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Distinct Mechanisms for Cross-Protection of the Upper Versus Lower Respiratory Tract Through Intestinal Priming[1]

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A main feature of the common mucosal immune system is that lymphocytes primed in one mucosal inductive site may home to distant mucosal effector sites. However, the mechanisms responsible for such cross-protection remain elusive. To address these we have used a model of local mucosal infection of mice with reovirus. In immunocompetent mice local duodenal priming protected against subsequent respiratory challenge. In the upper respiratory tract this protection appeared to be mainly mediated by specific IgA- and IgG2a-producing B cells, whereas ex vivo active effector memory CTL were found in the lower respiratory tract. In accordance with these findings, clearance of reovirus from the lower respiratory tract, but not from the upper respiratory tract, of infected SCID mice upon transfer of gut-primed lymphocytes depended on the presence of T cells. Taken together this study reveals that intestinal priming leads to protection of both the upper and lower respiratory tracts, however through distinct mechanisms. We suggest that cross-protection in the common mucosal immune system is mediated by trafficking of B cells and effector memory CTL. ***The Journal of Immunology*****, 2002, 169: 3920–3925.**

Transfer of either Peyer's patch (PP)[3] cells or cells from bronchus-associated lymphoid tissue to homologous, lethally irradiated recipients results in repopulation of both the intestinal and the bronchial lamina propria (LP) (1, 2). These findings led to the hypothesis of a common mucosal immune system (3), with a proposal that cells primed in one mucosal inductive site may home to many different, if not all, mucosal effector sites. Numerous studies have tested this concept, particularly attempts at developing new mucosal vaccines. Thus, it has been well elaborated in animal models (4–6) and human studies (7, 8) that intestinal priming can lead to protection of the lower respiratory tract. Similarly, it is now established that oral (5, 9) or intranasal (i.n.) (10) vaccination can elicit production of specific Ab in the female genital tract.

However, the mechanisms underlying cross-protection of distant mucosal sites after local priming remain largely elusive. It is, for example, not clear whether this protection is due to emigration of locally primed cells or whether passage/transportation of Ag from the site of entry to distant mucosal inductive sites is responsible for cross-protection. Furthermore, it was recently shown that after systemic immunization, effector memory T cells (11) emigrate and stably populate nonlymphoid compartments, including mucosal sites such as the intestinal LP and lung or nonmucosal sites such as the liver and kidneys (12). However, it has not been ascertained to date whether this generalized distribution of effector memory T cells to nonlymphoid sites also occurs after local mucosal priming and, thus, whether these relocation patterns also apply for cell migration within the common mucosal immune system.

In this study we sought to elucidate mechanisms providing for a common mucosal immune system. Using models of local mucosal reovirus infection, we show that cross-protection of the upper and lower respiratory tracts after intestinal priming is achieved through relocation of locally primed B and T lymphocytes to distant mucosal compartments. However, differences in the quality and kinetics of the immune responses in the upper vs the lower respiratory tract after intestinal priming were observed.

Materials and Methods

Mice and media

Male C3HeB/FeJ (referred to as C3H) and BALB/c/ByJ were purchased from The Jackson Laboratory (Bar Harbor, ME). Conventionally reared C.B-17 SCID mice were bred in the germfree animal facility of Department of Biology, University of Pennsylvania (Philadelphia, PA). All mice were used at the age of 8–14 wk. All animal experiments were conducted in accordance with the guidelines of University Laboratory Animal Resources, University of Pennsylvania.

L-929 fibroblasts were grown in medium 199 (Life Technologies, Grand Island, NY) containing 5% FCS (Life Technologies), 2 mM L-glutamine (Life Technologies), 1000 U/ml penicillin, and 0.1 mg/ml streptomycin (Life Technologies). For organ fragment cultures, Kennett's HY medium (Life Technologies) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, and gentamicin was used.

Virus and infection

Third-passage stocks of reovirus serotype 1, strain Lang, were produced by expanding a single plaque through three passages in L-929 fibroblasts, followed by extraction of virus with freon and purification by CsCl gradient centrifugation (13). For i.n. infection mice were lightly anesthetized with 0.1 mg/g body weight avertin (2,2,2-tribromoethanol; Aldrich, Milwaukee, WI), and 1–2.5 $\times$ 10^7 PFU reovirus was applied to both nostrils in a total volume of 25 μl saline/0.5% gelatin. For intraduodenal (i.du.) and intratracheal (i.t.) infection, mice were deeply anesthetized with 0.4 mg/g body weight avertin, and 50 μl saline/0.5% gelatin containing 1–2.5 $\times$ 10^7

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[3] Abbreviations used in this paper: PP, Peyer's patch; IEL, intraepithelial lymphocyte; i.du., intraduodenal(ly); i.n., intranasal(ly); i.t., intratracheal(ly); LN, lymph node; LP(L), lamina propria (lymphocytes); NALT, nasal-associated lymphoid tissue; SG, salivary gland.

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PFU reovirus was directly injected into the duodenum or trachea, respectively, by a small surgical procedure.

Virus titration

Viral titers in various tissues were determined in a standard virus plaque assay. Mice were sacrificed and perfused through the right ventricle with 20 ml PBS. Thereafter, tissues were homogenized in 3 ml saline/0.5% gelatin, and serial dilutions were incubated on monolayers of L-929 fibroblasts in six-well tissue culture plates (Costar, Cambridge, MA) for 45 min at 37°C and thereafter overlaid with 3 ml 1% agar in complete medium 199 and cultured at 34°C. Plaques were counted after 7 days of incubation.

Ab production in organ fragment cultures and serum

C3H mice (n = 3–4/time point) were sacrificed and perfused with 20 ml PBS. Thereafter, the entire small intestine, mesenteric lymph node (LN), mediastinal LN, and upper right lung lobe were surgically removed. PP were visually detected and excised from small intestine. Submandibular LN, which probably drain nasal-associated lymphoid tissue (NALT) (14), and NALT were isolated after removal of the mandible. Palatine salivary glands (SG), which are located above the maxilla and are a major effector site in the upper respiratory tract, were isolated after removal of the hard palate. Organ fragment cultures were established, and reovirus-specific IgA and IgG2a Ab were measured by RIA using reovirus-coated polyvinyl plates and ^{125}I-labeled anti-IgA or anti-IgG2a Ab (both from Southern Biotechnology Associates, Birmingham, AL) as described in detail recently (14).

Ex vivo CTL analysis

C3H mice were sacrificed 10 and 30 days after i.du. or i.n. infection and perfused with 20 ml PBS. Thereafter, cells were isolated from spleen, PP, cervical LN, or mesenteric LN by mechanical disruption. Intraepithelial lymphocytes (IEL) and intestinal LP cells were isolated by elution from small intestinal epithelium with PBS/1 mM EDTA (for IEL) and subsequent collagenase (1 mg/ml) digestion (for intestinal LP cells), followed by discontinuous Percoll gradient centrifugation at 600 × g for 20 min using 70 and 40% Percoll. Cells from lung interstitium, liver, submandibular SG, and palatine SG were isolated by collagenase digestion and Percoll gradient centrifugation as described above. Ex vivo CTL activity was determined in a standard ^{51}Cr release cytotoxicity assay. L-929 fibroblasts infected with reovirus (or uninfected for control) were labeled with 100 μCi ^{51}Cr (NEN, Boston, MA) and then incubated with effector cells at different E:T cell ratios (3000 target cells/well). After 5 h of incubation in V-bottom microtiter plates (Costar, Cambridge, MA) at 37°C, 100 μl supernatant fluids were collected and mixed with 1 ml scintillation fluid (Cytoscint; ICN, Costa Mesa CA), and beta emission was measured on an LS6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

One lytic unit is defined as the number of CD8$^+$ cells required to achieve 25% lysis and was calculated for all cell types used, considering the different E:T cell ratios and percentages of CD8$^+$ cells as determined by FACS.

Cell transfers to SCID mice

Donor BALB/c mice were primed i.du. as described above. On day 7 after infection, donor mice were sacrificed and perfused with 20 ml PBS, and single-cell suspensions of PP and mesenteric LN were injected i.p. into recipient SCID mice (10^7/mouse) infected with reovirus i.n. or i.t. 2 days before transfer of cells. For some experiments subsets of cells were depleted by MACS using biotinylated anti-CD8α (clone 53.6-7; BD PharMingen, San Diego, CA) and streptavidin microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

Statistical analysis

Two-tailed unpaired Student's t test was applied for all statistical analyses.

Results

Duodenal priming protects from nasal or tracheal challenge

We tested whether local gastrointestinal priming with reovirus would protect from a subsequent respiratory challenge. C3H mice were infected i.du. with reovirus serotype 1, strain Lang. Primed mice were challenged via the respiratory route 30 days later with the same virus and compared with an unprimed group of mice that received only a respiratory challenge. After primary i.du. infection (priming), reovirus was present in the gastrointestinal tract from where it was cleared within 7–14 days (Fig. 1, *A* and *B*). Importantly, after i.du. infection no replicating virus was found in either the upper or the lower respiratory tract, demonstrating a localized gut infection. Upon upper respiratory challenge by i.n. infection, viral titers were clearly lower, and clearance was achieved more rapidly in i.du. primed mice compared with unprimed, i.n. infected control mice (Fig. 1*C*). Similarly, i.du. priming conferred complete protection from lower respiratory challenge by i.t. reovirus infection (Fig. 1*D*).

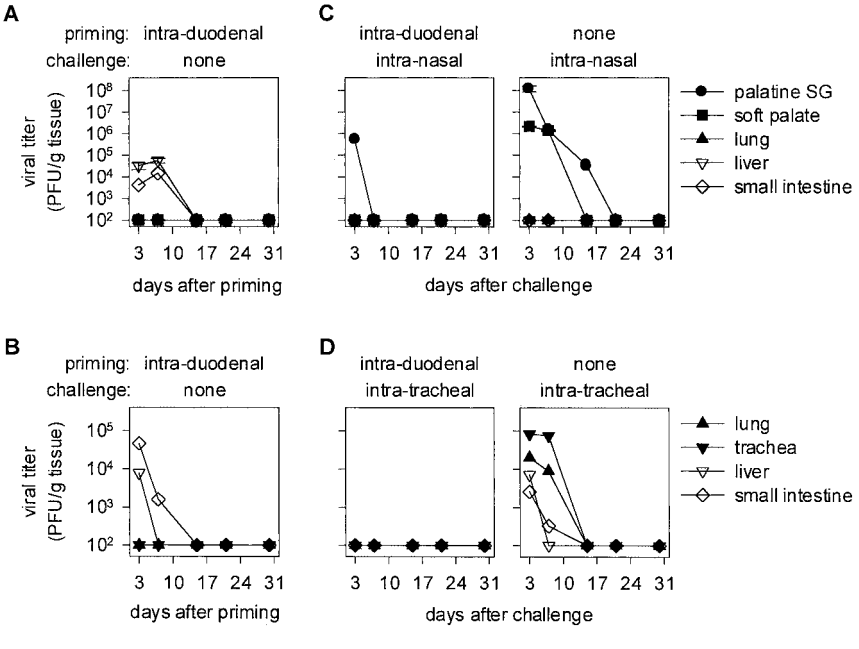

FIGURE 1. Intestinal priming of C3H mice protects from nasal or tracheal challenge. *A* and *B*, Immunocompetent C3H mice were primed i.du. with reovirus, and tissue viral titers were determined at various time points. Thirty days after priming, mice were challenged i.n. (*C*) or i.t. (*D*) with reovirus and compared with a group of unprimed mice that received only i.n. or i.t. challenge. Tissue titers of reovirus (PFU per gram of tissue ± SD) were determined in a standard virus plaque assay. Two individual experiments are shown; each experiment was performed twice.

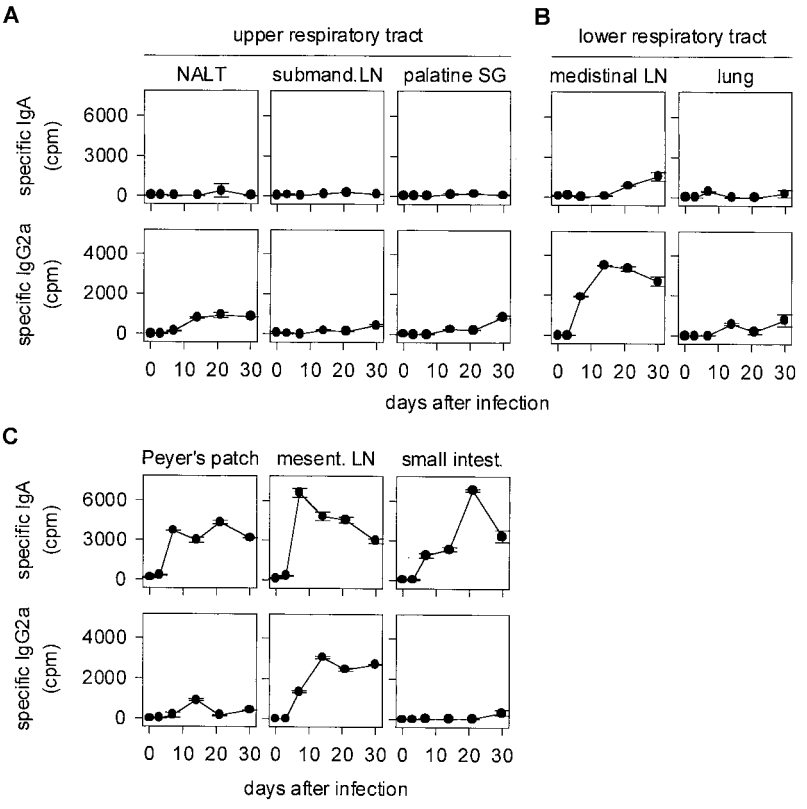

FIGURE 2. Distinct kinetics of production of reovirus-specific IgA and IgG2a in the upper vs the lower respiratory tract after intestinal priming. Immunocompetent C3H mice were primed i.du. with reovirus. Levels of reovirus-specific IgA and IgG2a (counts per minute ± SD) in organ fragment culture supernatants from the upper respiratory tract (*A*; NALT, submandibular LN, and palatine SG), the lower respiratory tract (*B*; mediastinal LN and lung), and the gastrointestinal tract (*C*; PP, mesenteric LN, and small intestine) were determined at various time points by RIA. Data were pooled from two experiments; two of four experiments are shown.

Distinct kinetics of reovirus-specific IgA and IgG2a production in the upper vs the lower respiratory tract

To determine whether B cells might contribute to this cross-protection, production of reovirus-specific IgA and IgG2a in the respiratory tract was monitored by kinetic analysis of organ fragment culture supernatant fluid. Intraduodenal priming did not induce appreciable production of reovirus-specific IgA in NALT, submandibular LN, or palatine SG over a period of 30 days (Fig. 2*A*). This lack of Ab production was not due to failed i.du. priming, since fragment cultures of PP, mesenteric LN, and small intestine showed induction of robust amounts of reovirus-specific IgA (Fig. 2*C*). Similarly, only weak IgG2a responses were detected in the upper respiratory tract, even though late in the course of i.du. infection some anti-reovirus IgG2a was induced in NALT and palatine SG (Fig. 2*A*). Surprisingly, i.du. infection resulted in the production of some IgA and significant amounts of IgG2a in the mediastinal LN that drains the lower respiratory tract (Fig. 2*B*). In the lungs, however, intestinal priming did not lead to the production of any IgA and only minute amounts of IgG2a (Fig. 2*B*).

Interestingly, after upper respiratory challenge by i.n. delivery of reovirus, specific Ab responses with typical characteristics of a memory response unfolded in the upper respiratory tract of i.du. primed mice (Fig. 3*A*). These characteristics included rapid onset of both IgA and IgG2a production, peaking early within 3–7 days, whereas in unprimed, i.n. infected mice maximal levels of IgA and IgG2a secretion were only reached 7–14 days postinfection. Most strikingly, in almost all tissues of i.du. primed mice, significant amounts of specific Ab were detected by day 3 after challenge,

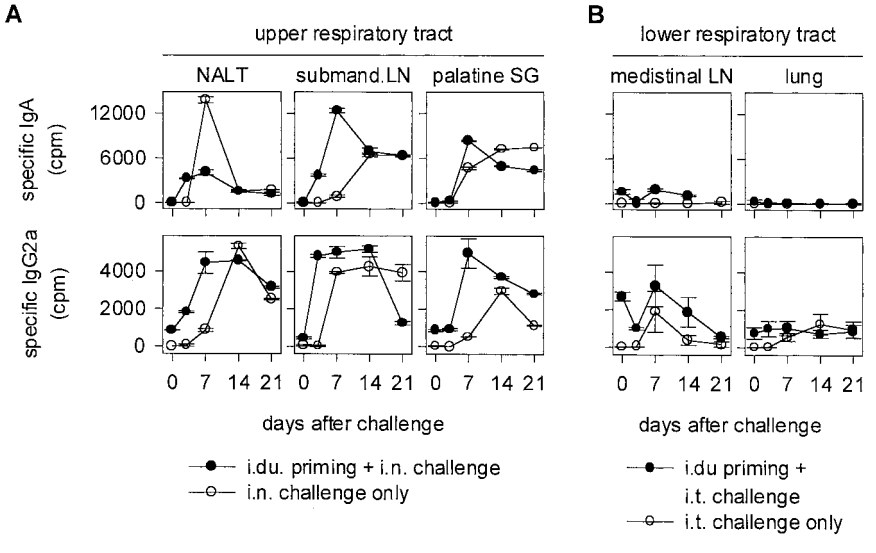

FIGURE 3. Distinct kinetics of production of reovirus-specific IgA and IgG2a in the upper vs the lower respiratory tract after respiratory challenge of i.du primed mice. Immunocompetent C3H mice were primed i.du. with reovirus and challenged 30 days thereafter via the respiratory tract. Levels of reovirus-specific IgA and IgG2a (counts per minute ± SD) in organ fragment culture supernatants from the upper respiratory tract (*A*; NALT, submandibular LN, and palatine SG) after i.n. challenge and the lower respiratory tract (*B*; mediastinal LN and lung) after i.t. challenge were determined at various time points by RIA. Data were pooled from two experiments; two of four experiments are shown.

whereas at this time none of the tissues of unprimed, i.n. infected mice showed any specific IgA or IgG2a. In contrast, no accelerated onset of Ab production was observed in i.du. primed mice, compared with unprimed mice, after i.t. challenge (Fig. 3*B*). Only minute amounts of specific IgA and a minor boost of specific IgG2a production in mediastinal LN were detected, and no appreciable production of IgA or IgG2a ensued from i.t. challenge in the lungs (Fig. 3*B*).

Effector memory CTL relocate to the lungs, but not palatine SG, after intestinal priming

Next we analyzed the distribution of ex vivo active effector memory CTL (11, 12) after local intestinal or upper respiratory infection of C3H mice. As shown in Fig. 4*A*, after i.du. infection, reovirus-specific CTL displaying strong ex vivo killing activity were detectable in the lungs over a period of 30 days. In contrast, no CTL were found in palatine SG at any time after i.du priming, even though i.n. priming induced strong ex vivo active CTL in palatine SG (Fig. 4*A*). To accommodate differences due to varying E:T cell ratios and percentages of $CD8^+$ cells, lytic units in individual tissues were calculated (Fig. 4*B*). After i.n. infection the strongest CTL responses were observed in the respiratory tract (lung and palatine SG), and some activity was found in the gut, whereas after i.du. priming the highest killing activities were found in the gastrointestinal tract and lungs.

Different cell types are responsible for clearance of reovirus from the upper vs the lower respiratory tract of SCID recipients

These findings were supported using an adoptive transfer model. Immunocompetent BALB/c mice were primed i.du. with reovirus and single-cell suspensions of PP transferred to SCID mice infected i.n. or i.t. 2 days before cell transfer. Successful repopulation of the SCID recipients was confirmed by FACS analysis and the appearance of specific IgG Ab in serum (data not shown). As shown in Fig. 5*A*, PP cells readily cleared reovirus from the upper and lower respiratory tracts of infected SCID mice (Fig. 5*A*). Transfer of mesenteric LN cells yielded similar results (data not shown). To assess the role of B cells vs T cells in clearing reovirus from the respiratory tract, subsets of cells were depleted from PP by MACS, and viral titers were determined 10 days after transfer.

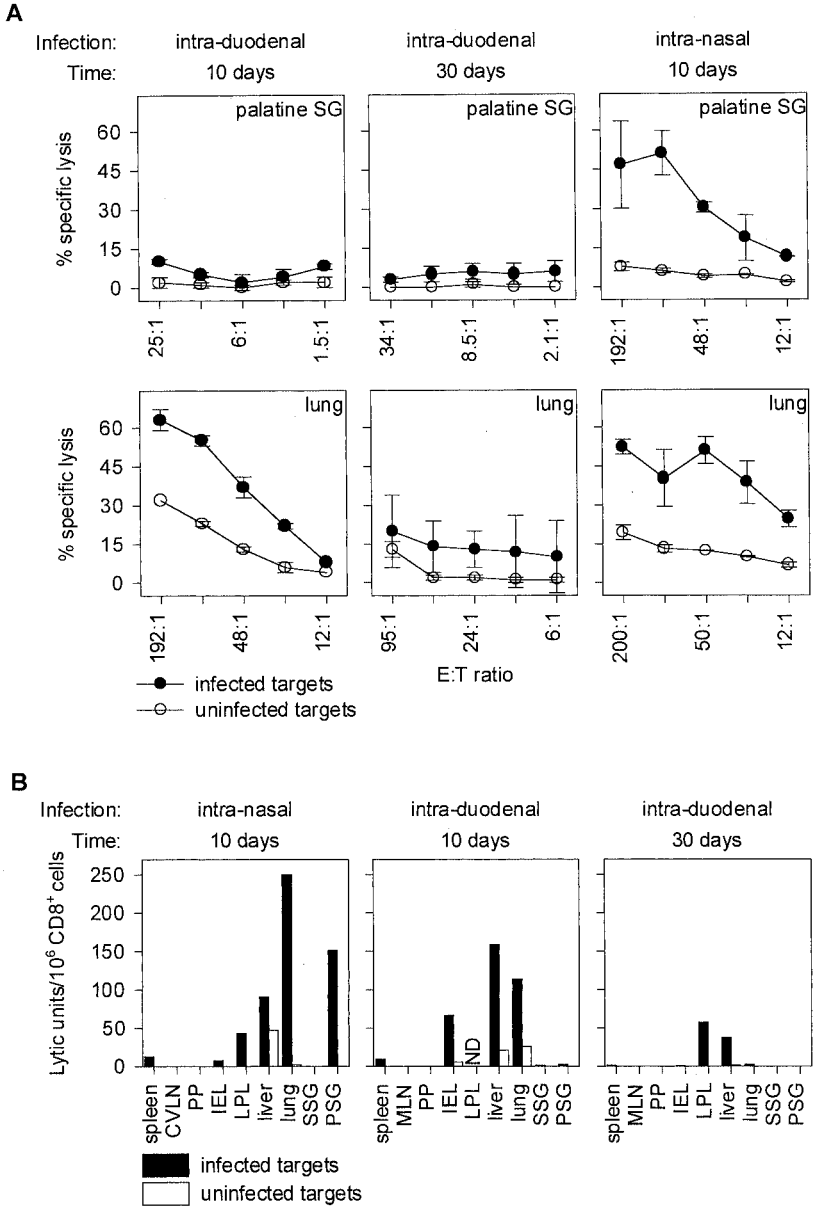

FIGURE 4. Effector memory CTL relocate to the lower, but not the upper, respiratory tract after i.du. priming. *A*, Immunocompetent C3H mice were infected i.du. or i.n. with reovirus. Ten or 30 (i.du. primed only) days later cells were isolated from lungs and palatine SG and used ex vivo in a standard ^{51}Cr release cellular cytotoxicity assay at different E:T cell ratios. The percent specific lysis ± SD is shown. *B*, Quantification of ex vivo lytic activity in various tissues. One lytic unit is defined as the number of $CD8^+$ cells needed to achieve 25% specific ^{51}Cr release. MLN, mesenteric LN; SSG, submandibular SG; PSG, palatine SG; ND, not done. Data from three of five similar experiments are shown.

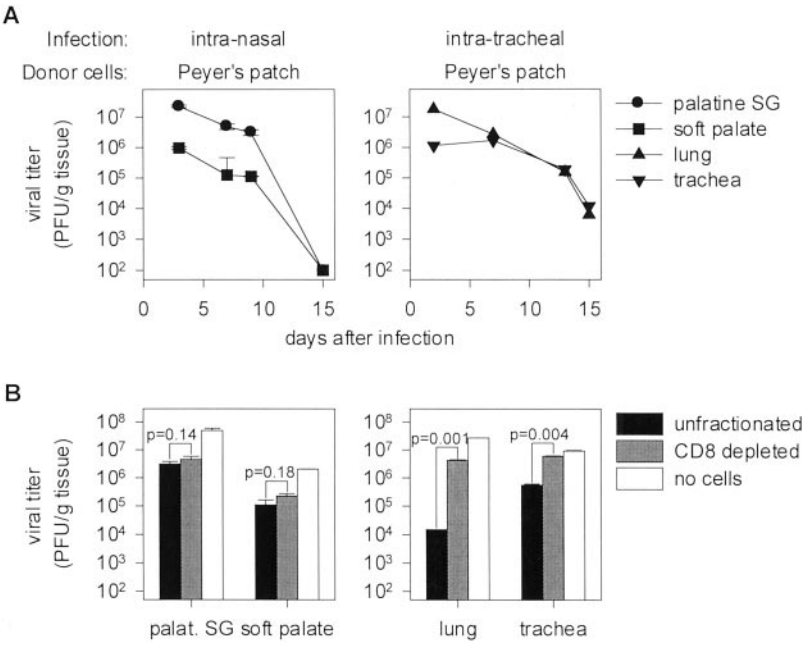

FIGURE 5. Distinct cell types are responsible for clearance of reovirus from the upper vs the lower respiratory tract of infected SCID mice. PP were isolated from BALB/c mice 7 days after i.du. priming and transferred to i.n. or i.t. infected SCID mice. *A*, Tissue titers of reovirus (PFU per gram of tissue ± SD) were measured in a standard virus plaque assay. Data were pooled from two experiments; each experiment was repeated at least twice. *B*, $CD8^+$ cells were depleted by MACS and transferred to i.n. or i.t. infected SCID mice. Tissue titers of reovirus were determined 10 days after cell transfer. Statistical analysis was performed using two-tailed Student's *t* test. Data were pooled from two experiments.

CD8-depleted PP cells cleared reovirus from palatine SG or soft palate tissue of i.n. infected SCID mice with similar efficacy (Fig. 5*B*) and kinetics (data not shown) as unfractionated cells. In marked contrast, depletion of $CD8^+$ cells from PP significantly delayed the clearance of reovirus from lung and trachea of i.t. infected SCID recipients (Fig. 5*B*). Nevertheless, after 21 days recipients of CD8-depleted lymphocytes also had successfully eradicated reovirus from lungs and trachea (data not shown). Likewise, depletion of $CD8^+$ cells from mesenteric LN delayed clearance of virus from the lower respiratory tract. In contrast, depletion of $B220^+$ cells did not have any effect (data not shown).

Discussion

We did not detect infective virus in the upper or lower respiratory tract after intestinal infection, indicating that i.du. delivery resulted in a gut-restricted infection with no or only very limited systemic dissemination of viable virions (Fig. 1, *A* and *B*). Nevertheless, this gut priming conferred protection from subsequent challenge of the respiratory mucosa. Lack of Ab responses in the upper respiratory tract after intestinal priming (Fig. 2*A*) also made it unlikely that migration/redistribution of Ag from the gut to the upper respiratory tract and subsequent priming of lymphocytes in local inductive sites such as NALT (14) were responsible for protection from challenge. Together these data strongly suggest that relocation of B and/or T cells from the gut to the upper and lower respiratory tracts was the major mechanism for cross-protection.

However, we found significant differences in the kinetics and quality of immune responses in the upper vs lower respiratory tracts after intestinal priming. First, the kinetics of Ab production were markedly different. Intestinal priming did not elicit any appreciable Ab secretion in the upper respiratory tract; in contrast, significant production of IgG2a was induced in mediastinal LN by this route of infection. After respiratory challenge, however, strong and rapid memory responses developed in the upper respiratory tracts whereas no accelerated kinetics and only a slight boost of responses were observed in the lower respiratory tract. This suggests an important role for B cells in protection of the upper respiratory tract. We propose that gut-primed (memory) B cells migrated to the upper respiratory tract and accounted for the rapid production of specific IgA and IgG2a. Alternatively, it might be speculated that the rapid recall responses were due to recirculating, rather than residing, memory B cells in the upper respiratory tract. Second, different predominating isotypes of Ab were observed in the upper vs the lower respiratory tract. All tissues from the upper respiratory tract, notably also the submandibular LN, produced both specific IgA and IgG2a; in contrast, IgA was a minor isotype in the lower respiratory tract. This is in line with findings presented by Sangster et al. (15) showing a dichotomy in Ab responses in LN draining the upper vs lower respiratory tract after i.n. Sendai virus infection. Importantly, Palladino et al. (16) demonstrated a more important functional role for IgG compared with IgA Ab in protection of the lungs from influenza infection. Transudation of IgG Ab from the central circulation to the lungs is known to contribute a major proportion of specific Ab at this site, and consequently, even parenteral immunization leads to significant protection of the lower respiratory tract (reviewed in Ref. 17). To more clearly address the role of IgG2a Ab in the upper vs the lower respiratory tract we are currently studying the potential of passively transferred monoclonal, neutralizing reovirus-specific IgG2a Ab to prevent or resolve reovirus infection in SCID mice. Third, the homing of effector memory CTL to the upper vs the lower respiratory tract after intestinal priming was significantly different. CTL effector activity was found in the lungs as early as 10 days after i.du. priming despite the absence of infection at this site. By contrast, effector memory CTL only appeared in infected palatine SG. This along with our results from the adoptive transfer model (Fig. 5*B*) suggest a more prominent role for CTL in surveillance of the lower respiratory tract. Altogether we demonstrate that intestinal priming leads to protection of both the upper and lower respiratory tracts, but that distinct cell types and/or different Ab isotypes account for protection. Our data implicate that the lungs are not a classical site of the common mucosal immune system, but, rather, are part of the systemic immune system. In contrast, we have shown recently that the immune components of the upper respiratory tract share many characteristics with the mucosal immune system of the gut and can thus be considered a more typical mucosal site (14).

Consequently, protection of the upper respiratory tract by gut-primed cells may require specific homing of cells to sites in the upper respiratory tract, whereas transition of gut-primed cells into

the systemic circulation may be sufficient to protect the lower respiratory tract. It remains to be established what mechanisms are operative in other situations of cross-protection, such as protection of the genital tract after nasal priming (10) or possible protection of the gastrointestinal tract after local nasal priming.

Interestingly, we observed memory IgG2a responses in NALT, submandibular LN, and palatine SG after i.n. challenge of i.du. primed mice (Fig. 3*A*) even though only marginal IgG2a responses were induced in the primary mucosal inductive sites of the gut, i.e., the PP, by i.du. priming. However, i.du. priming induced significant production of IgG2a in the mesenteric LN (Fig. 2*C*) (18). Thus, these findings demonstrate that the mesenteric LN may not only serve their acknowledged function as amplifiers of responses induced in PP, but, probably due to a distinct connection to the systemic immune system, may support generation of isotypes that are more important for B cells emigrating to systemic or distant mucosal sites rather than for those destined for homing to the intestinal LP.

It remains largely elusive which homing receptors control cell trafficking within the common mucosal immune system. It is established that $\alpha_4\beta_7$ integrins and CCR9 (reactive with MadCAM-1 and TECK/CCL25, respectively) are responsible for homing of gut-primed B cells, including IgA-producing B cells, and T cells to the intestinal LP (19–21). Csencsits et al. (22) have reported significant differences in the expression of integrins in NALT vs PP, suggesting that different receptors may control homing to mucosal sites distant from the gut. In line with these findings we did not find preferential expression of $\alpha_4\beta_7$ or $\alpha_E\beta_7$ by lymphocytes in NALT or the lungs of immunocompetent mice or SCID mice after transfer of gut-primed lymphocytes (data not shown). Recently, Pan and colleagues (23) described a chemokine receptor, termed MEC/CCL28, that is expressed on epithelial cells of bronchi, salivary glands, colon, and mammary gland. Intriguingly, CCL28 shares high sequence homology with TECK/CCL25 and CTACK/CCL27, which are all involved in homing to mucosal sites (reviewed in Ref. 24). This suggests a possible network of integrin and chemokine receptors that may control trafficking of cells within the common mucosal immune system. It will be particularly important to determine whether the same receptors control homing of B vs T cells and in this respect to analyze the expression of homing receptors on effector memory CTL. Having demonstrated the distinct functional roles and migration characteristics of gut-primed B and T cells in protection of the upper and lower respiratory tracts, our model of local reovirus infection may represent a promising approach to address these open questions.

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