role in producing IL-23 in response to C. rodentium infection, are also partially LTβR dependent when examined in the context of competitive mixed BM chimeras [13]. Collectively, these findings...
support the idea that LTβR signaling in radio-resistant and -sensitive compartments is crucial for intestinal homeostasis to limit mucosal damage caused by bacterial invasion.

Whereas the importance of LTβR signaling in host defense against C. rodentium infection in the colon is well characterized, the role of DC-intrinsic LTβR signaling in viral clearance within the small bowel is less clear. RV infection is a well-defined model system for studying viral infection in the small intestine, as RV predominantly infects and replicates within mature epithelial cells on the tip of the small intestinal villi [15]. Before the introduction of RV vaccines, RV was a major cause of severe dehydrating diarrhea in infants and children <5 y old [15]. Previous studies have shown that *Lta* /− mice have prolonged intestinal RV infection corresponding with a defect in anti-RV IgA production, and remarkably, these lymphoid-tissue deficient mice do eventually mount an IgA response and can clear the virus [16]. However, this study did not dissect a role for LTβR versus TNFR signaling nor whether the key LTα responding cell type was a DC or an epithelial cell.

Although RV infection in adult mice is asymptomatic, viral particle shedding in the feces is detectable and correlates with the presence and replication of the virus. The clearance of RV in adult mice is dependent on cellular and humoral responses, as T cell-deficient mice (αβTCR−/−, αβ/γδTCR−/−, β2m−/−, and anti-CD8α mAb-treated C57BL/6) and IgA−/− mice have varying degrees of delayed viral clearance. However, even these severely immunocompromised mice can eventually resolve the RV infection [17–19], with the exception of recombination activating gene 2 (Rag2)−/− mice that become chronically infected and continuously shed viral antigen [17]. Although most immunocompromised mice can clear RV, it is nevertheless a very useful model for studying the dynamics of CD8+ T cell priming to a small intestinal tropic virus. Here, we focus on RV infection in adult mice to discern a role for DC-intrinsic LTβR signaling on CD8+ T cell priming in the gut. To evaluate this, we generated *Lbr* −/− → WT BM chimeric mice and monitored SILP T cell responses as a readout of DC function, as DCs have been shown to be important for priming naïve T cells in the gut-associated lymphoid tissues [20]. Collectively, the results from our study indicate that loss of LTβR signaling in the myeloid compartment shifts the gut microenvironment from a Th17- to a Th1-dominant state; yet, this alteration in cytokine production does not affect the local anti-RV IgA response.

**MATERIALS AND METHODS**

**Mice**

WT C57BL/6 (Charles River Laboratories, Senneville QC, Canada) and *Lbr* −/− mice (gift of Dr. Rodney Newberry, Washington University, St. Louis, MO, USA) were housed in the University of Toronto Division of Comparative Medicine under specific pathogen-free conditions. The day before oral gavage with RV, the mice were transferred to Biosafety Level 2 facilities for the duration of all studies. All experiments were approved by the University Animal Care Committee.

**BM chimeras**

BM cells (2–4 × 10^6), collected from femurs and tibia of WT C57BL/6 mice or *Lbr* −/− mice, were injected intravenously into C57BL/6 mice that had been lethally irradiated (2 × 550 cGy). Recipient mice were left for 8–10 wk to reconstitute and were given water supplemented with neomycin sulfate (2 g/l; BioShop, Burlington, ON, Canada) for the first 2 wk.

**Virus preparation**

The virulent WT, noncell culture-adapted murine RV strain ECw (gift of Dr. Harry Greenberg, Stanford University, Stanford, CA, USA) was used to infect mice. Stocks of RV were prepared as intestinal homogenates, and the DDo of the ECw virus stock was determined for C57BL/6 neonatal mice, as described previously [21].

**Virus inoculation and sample collection**

Mice were orally gavaged with 10^9 DDo ECw in 100 μl HBSS containing 1 mM CaCl_2 and 0.5 mM MgCl_2 after oral administration of 100 μl 1.33% sodium bicarbonate to neutralize stomach acidity. Fecal pellets were collected on the day of challenge and for the following days. Fecal samples were stored frozen at −20°C until assayed. For use in the ELISAs, 10% (wt/vol) stool suspensions were prepared with PBS containing 0.1% sodium azide (Merck Millipore, Billerica, MA, USA). Serum samples were collected with Microvette capillary blood collection tubes (Sarstedt, Nürnbergb, Germany), according to the manufacturer’s instructions. We compared WT → WT versus *Lbr* −/− → WT BM chimeric mice that were cocomed (2 wk before RV infection) versus not cocomed, in terms of responses to RV infection, and did not observe any difference in results, including kinetics of viral antigen shedding, local and systemic IgA production, and cytokine production by CD8+ and CD4+ T cells.

**Detection of viral antigen and virus-specific IgA by ELISA**

ELISA was performed as previously described to detect RV antigen and RV-specific fecal/serum IgA [21], with modifications: using anti-RV mAb (AbD Serotec, Raleigh, NC, USA), followed by HRP-conjugated anti-mouse IgG2b antibody (SouthernBiotech, Birmingham, AL, USA) to detect RV antigen and HRP-conjugated anti-mouse IgA (SouthernBiotech) to detect anti-RV IgA. The OD was read at 450 nm.

**Isolation of LP cells from the small intestine**

Preparation of SILP lymphocytes was described previously [22], with the modification of using Collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and DNase I (Roche, Indianapolis, IN, USA) in the digestion step instead of collagenase. Microvette capillary blood collection tubes (Sarstedt, Nürnbergb, Germany), according to the manufacturer’s instructions. We compared WT → WT versus *Lbr* −/− → WT BM chimeric mice that were cocomed (2 wk before RV infection) versus not cocomed, in terms of responses to RV infection, and did not observe any difference in results, including kinetics of viral antigen shedding, local and systemic IgA production, and cytokine production by CD8+ and CD4+ T cells.

**Antibodies and staining**

Antibodies against CD3ε, CD8α, CD4, and IFN-γ were purchased from eBioscience (San Diego, CA, USA). Antibodies against Ki67 and IL-17A were obtained from BD Biosciences (San Jose, CA, USA). LIVE/DEAD fixable Aqua was purchased from Life Technologies (Thermo Fisher Scientific, Grand Island, NY, USA). After LIVE/DEAD Aqua staining, cells were washed and then blocked with purified anti-FcγRIII mAb (2.4G2). All surface stains were performed in PBS with 2% FBS. ICs was performed using a Cytofix/ Cytoperm Kit (BD Biosciences). All stained samples were acquired on a FACSCanto or LSRII (BD Biosciences) as appropriate. FlowJo software (Tree Star, Ashland, OR, USA) was used for FACs data analysis.
ICS
To enumerate the number of cytokine-secreting T cells, ICS was performed, as described previously [22]. In brief, lymphocytes were incubated for 6 h at 37°C in complete medium, supplemented with recombinant human IL-2 (100 U/ml; R&D Systems, Minneapolis, MN, USA) and GolgiPlug (1 μl/ml; BD Biosciences). Cells were stimulated with PMA (20 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), VP6[33][36] peptide (VPVFPFPGM; 2 μg/ml; Genemed Synthesis, San Antonio, TX, USA), or VP7[33][36] peptide (IVYRLFLV; 2 μg/ml; Genemed Synthesis) [22].

Statistics
Comparisons of data were analyzed by Mann-Whitney nonparametric test with the GraphPad Prism 6.0 program. Data from such experiments are presented as means ± SEM; P < 0.05 was considered significant.

RESULTS AND DISCUSSION

Ltbr−/− chimeric mice clear RV with normal kinetics
As mentioned, even highly immunocompromised adult mice can clear RV infections as a result of redundant humoral and cellular mechanisms. To ascertain if clearance of RV infection is affected by myeloid cell-intrinsic LTβR deficiency, we generated Ltbr−/− → WT BM chimeras. At 8–10 wk post-BM reconstitution, Ltbr−/− → WT and WT → WT chimeras were inoculated with murine RV by oral gavage, respectively. We found that the kinetics of fecal viral shedding in the Ltbr−/− → WT chimeras were similar to those in the WT → WT chimeras (Fig. 1). Thus, as expected, LTβR signaling in the myeloid compartment is dispensable for viral clearance in the intestine. It is possible that neonatal mice, which do in fact exhibit diarrhea following RV infection [23], may be more susceptible to the absence of LTβR in the myeloid compartment in terms of their ability to clear an RV infection. However, the Ltbr−/− → WT chimera approach does not allow us to answer this question, and intact Ltbr−/− mice lack secondary lymphoid tissues [4], thus introducing a major confounding variable.

Ltbr−/− chimeric mice generate more IFN-γ-secreting CD8+ T cells during primary RV infection
The RV system is an excellent system for examining CD8+ T cell responses within the small intestinal environment [22]. Although Ltbr−/− → WT chimeric mice can clear RV with comparable kinetics compared with WT → WT chimeric mice, nevertheless, we followed the RV-specific CD8+ T cell response to determine if a DC-intrinsic LTβR signaling pathway is required for CD8+ T cell priming, expansion, and/or effector function in response to a mucosal viral infection. In the current study, we examined the anti-RV CD8+ T cell response at 7 d.p.i. in the SILP, as this was reported previously as the peak of the intestinal RV-specific CD8+ T cell response [22]. A gating strategy for total CD4+ and CD8+ T cells in the SILP is depicted in Supplemental Fig. 1. At 7 d.p.i., the proportion of CD8+ T cells as a frequency of total mononuclear cells was significantly higher in the SILP of Ltbr−/− → WT chimeras compared with WT → WT control chimeric mice (Fig. 2A). Likewise, the percentage of SILP CD8+ T cells as a frequency of total mononuclear cells that were positive for intracellular Ki-67 staining (an indicator of cell proliferation) was higher in the Ltbr−/− → WT chimeras (Fig. 2B). However, there was no difference in SILP CD8+ T cell proliferation when measured as a frequency of the total CD8+ T cell population (Fig. 2C), suggesting that the observed increase in frequency of proliferating CD8+ T cells is a result of an over-representation of CD8+ T cells within the SILP rather than increased proliferation within the SILP. These results suggested that LTβR deficiency in the myeloid compartment has the capacity to affect CD8+ T cell accumulation but not expansion in the SILP after viral infection.

We next examined the quality of the CD8+ T cell response in Ltbr−/− → WT versus WT → WT chimeric mice. In vitro polyclonal stimulation revealed that IFN-γ secretion was more robust in SILP CD8+ T cells derived from Ltbr−/− → WT chimeras compared with SILP CD8+ T cells derived from WT → WT chimeras when examined as a frequency of total CD8+ T cells or total mononuclear cells in the SILP (Fig. 3A–C). To examine SILP CD8+ T cell responses to specific antigens, we then measured intracellular IFN-γ production from CD8+ T cells stimulated with VP6[33][36], one of the immunodominant RV epitopes recognized by H-2b-restricted CD8+ T cells [22]. Although the percentage of IFN-γ-producing CD8+ T cells was comparable between Ltbr−/− → WT and WT → WT chimeras when expressed as a frequency of the total CD8+ T cell population, the percentage of IFN-γ-secreting CD8+ T cells was higher in the Ltbr−/− → WT chimeras when expressed as a frequency of total mononuclear cells in the SILP (Fig. 3A, D, and E). Similar results were obtained when CD8+ T cells were stimulated with VP7[33][40], another immunodominant epitope recognized by H-2b-restricted CD8+ T cells (Fig. 3F and G) [22, 24]. The overall increase in VP6[33][36] and VP7[33][40] specific IFN-γ-producing CD8+ T cells is likely a result of the observed increase in SILP CD8+ T cells present in the Ltbr−/− → WT chimeras (Fig. 2A). Therefore, LTβR signaling in the myeloid...
compartment is dispensable for VP6357 and VP733-specific CD8+ T cell responses after RV challenge. However, LTβR signaling in the myeloid compartment may be required to regulate other RV-specific CD8+ T cell IFN-γ responses besides those induced by VP6357 and VP733 epitopes, as reflected by the increase in CD8+ T cell IFN-γ production induced by polyclonal stimulation (Fig. 3A–C).

Although the expansion of antigen-specific SILP CD8+ T cells in Ltbr−/− → WT chimeric mice was normal, we observed a significant increase in the percentage of total CD8+ T cells in the SILP of Ltbr−/− → WT chimeric mice after viral challenge. Given that competitive BM chimeras have revealed that LTβR signaling plays a role in maintaining SILP-resident CD103+CD11b+ cDCs [13], it is possible that LTβR-dependent CD103+CD11b+ cDCs have the capacity to constrain the CD8+ T cell population within the SILP (or alternatively, a possible compensatory increase of CD103+CD11b− cDC subset could lead to an increase in gut-homing CD8+ T cells after viral inoculation). Future studies examining the role of specific SILP DC subsets and macrophages in the context of mucosal viral infection would shed further light on mechanisms of T cell priming in the gut.

**Polyclonal CD4+ T cell cytokine profiles are skewed in the Ltbr−/− chimeric mice at steady state and after RV infection**

It has been shown that depletion of CD4+ T cells results in a delay in the generation of RV-specific intestinal IgA, which is the principal effector of long-term protection against RV reinfection [15, 25, 26]. Therefore, we evaluated polyclonal CD4+ T cell responses to RV infection in Ltbr−/− → WT chimeras at steady state and during RV infection. Unlike CD8+ T cells, the percentage of CD4+ T cells in the SILP was comparable between Ltbr−/− → WT and WT → WT chimeras at steady state and at 7 d.p.i. (Fig. 4A). However, the proliferation of CD4+ T cells was increased significantly in the Ltbr−/− → WT chimeras at 7 d.p.i. compared with WT → WT chimeras (Fig. 4B), indicating that loss of LTβR signaling in the myeloid compartment can increase the expansion of SILP CD4+ T cells after viral infection.

Whereas CD4+ T cells from WT → WT chimeras did not exhibit augmented IFN-γ production after RV infection, CD4+ T cells from RV-infected Ltbr−/− → WT chimeras exhibited elevated IFN-γ production compared with uninfected Ltbr−/− → WT controls (Fig. 4C and D). On the other hand, IL-17 production by CD4+ T cells was significantly reduced in Ltbr−/− → WT chimeras compared with WT → WT chimeras, whether at steady state or after RV infection (Fig. 4C and E). These results suggest that although primary RV infection does not augment IL-17 production by CD4+ T cells, the frequency of IL-17-producing T cells in the SILP of resting and infected mice was partially dependent on myeloid cell-intrinsic LTβR signaling. The reason(s) for increased IFN-γ production by CD4+ T cells in the LTβR deficient setting is unclear but may be related to the complexity of the LT network. Specifically, the LTαβ-LTβR and the LIGHT-HVEM-BTLA systems form an integrated circuit, controlling intercellular communication between T cells and DCs [2], with LIGHT serving as a key factor controlling the HVEM-BTLA switch between positive and inhibitory signaling. It has been proposed that the induction of LIGHT during T cell activation and its occupancy of HVEM displaces BTLA and alleviates inhibitory signaling [2]. Therefore, the loss of LTβR expression within the myeloid compartment could promote preferential binding of

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**Figure 2. Frequency and proliferation status of CD8+ T cells in RV-infected Ltbr−/− chimeric mice.** WT → WT and Ltbr−/− → WT (KO → WT) chimeric mice were euthanized at 7 d.p.i., UI, Uninfected; RV, RV infected. After PMA/ionomycin in vitro restimulation for 6 h, SILP CD8+ T cells were analyzed by flow cytometry. (A) Percentage of total CD8+ T cells as a frequency of SILP mononuclear cells. (B) Percentage of Ki67+ CD8+ T cells as a frequency of SILP mononuclear cells. (C) Percentage of Ki67+ as a frequency of total SILP CD8+ T cells. Each point represents an individual mouse and data are pooled from 3 independent experiments. Data are presented as average ± sem. Mann-Whitney nonparametric test; **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 3. Assessment of IFN-γ-producing CD8+ T cells in LtbR−/− chimeric mice induced by mitogen or viral peptide stimulation. WT → WT and LtbR−/− → WT chimeric mice were euthanized at 7 d.p.i. (A) Representative flow cytometry data showing IFN-γ-producing SILP CD8+ T cells after PMA/ionomycin (Iono; upper) and VP6357–366 peptide (lower) in vitro restimulation for 6 h (pregated on CD8+ T cells). (B and C) After PMA/ionomycin in (continued on next page)
LIGHT (expressed by T cells) with HVEM, thus maintaining T cell activation. The complex relationship between LTα1β2-LTβRa and the LIGHT-HVEM-BTLA systems in the context of SILP-resident DC:T cell interactions requires further examination.

Ltbr2/2 chimeric mice generate a normal intestinal IgA response to RV

Given that we observed a reduction in SILP Th17 cells in the Ltbr2/2 → WT chimeras (Fig. 4E), and Th17 cells have been implicated in promoting antigen-specific IgA responses [27], we speculated that there might be a delay and/or reduced production of antigen-specific IgA in response to RV infection in Ltbr2/2 → WT chimeric mice. Although the initiation of systemic RV-IgA in the Ltbr2/2 → WT chimeras was slightly delayed, IgA levels increased quickly, achieving levels comparable with the WT → WT chimeras (Fig. 5A). Moreover, intestinal RV-specific IgA production was comparable between the Ltbr2/2 → WT and WT → WT chimeras at all of the time points examined (Fig. 5B). These results suggest that antigen-specific IgA, in response to mucosal viral infection, can be generated in mice lacking LTβR signaling in the myeloid compartment.

Th17 cells have been shown to be responsible for inducing the switch of germinal center B cells toward the production of...
high-affinity TD IgA [27]. Moreover, IL-17 produced by Th17 cells increases polymeric Ig receptor expression on IECs and increases the rate of secretory IgA production into the lumen [28]. Herein, our results demonstrate that in spite of abrogated SILP Th17 cell homeostasis in Ltbr−/− mice, decreased production of IL-17 by Th17 cells did not alter local antiviral IgA production. It is possible that the residual IL-17 production was sufficient to induce the antiviral IgA response in a TD manner. Alternatively, cytokines and growth factors, such as APRIL and BAFF, which play an important role in TI IgA class-switch recombination within isolated lymphoid follicles of the SILP, may provide an independent mechanism for promoting RV-specific mucosal IgA [29, 30]. This TI IgA induction can be maintained by regulatory T cells [31, 32], LTα1β2-expressing RORγt+ ILCs [29], and APRIL- and BAFF-expressing plasmacytoid DCs in the mesenteric lymph nodes [33], which we did not evaluate in this study. Lastly, soluble LTα3, derived from RORγt+ ILCs, could promote TD IgA production via TNFRI/TNFRII signaling [29].

In summary, we show that Ltbr−/− → WT chimeric mice are capable of mounting a primary CD8+ T cell response against RV infection. Unlike its critical role in C. rodentium infection, LTβR signaling in the myeloid compartment is not absolutely required for RV clearance in the small bowel, suggesting that the role of DC-intrinsic LTβR signaling varies with the type of mucosal challenge as a result of factors, such as the site of infection (large bowel vs. small bowel), the types of pathogen-associated molecular patterns (bacterial vs. viral), and the severity of disease after challenge (fatal vs. asymptomatic). We previously showed that DC-intrinsic LTβR signaling is required for CD8+ T cell responses to both self and foreign protein antigens [34, 35]. Presumably the presence of viral-derived innate signals over-ride a requirement for DC-intrinsic LTβR signaling to prime CD8+ T cells, as has been noted in the case of Influenza virus infection [36].

The reduction of IL-17-producing CD4+ T cells and the comparable local RV-specific IgA level in Ltbr−/− → WT chimeras suggest that the local humoral anti-RV response does not require optimal levels of IL-17. Recently, it has been shown that IL-22, a member of the IL-10 family of cytokines, is essential for protection against RV [37]. Moreover, the main source of IL-22 production after these challenges is intestinal ILC3 [12, 38]. It is possible that IL-22 may play a more important role than IL-17 in responses to RV, and it would be of interest to determine if RV-induced IL-22 production by ILC3 is influenced by the LT pathway. Further studies could focus on the role of LTβR signaling within the radio-resistant compartment (IECs and stromal cells) during intestinal viral responses, vis-avis IL-22 production.

Although the current 2 licensed, live oral RV vaccines, RotaTeq (Merck, West Point, PA, USA) and Rotarix (GlaxoSmithKline, Research Triangle Park, NC, USA), prevent up to 74% of severe RV episodes [39], the lower vaccine efficacy in resource-poor countries, as well as the risk of intussusception after vaccination are significant problems currently without a solution [40]. One reason for the low vaccine efficacy in the low-income countries is a result of reduced immune responses in infants because of comorbidities or malnutrition, including micronutrient deficiency [41]. An understanding of how different dietary conditions affect the SILP cytokine milieu during the priming phase of RV infection, and how such cytokines impact the formation and potency of CD8+ effector/memory T cells, may provide a better RV vaccine design.

**AUTHORSHIP**

T.S. did the majority of the experiments and prepared the manuscript. T.S., O.L.R., and J.L.G. designed all of the experiments. O.L.R., C.L., and T.S. standardized and optimized ELISAs and the protocol for isolation of SILP lymphocytes. D.J.P. and J.L.G. provided advice for the experiments and preparation of the manuscript. J.L.G. directed the research.

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**DISCLOSURES**

The authors declare no conflicts of interest.
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