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A Role for IL-15 in the Migration of Effector CD8 T Cells to the Lung Airways following Influenza Infection

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The cytokines generated locally in response to infection play an important role in CD8 T cell trafficking, survival, and effector function, rendering these signals prime candidates for immune intervention. In this paper, we show that localized increases in the homeostatic cytokine IL-15 induced by influenza infection is responsible for the migration of CD8 effector T cells to the site of infection. Moreover, intranasal delivery of IL-15–IL-15Rα soluble complexes (IL-15c) specifically restores the frequency of effector T cells lost in the lung airways of IL-15–deficient animals after influenza infection. Exogenous IL-15c quantitatively augments the respiratory CD8 T cell response, and continued administration of IL-15c throughout the contraction phase of the anti-influenza CD8 T cell response magnifies the resultant CD8 T cell memory generated in situ. This treatment extends the ability of these cells to protect against heterologous infection, immunity that typically depreciates over time. Overall, our studies describe what to our knowledge is a new function for IL-15 in attracting effector CD8 T cells to the lung airways and suggest that adjuvanting IL-15 could be used to prolong anti-influenza CD8 T cell responses at mucosal surfaces to facilitate pathogen elimination. The Journal of Immunology, 2011, 186: 174–182.

C urrent influenza vaccines rely on the induction of a neutralizing Ab response to viral coat proteins that are subject to variation from antigenic drift and shift (1). An alternate vaccination strategy is to immunize against conserved, internal viral proteins recognized by CD8 T cells, a strategy that confers protection against multiple serotypes, or heterosubtypic immunity (2–4). Such a “universal” vaccine could provide cross-protection against the emerging and imminent threats of avian H5N1 and swine-origin H1N1 pandemics. However, despite such promise in generating a cross-protective vaccine, the number of memory T cells (Tmems) in the lung airways that differentiate from local effector cell precursors declines over time, eventually plateauing ~3 mo postinfection (p.i.) (5). Curiously, protective heterosubtypic immunity declines concomitantly, despite the large reservoir of Tmems in the spleen that continues to migrate to the lung airways and maintains a local steady-state pool of Tmems (3, 6). Overall, these data suggest that the effector T cells (Teffs) that gain access to the airways early p.i. and differentiate to Tmems in situ are responsible for the limited heterosubtypic immunity. Thus, understanding how to augment the initial seeding pool of Teffs in the lung airways may represent a unique methodology to increase the resultant pool of Tmems that develops in the respiratory tract and to coordinately prolong protection after a challenge infection.

IL-15 is a common γ-chain cytokine sharing overlapping signaling and biological properties with IL-2 as a result of their mutual usage of the IL-2/-15β and common γ-chain (γc) receptor subunits (7, 8). Cells coexpressing IL-15 with IL-15Rα on their surfaces present the cytokine in trans to responsive IL-2/-15β/γc–expressing lymphocytes, a process referred to as transpresentation (9). Although production of IL-2 is confined to periods of lymphocyte activation, the constitutive expression of IL-15 maintains the homeostatic proliferation of lymphocytes, most notably memory CD8 T cells, in the steady-state through the sustained expression of bcl-2 (10–13). Pathogenic triggers such as type I IFNs, however, can induce the expression of IL-15, and Teffs upregulate expression of IL-2/-15β after activation (12, 14, 15). In addition, systemic administration of IL-15 or soluble IL-15–IL-15Rα complexes (IL-15c) during the contraction phase of the immune response against Listeria monocytogenes and Mycobacterium bovis prolongs Teff survival and results in the preferential accumulation of short-lived KLRG-1hi Ag-specific Teffs (16). Taken together, these observations suggest that infection-induced alterations in IL-15 expression could modulate regional CD8 Teff responses.

Recent studies indeed demonstrated that a subset of dendritic cells (DCs) in the lung transpresent IL-15 to Teffs that have migrated to the respiratory tract after influenza infection (17). Removal of IL-15–producing DCs from the respiratory tract or IL-15 blockade decreases the frequency of Ag-specific CD8 Teffs recovered from the lung. Although the attrition of Teffs in the lung is accelerated in the absence of IL-15, it remains unclear whether this is solely due to the loss of a pertinent survival signal or also the impaired ability of Teffs to traffic to the lung airways, because IL-15 is chemotactic in certain inflammatory milieus (18, 19). In this article, we demonstrate that influenza infection potentiates IL-15 levels in the respiratory tract that drives the migration of Teffs into the lung airways. Moreover, the therapeutic provision of IL-15 during this effector phase of the immune response translates into a greater proportion of locally derived Teffs that differentiate into Tmems in situ. Overall, our studies describe a function for IL-15 in T cell migration in which IL-15c can be used to augment respiratory CD8 T cell effector responses to extend the duration of
heterosubtypic immunity and provide long-term immunological protection to subsequent influenza virus infections.

Materials and Methods
Mice, viruses, and infection

C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD) or Taconic Farms (Germantown, NY), and age- and sex-matched IL-15−/− and C57BL/6-Tg(TcraTcrb)100MjbJ (OT-I) mice were generally provided by Dr. Leo Lefrancóis (University of Connecticut, Farmington, CT) or obtained from Taconic Farms. Influenza viruses A/HK-x31 (C3H/HN2) and A/Puerto Rico 8 (PR8, H1N1) were generously provided by Dr. S. Mark Tompkins (University of Georgia, Athens, GA). The recombiant x31-OVA expressing the CD8 H2-Kb restricted SIINFEKL epitope was generously provided by Dr. Peter Dougherty (St. Jude Children’s Research Hospital, Memphis, TN) (20). Animals were infected with either 107 PFU x31 or 5 × 107 PFU PR8 intranasally (i.n.) in 50 μl PBS.

Quantitative RT-PCR

Total RNA was purified from tissues of naive or 107 PFU x31-infected C57BL/6 mice using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Reverse transcriptions were primed with random primers and performed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Quantitative PCR assays were done using the ABI TaqMan Gene Expression Master Mix from ABI 7500 Real Time PCR System (Applied Biosystems). Only ratios with an SE < 0.2 log (95% confidence limits) were considered for determination of induction levels. Quantitative real-time RT-PCR was performed using IL-15–FAM (#mm00434210_m1) and 18s–VIC (#4319413E) assays (Applied Biosystems). Thermal cycling conditions were 30 min at 48˚C, 10 min at 95˚C, and 40 cycles of denaturation (95˚C for 15 s) and annealing (60˚C for 60 s). Samples were analyzed in triplicate, normalized against 18s, and expressed relative to mock-infected animals. The frequency and number of NP-specific cells in each cohort of animals was determined by gating on CD8+, dump− lymphocytes.

Adaptive transfers and migration and proliferation assays

CD8 T cells were enriched to >88% purity from spleens or lung tissue by negative enrichment per the manufacturer’s instructions (Dynal/Invitrogen, Carlsbad, CA). For in vivo migration assays, cells from the indicated tissues were placed in the top insert of a 0.4-μm chemotaxis Transwell (Fisher Scientific, Waltham, MA). The bottom chamber contained either warm media alone or supplemented with 100 ng/ml recombinant murine IL-15–IL-15Rα Fe-chimeric complexes (R&D Systems, Minneapolis, MN). Percent migration was calculated as number of NP-tet+ CD8 T cells in the bottom chamber divided by number of NP-tet+ CD8 T cells in the input sample. For in vivo migration assays, IL-15-cs containing 1.5 μg recombinant murine IL-15 and 7 μg IL-15Rs Fe-chimeras (R&D Systems) were generated by incubation at 37˚C for 20 min followed by incubation at 4˚C for 10 min and delivered i.n. in 72.5 μl PBS. Similarly, IL-2c and IL-7c containing 1.5 μg recombinant murine IL-2 (eBioscience) or IL-7 (PeproTech, Rocky Hill, NJ) and 7 μg mAb (R&D Systems) were generated by incubation at 37˚C for 20 min followed by incubation at 4˚C for 10 min and delivered i.n. in 72.5 μl PBS. IL-15c was also used to treat animals throughout the contraction phase of the anti-influenza response and was delivered i.m. in 36.25 μl PBS and CD11c deficient mice alone or supplemented with 100 ng/ml recombinant murine IL-15–IL-15Rα Fe-chimeric complexes (R&D Systems, Minneapolis, MN). Percent migration was calculated as number of NP-tet+ CD8 T cells in the bottom chamber divided by number of NP-tet+ CD8 T cells in the input sample. For in vivo migration assays, IL-15-cs containing 1.5 μg recombinant murine IL-15 and 7 μg IL-15Rs Fe-chimeras (R&D Systems) were generated by incubation at 37˚C for 20 min followed by incubation at 4˚C for 10 min and delivered i.n. in 72.5 μl PBS.

Plaque assays

Plaque assays were performed as previously described (22). In brief, lungs from x31-immune wild type (WT) and IL-15−/− mice challenged with PR8 were lysed at the indicated times in 1 ml 1× MEM+ 1 mM TPCK-treated trypsin. Confluent monolayers of Madin-Darby canine kidney cells were incubated with 10-fold serially diluted 10% homogenate for 1 h at 37˚C. The inocula were removed and cells were washed with PBS. Medium was removed and weighed in MEM containing 0.2% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA) (22), 0.04 M HEPES, 0.02 mM l-glutamine, 0.15% NaHCO3 (w/v), and 1 μg/ml TPCK-trypsin. Seventy-two hours p.i., monolayers were fixed with cold methanol/acetic acid (60:40%) and stained with crystal violet.

Statistics

Where appropriate, an unpaired two-tailed Student t test was applied using Prism GraphPad software (CA). The p values are indicated in the figure legends where statistical significance was found. For multiple comparisons, an ANOVA was applied with Tukey’s post hoc analysis using Prism GraphPad software. The p values less than 0.05 were considered significant.

Results

Infection with influenza virus induces localized IL-15 expression in the respiratory tract

IL-15 is constitutively expressed systemically by DCs and macrophages but can also be expressed in mucosal tissues by epithelial cells (10, 23, 24). Moreover, cellular expression of IL-15 is regulated by various pathogenic triggers (15). Namely, the IL-15 promoter contains a type I IFN regulatory element (14, 15), and IL-15 expression is increased in response to type I IFNs (12), which are abundant after influenza infection (25). Indeed, influenza infection has been shown to induce the expression and transspiration of IL-15 in the lungs at day 6 p.i. (16); however, we sought to more carefully characterize the kinetics of IL-15 expression after respiratory infection to better determine how pathogen-induced IL-15 impacts local CD8 T cell
values are represented as mean ± SD compared with mock-infected mice. *p < 0.05 by ANOVA with Tukey’s post hoc analysis; n = 3.

FIGURE 1. Influenza infection induces the local expression of IL-15 in the lung airways. Gene expression levels were quantified from BAL, lung tissue, spleen, and MdLNs by quantitative RT-PCR at the indicated time points p.i. with x31, and are expressed as relative expression greater than baseline in naive animals normalized to 1 using a cycle threshold method. Values are represented as mean ± SD compared with mock-infected mice. *p < 0.05 by ANOVA with Tukey’s post hoc analysis; n = 3.

Because IL-15 protein has been historically difficult to detect in biological solutions, we first monitored IL-15 mRNA expression in the lung airways (via BAL), lung parenchyma, lung-draining mediastinal lymph nodes (MdLNs), and spleens of C57BL/6 mice after i.n. infection with influenza A/Hong Kong x31 (x31). By 3 d p.i., there was a 4-fold induction in the relative expression of IL-15 in the lung airways of infected animals when compared with naive (i.e., mock-infected) animals, and IL-15 mRNA levels remained increased until day 7 p.i. (Fig. 1). Although IL-15 transcription in the airways began to decline by day 10 p.i., it remained 3-fold greater than the levels in naive mice. Expression of IL-15 also increased in the lung parenchyma, MdLNs, and spleen after infection, albeit to a much slighter degree. In addition, a newly developed ELISA that specifically detects soluble IL-15–IL-15Rα complexes (IL-15c) confirmed that expression of IL-15c in the lung lysate was greatly enhanced and quantitatively much greater than the serum of influenza-infected animals on day 3 p.i. when compared with naive mice (Supplemental Fig. 1). Therefore, infection with influenza virus initiates an inflammatory cascade that results in a local increase in the expression of IL-15, suggesting that this cytokine may specifically regulate anti-influenza CD8 Teff responses in the lung airways.

IL-15 is responsible for the early accumulation of anti-influenza Teffs in the lung airways

IL-15 prolongs the survival of activated CD8 Teffs during the contraction phase of the immune response to systemic viral infection, and systemically administered IL-15 can specifically restore the numbers of Teffs lost in IL-15−/− mice (16, 26–28). Recent data also suggest that IL-15 modulates local respiratory CD8 Teff responses via sustained survival signals provided by lung-resident DCs (17). However, given that transient augmentation of IL-15 mRNA expression immediately precedes the initial influx of Teffs to the lung airways (Fig. 1; Teffs first detectable ∼5.5 d p.i.; data not shown), the documented chemotactic properties of IL-15, and the fact that lung parenchymal cells (including influenza-infected lung epithelial cells) can also express IL-15 (29), we hypothesized that, in addition to supporting the survival anti-influenza CD8 Teffs, IL-15 could also participate in the recruitment of the Teffs to the site of infection. To that end, IL-15−/− and WT animals were infected i.n. with x31, and the kinetics of the CD8 T cell response against influenza NP was assessed using MHCI class I tetramers on lymphocytes isolated from the BAL, lung parenchyma, spleen, and MdLNs. Because IL-15−/− animals have half the total number of CD8 T cells as WT animals (11, 30), and correspondingly half the number of NP-specific precursors as WT mice (but the same frequency; Supplemental Fig. 2A–C), the anti-NP CD8 T cell response is expressed as a percentage of total CD8 T cells to accurately compare rates of expansion.

Interestingly, the lung airways of IL-15−/− mice harbored only half the frequency of influenza-specific Teffs at day 7 p.i. (Fig. 2A) and only 25% at day 12 p.i. (Fig. 2B). This reduction in the frequency of NP Tet+ CD8 T cells was observed as early as day 6 (∼40%) but resolved by day 15 p.i. (data not shown). Moreover, when the total number of NP-specific cells recovered from the airways of IL-15−/− at day 6.5 p.i. was assessed, the loss of NP-specific Teffs in IL-15−/− mice was greater than the 50% numerical reduction resulting from their reduced NP-specific precursor number (Supplemental Fig. 2D). Only the CD8 and NK cell pools were numerically deficient in the BAL from infected IL-15−/− mice, and the numbers of B220+ B cells and populations of CD11b and CD11c+ cells were unaffected (data not shown). Furthermore, these deficiencies in CD8 Teff accumulation were specific to the lymphocytes recovered from the BAL, as the percentage of influenza-specific Teffs in the lymphoid tissues of IL-15−/− mice was not compromised (Fig. 2A, 2B). The frequency of NP-specific CD8 T cells was also reduced in the lung parenchyma of IL-15−/− animals at day 12 p.i., though not to significant levels. Thus, an IL-15 deficiency resulted in a specific reduction in the frequency of influenza-specific CD8 Teffs at the site of infection.

To confirm our findings that the absence of IL-15 selectively reduces influenza-specific CD8 T cells in the lung airways with equal Ag-specific precursors, we adoptively transferred 500 OVA-specific CD8 T cells from congenic (CD45.1+) OT-I mice into WT and IL-15−/− CD45.2+ mice. Recipient animals were subsequently infected with x31-OVA, and on day 12 p.i., lymphocytes were isolated from the BAL, lung, and lymphoid tissues, and the level of donor OT-I expansion was calculated. The quantity of OT-I cells recovered from the spleen, MdLNs, and lung parenchyma was similar between WT and IL-15−/− recipients (Fig. 2C). We did observe, however, a 45% reduction in the number of donor OT-I cells isolated from the BAL of IL-15−/− mice. These data confirm our findings that the absence of IL-15 results in a tissue-specific reduction of Ag-specific CD8 T cells in the lung airways of influenza-infected animals.

Delivery of exogenous IL-15c i.n. enhances the accumulation of influenza-specific CD8 Teffs at the site of infection

Although i.v. administration of γc cytokines can affect CD8 T cell responses systemically, it is unclear whether such cytokine therapies can be modified to spatially limit their function. Because an IL-15 deficiency resulted in the selective reduction of CD8 Teffs in the respiratory tract, we sought to determine whether administration of exogenous IL-15 to the lung airways could rescue this defect. To that end, we i.n. delivered either PBS or IL-15–IL-15Rα complexes (IL-15c) to WT or IL-15−/− mice at day 12 p.i. with x31. Delivery of cytokine–receptor complexes increases the cytokine’s potency in vivo, presumably by increasing its half-life (31, 32). Twelve hours posttreatment with IL-15c, cells from the BAL were collected and analyzed for tetramer reactivity. As shown previously, the frequency of NP Tet+ CD8 T cells isolated from the lung airways of IL-15−/− mice was reduced by 50% compared with WT animals; however, this percentage was restored to WT levels when exogenous IL-15c was administered to the respiratory tract of these mice (Fig. 3A).

IL-15 is known to prolong the survival of CD8 Teffs mainly through downstream signaling events that result in the enhanced expression of the antiapoptotic molecule Bcl-2 (26). Accordingly, it has recently been demonstrated that, after influenza infection, IL-15 transpresented to CD8 Teffs in the lung airways is able to...
FIGURE 2. IL-15 deficiency results in a reduced frequency of influenza-specific CD8 Teffs in the lung airways. Lymphocytes from the indicated tissues were isolated and analyzed for tetramer reactivity. Representative flow plots for WT and IL-15−/− animals are shown for day 7 (A) and 12 (B) p.i. with x31 i.n. The mean percentage tetramer-positive among CD8 T cells for WT (shaded bars) and IL-15−/− (open bars) (A) on day 7 p.i. is represented ± SEM (**p = 0.0282; n = 3 mice/group) and (B) on day 12 p.i. is represented ± SEM (**p = 0.0011; n = 3 mice/group). Data are representative of two independent experiments. C, Five hundred naive CD45.1+ OT-I CD8 T cells were adoptively transferred into WT or IL-15−/− CD45.2+ recipients subsequently infected with x31-OVA. Representative flow plots from the BAL, lung, and spleen are shown (left). The mean number of donor OT-I CD8 Teffs isolated from indicated tissues at day 12 p.i. is plotted ± SEM (n = 4–5 mice/group) on right. Data are representative of three independent experiments.

Because of the documented chemotactic properties of IL-15, the concentrated regional expression of IL-15 after influenza infection, and the failure of Teffs to accumulate in the lung airways of IL-15−/− mice after influenza infection, we hypothesized that IL-15 participates in recruiting influenza-specific CD8 Teffs to the site of infection. To test this hypothesis, we first tested whether locally instilled exogenous IL-15 could induce the chemotactic migration of NP-specific CD8 Teffs to the lung airways. WT animals were infected with x31, and 6.5, 12, or 40 d later, we i.n. administered either PBS or IL-15c. We monitored alterations in the accumulation of NP-specific CD8 Teffs in the lung airways 12 h post-treatment to distinguish the effects of IL-15c on migration from its potential effects on proliferation or protection from apoptosis. Administration of exogenous IL-15c increased the number of NP-specific CD8 T cells in the lung airways 8-fold (or ~70 × 10^3 more cells) compared with PBS-treated mice at day 12 p.i. (Fig. 3C), whereas ~50 × 10^3 NP-specific CD8 Teffs were correspondingly lost from the splenic pool of Teffs (data not shown), suggesting that this Teff reservoir may be recruited to the BAL in response to IL-15c treatment. Moreover, local delivery of IL-15c induced the migration of activated CD44hi CD8 and CD4 T cells to the lung airways (Fig. 3C), whereas naive (CD44lo) CD8 and CD4 T cells and B220+ cells did not accumulate (data not shown). The specific augmentation in the number of influenza-specific CD8 Teffs could only be observed as early as day 6.5 p.i. (Fig. 3B) because few NP-tet+ CD8 T cells could be recovered from the BAL at earlier time points, but this effect was abrogated by the memory phase of the response (day 40 p.i.; Fig. 3D). These data indicate that either Tmems are refractory to IL-15–mediated migratory signals or are incapable of accumulating in measurable numbers considering that the vasculature beds may be less permissive for lymphocyte entry in the absence of inflammation, irrespective of a chemotactic signal. IL-15 was specifically responsible for eliciting the migration of influenza-specific CD8 Teffs to the lung airways because the i.n. instillation of complexes of the common γc cytokines IL-2 or IL-7 failed to induce the trafficking of CD8 Teffs to the lung airways (Fig. 3E).

Recently, it has been reported that pulmonary DCs promote the survival of influenza-specific CD8 Teffs through transpresentation of IL-15 (17). In our studies, it was possible that, in addition to the effect of IL-15 on the migration of CD8 Teffs, IL-15–dependent proliferation and/or enhanced survival were also contributing to the observed accumulation of CD8 Teffs in the lung airways after IL-15c treatment. To test whether administration of IL-15c affects CD8 Teff proliferation, we infected animals with x31; 12 d later, we pulsed animals with BrdU and simultaneously administered PBS, IL-15, IL-7, or IL-2 complexes i.n. Compared with PBS-treated animals, treatment with any of the γc cytokine complexes did not augment the levels of BrdU incorporation by BAL resident anti-influenza Teffs (Fig. 3F, 3G). To test whether IL-15c enhanced the survival of Teffs otherwise destined to die, CD8 Teffs isolated from the lung airways 12 h after γc cytokine complex treatment were stained with 7-AAD, a fluorescent dye excluded from viable cells. Analysis of NP-specific CD8 T cells from IL-15c, as well as IL-7 and IL-2 complexes, revealed that all three cytokine treatments enhanced the survival of CD8 Teffs because ~50% of these CD8 T cells excluded 7-AAD (Fig. 3F, 3G). In accordance with previous studies, these data confirm that IL-15 provides sustained survival signals to influenza-specific Teffs in...
FIGURE 3. Exogenous IL-15 results in the accumulation and enhanced survival of influenza-specific CD8 Teffs at the site of infection. A, Twelve days p.i. with x31, WT or IL-15−/− received PBS or IL-15–IL-5Rα complexes (IL-15c) as indicated below horizontal line. BAL was collected 12 h post-treatment, and isolated lymphocytes were analyzed for tetramer reactivity. The mean percent NP-tetramer+ among CD8 T cells is represented ± SEM. *p = 0.0291; n = 4 mice/group. Data are representative of two independent experiments. B, 6.5 or (C) 12 d after x31 infection, animals received either PBS or IL-15c. Twelve hours later, BALs were analyzed for CD4, CD8, and CD44 expression and tetramer reactivity. Mean cell number is plotted ± SEM in corresponding right panels. *p = 0.0024; n = 3 mice/group. Data are representative of three independent experiments. D, Forty days after x31 infection, animals received either PBS or IL-15c. Twelve hours later, BALs were analyzed for CD4, CD8, and CD44 expression and tetramer reactivity. Mean cell number is plotted ± SEM. E, Twelve days after x31 infection, animals received PBS (black bar), IL-15c (open bar), IL-7–anti–IL-7 mAb complexes (IL-7c; dark shaded bar), or IL-2–anti–IL-2 mAb complexes (light shaded bar) i.n. Twelve hours later, BALs were analyzed for tetramer reactivity. Mean cell
the absence of proliferation (17). However, IL-2 and IL-7 complexes were also effective at enhancing the survival of influenza-specific Teffs without affecting Teff accumulation in the lung airways, as observed with IL-15c treatment (Fig. 3E). Thus, these data show that exogenous IL-15c delivered to the lung airways uniquely induced the migration of CD8 Teffs to the site where interaction with IL-15 also augmented survival in situ.

**IL-15 induces the migration of influenza-specific CD8 Teffs from the systemic Teff pool**

Our data demonstrate that localized in vivo administration of exogenous IL-15c recruited influenza-specific CD8 Teffs to the lung airways; however, it is unclear whether this IL-15R-induced migration was the result of direct chemotaxis to the IL-15c, modification of chemokine receptor expression by IL-15c treatment, or an alteration of a secondary chemotactic signal by an accessory cell affected by the IL-15c treatment. To begin to distinguish between these possibilities, we monitored the direct effects of IL-15c on Teff migration in an in vitro chemotaxis assay. Single-cell suspensions from the lung parenchyma, BAL, spleen, MLNs, and nondraining inguinal lymph nodes of day 12 x31-infected animals were placed in the top chamber of a transwell chemotaxis chamber, whereas media or media supplemented with IL-15c were placed in the bottom. After 90-min incubation, cells in the bottom chamber were collected, counted, and the percentage of NP-specific CD8 T cells that had migrated toward the IL-15c was determined. Although NP-specific CD8 Teffs isolated from both lymph nodes migrated fairly efficiently toward the IL-15c, the Teffs isolated from the lung parenchyma and spleen exhibited a 10-fold increase in migration to IL-15c (Fig. 4A). However, the Teffs isolated from the BAL migrated poorly toward IL-15c, presumably because they have downregulated expression of IL-2/15Rβ (CD122; Fig. 4B) after IL-15–induced migration and arrival at their terminal destination.

Overall, how Teffs enter the lung airways is not well understood because two routes of entry are available to them. Lymphocytes can enter the lung airways either by traversing the lung parenchyma or by bypassing the lung tissue and entering directly from the circulation (35). Because CD8 Teffs isolated from both the spleen and the lung tissue readily migrated to IL-15c in vitro, we asked whether influenza-specific CD8 Teffs from one anatomical location preferentially migrated to IL-15c in vivo. To pursue this question, donor CD8 Teffs were enriched from the lung or the spleen of day 12 x31-infected CD45.2 WT donor mice and independently transferred into congenic CD45.1+ recipients that had migrated toward the IL-15c was determined. Although NP-specific CD8 Teffs isolated from both lymph nodes migrated fairly efficiently toward the IL-15c, the Teffs isolated from the lung parenchyma and spleen exhibited a 10-fold increase in migration to IL-15c (Fig. 4A). However, the Teffs isolated from the BAL migrated poorly toward IL-15c, presumably because they have downregulated expression of IL-2/15Rβ (CD122; Fig. 4B) after IL-15–induced migration and arrival at their terminal destination.

Indicate that circulating Teffs are partially dependent on IL-15 for their immigration into the lung airways and those Teffs that have mobilized into the lung parenchyma either do not migrate to IL-15 or use IL-15–independent mechanisms to traverse into the airways.

**Mucosal delivery of IL-15c during the effector phase of the anti-influenza CD8 T cell response enhances the resultant Tmem pool**

Because IL-15 recruits influenza-specific CD8 Teffs to the lung airways and prolongs their survival, adjuvanting IL-15 in the respiratory mucosa could numerically boost the CD8 Tmems generated from Teffs maintained at this site. WT mice infected with x31 were given IL-15c or PBS i.n. every other day between day 10 and 20 p.i., to test this hypothesis. Animals were rested until memory (day 31 p.i.) when cells from the lung airways and other indicated tissues were collected, counted, and analyzed for the presence of NP-specific CD8 Teffs. Animals receiving IL-15c retained a greater frequency (2 x + number of NP-tet+ CD8 T cells in the Balb/c input sample. Mean percentage migration is calculated as number of NP-tet+ CD8 T cells divided by number of NP-tet+ CD8 T cells in the input sample. Mean percentage migration is plotted + SEM among three replicates for each tissue sample. B, The mean percentage of CD122+ cells among day 12 CD8 Teffs was determined and plotted + SEM (n = 3 mice/group).

**FIGURE 4. IL-15 is chemotactic for influenza-specific CD8 Teffs in vitro. A, 1 x 106 bulk cells isolated from the indicated tissues of WT animals infected 12 d previously with x31 were placed in the upper chamber of a 0.5-μm Transwell with media alone or supplemented with IL-15c in the bottom chamber. Percentage migration was calculated as number of NP-tet+ CD8 T cells in the bottom chamber divided by number of NP-tet+ CD8 T cells in the input sample. Mean percentage migration is plotted + SEM among three replicates for each tissue sample. B, The mean percentage of CD122+ cells among day 12 CD8 Teffs was determined and plotted + SEM (n = 3 mice/group).**

number is plotted + SEM (n = 4 mice/group). *p < 0.0400. Data are representative of two independent experiments. F and G, At d 12 p.i., WT mice were pulsed with 2 mg BrdU i.p. and simultaneously received either PBS, IL-15c, IL-7c, or IL-2c i.n. Twelve hours later, BAL was collected and stained with 7-AAD, and subsequently intracellularly stained for BrdU. Representative flow plots (F) and mean percentage of BrdU+ (top) or 7-AAD+ (bottom) among NP-tet+ CD8 T cells (G) is plotted + SEM (n = 4 mice/group).
however, the lungs of IL-15c–treated animals had lower viral titers than those of PBS-treated animals (data not shown). More importantly, no detectable virus was isolated from IL-15c–treated mice as early as day 6 postchallenge, whereas WT mice did not clear virus until 2 d later (Fig. 6C and data not shown). Thus, by altering the dynamics of the CD8 Teffs initially seeding the respiratory tract, IL-15c quantitatively and qualitatively augmented the resultant Tmems developing in the lung airways, suggesting IL-15c therapy could be used as a means of prolonging hetero-subtypic immunity to influenza infection.

Discussion

The prevention of influenza epidemics is reliant on the generation of a potent and long-lived pool of CD8 Tmems capable of cross-protecting hosts from multiple strains of virus, which are continually evolving between and within humans and other mammalian and avian hosts. Unfortunately, the duration of the protection afforded by respiratory CD8 Tmems is limited because these cells are lost over time (3, 5). Given that the factors responsible for this attrition are ill defined, a reasonable methodology to prolong long-term immunity to influenza infection is to reset the initial frequency of Ag-specific CD8 Teff seeding the lung airways, which would coordinately increase the numerical output of Tmems. This methodology requires a deeper understanding of the factors and mechanisms used by Ag-specific CD8 Teffs to migrate into and survive within the lung airways after influenza infection for exploitation as a vaccine adjuvant.

In the 15 y since its discovery, IL-15 has been implicated in the development, activation, or maintenance of many cell types both inside and outside the immune system (10). The ability of IL-15 to mediate so many effects on divergent cell populations requires regulation of both IL-15 and its receptors. Our studies define what to our knowledge is a new function for IL-15, namely, modulating the trafficking of Teffs to the lung airways. This function of IL-15 may require regulation distinct from our traditional view of how IL-15 delivers signals for the survival and proliferation of CD8 Teffs and Tmems. In these scenarios, IL-15 is transpresented by DCs and macrophages to CD8 Tmems that express CD122 (36–38).

Although DCs can transpresent IL-15 in the lung airways after influenza infection, and this method supports Teff survival, substantial levels of IL-15 are produced by non-DC populations. Airway epithelial cells constitutively express IL-15 and IL-15Rα on their apical surfaces and are directly juxtaposed to the vascular endothelium (39). During inflammation, airway epithelial cells can be induced to express IL-15 on the basolateral surface, potentially making the cytokine available to cells in the vasculature (39). Viral infection also induces the expression of metalloproteases such as ADAM17, which can cleave IL-15Rα from the surface of cells to create soluble IL-15–IL-15Rα complexes, similar to our i.n. delivered IL-15c (40). Indeed, soluble IL-15c can be detected as early as day 3 p.i. in the lung, as well as in the serum (Supplemental Fig. 1). With IL-15 message levels continuing to increase locally in the

FIGURE 5. IL-15 induces the migration of influenza-specific CD8 Teffs in vivo. A, Methods schematic for B and C. B, 2.5 × 10⁶ CD8 T cells from the spleens and lungs of CD45.2+ mice 12 d after x31 infection were adoptively transferred i.v. into identically infected CD45.1+ recipients. One hour posttransfer, recipients received either PBS or IL-15c i.n.; 12 h later, BAL was collected and the number of donor (CD45.2+) NP-specific CD8 Teffs migrating into the lung airways was determined by flow cytometry. C, Mean number of donor cells recovered is plotted ± SEM. *p = 0.0224 (lung), 0.0019 (BAL, A), and 0.0002 (BAL, B); n = 6 mice/group. Data are representative of two independent experiments.

FIGURE 6. Mucosal delivery of IL-15c during the effector phase of the anti-influenza CD8 T cell response augments the resultant Tmem pool. Starting 10 d after x31 infection, animals received either PBS or IL-15c every other day for 10 d. Animals were rested for an additional 11 d until memory (day 31 p.i.) when cells from the indicated tissues were collected, counted, and analyzed for CD8 expression and NP-tetramer reactivity. Plotted are the mean frequencies (A) or mean numbers (B) of NP Ter+ CD8 T cells ± SEM. *p = 0.0224 (lung), 0.0019 (BAL, A), and 0.0002 (BAL, B); n = 6 mice/group. Data are representative of two independent experiments. C, On day 45 after x31 infection, WT and IL-15c−/− memory mice treated with PBS (squares) or IL-15c (triangles) as in A and naive (circles) animals were challenged with PR8; 6 d later, lung viral titers were determined by plaque assay (n = 3 mice/group).
lungs (in the absence of sustained or increasing levels of IL-15c), it is likely that these complexes are shed locally only during a distinct time frame, and subsequent responses to IL-15 in the lung are due to interaction with membrane-bound complexes (Fig. 1, Supplemental Fig. 1) (16). These two forms of IL-15 are likely important in creating a chemotactic gradient for migrating CD8 Teffs that instructs them to leave the circulation and enter the lung airways. The spatial and temporal expression of the soluble versus membrane forms of IL-15 could regulate the migration and prosurvival functions of IL-15, respectively. Ongoing studies will explore these possibilities.

How CD8 T cells “see” these complexes is unclear; IL-15 has dual binding sites for IL-15Rα; therefore, CD8 T cells responding to these IL-15 complexes could potentially receive signals through either IL-15Rα or CD122, which are both expressed on the surface of Ag-experienced CD8 T cells (10, 41). Studies thus far have focused only on IL-15 signaling through CD122; however, IL-15Rα can transmit signals either alone or in conjunction with γc and the newly identified transmembrane tyrosine kinase Axl (42). The differential coupling and signaling through these IL-15Rs may help integrate IL-15 signals into unique cascades that result in different biological functions, including survival, proliferation, and migration. Future studies in our laboratory will define how IL-15 signaling regulates Teff migration to the lung airways.

Although signaling through CD122 can enhance the survival of KLRG-1−/− Teffs (16, 43), and pulmonary DCs can provide survival signals to resident influenza-specific CD8 Teffs through IL-15 transpresentation (17), our data show for the first time, to our knowledge, that IL-15 also evokes the chemotactic migration of pulmonary CD8 Teffs to the site of infection. Anti-NP CD8 Teffs isolated from secondary lymphoid tissues are only minimally affected by an IL-15 deficiency, despite the fact that influenza infection increased IL-15 mRNA levels in these sites, whereas respiratory-bound anti-influenza effectors are numerically compromised. In addition, the timing of the influenza-induced IL-15 expression in the lung airways precedes and coincides with Teff influx, and the anti-influenza CD8 Teffs in the lung airways are enriched by 8-fold only 12 h after IL-15c treatment, a time frame more consistent with enhanced migration than survival (Fig. 3). Although our in vitro chemotaxis assays suggest that the enhancement of Teff migration is a direct effect of IL-15 signaling, IL-15 can induce the transcription of chemokine receptors of the CC family in human PBLs (44). Lymphocytes exposed to IL-15 also morphologically resemble motile T cells with polarized expression of chemokine receptors at the leading edge and adhesion molecules on the uropod (45). Moreover, IL-15 specifically increases the expression of LFA-1, an integrin required for lung-specific trafficking of T cells (6, 33); however, none of these IL-15–induced effects on T cell migration has been studied in an infection model.

Perhaps the most significant finding of our work, however, is that IL-15c instilled directly into the respiratory tract can augment regional CD8 Teff responses and coordinately prolongs the persistence of immunological memory to influenza infection. Memory mice treated with IL-15c during the effector phase exhibited more rapid clearance of a heterologous influenza infection (Fig. 6), even when challenged a full 25 d after treatment had discontinued, implying that augmenting the number of Teffs gaining access to the lung airways concordantly augments the resultant Tnems that differentiate in situ and mediate protection. Importantly, the use of IL-15 as a therapeutic or vaccine adjuvant may not be limited to influenza virus, because infection with dual recombinant vaccinia viral vectors expressing both gp160 and IL-15 resulted in a heightened CD8 Teff and Tnem response against this HIV Ag (46). Although γc cytokines, most notably IL-2, have been used as adjuvants with vaccines against specific cancers and infectious diseases (47), toxicity and half-life issues have precluded their success in clinical trials. However, by complexing IL-15 to its receptor and delivering i.n., direct communication between the cytokine and responding Teffs can be established locally and temporally regulated without affecting lymphocytes in other sites.

In summary, our experiments demonstrate for the first time, to our knowledge, that IL-15c can be used therapeutically to augment the number of Teffs that traffic to and survive in the lung airways, suggesting that IL-15c can be used as a vaccine adjuvant to boost site-specific immunity. The implications of continual enhanced recruitment of Teffs to the lung airways could be extended to multiple disease models, including cancer and other chronic infections, but is especially relevant to influenza infection, where the efficacy of CD8 Teff cell-mediated protection and viral clearance is linked to the number of virus-specific CTLs present directly in the mucosa before challenge (3, 5). Periodic recruitment and augmentation of T cells reactive to conserved influenza epitopes by an adjuvanted IL-15 boost could sustain Teffs above numerical thresholds and restore their protective capacity to both seasonal and pandemic strains of influenza.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1. Soluble IL-15/IL-15Ra complexes are released in response to influenza infection. At the indicated timepoints p.i., lung lysates (A) and serum (B) were collected from naïve IL-15⁻/⁻ and wild type or infected wild type animals and analyzed for the presence of IL-15/IL-15Ra complexes by ELISA as described in the Materials and methods section. Plotted are the means ± SEM (n=3 mice/group).
Supplemental Figure 2. **IL-15−/− mice have a reduced number but similar frequency of NP-specific naïve precursor CD8 T cells.** Total lymphocytes from spleen and all visible lymph nodes were taken from OT-I Rag2<sup>−/−</sup> mice, naïve wild type and IL-15<sup>−/−</sup> mice, and a memory wild type mouse (infected approximately 9 mo. previously). (A) Representative plots for lymphocytes previously gated on CD8<sup>+</sup> and negative for CD4, CD19, and CD11c following pMHC class I tetramer enrichment with both PE- and APC-labeled H-2D(b) tetramers. (B and C) Graphical representation of the NP-specific naïve precursor number (B) and frequency (C) among CD8 T cells. Each symbol represents an individual mouse, and lines represent mean values (*p=0.0179).