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Selective accumulation of differentiated CD8$^+$ T cells specific for respiratory viruses in the human lung

Godelieve J. de Bree,[1,2] Ester M.M. van Leeuwen,[2,3] Theo A. Out,[2]
Henk M. Jansen,[1] René E. Jonkers,[1] and René A.W. van Lier[2]

[1]Department of Pulmonology, [2]Department of Experimental Immunology, and [3]Department of Nephrology, Academic Medical Center, University of Amsterdam, 1100 DE Amsterdam, Netherlands

The lungs are frequently challenged by viruses, and resident CD8$^+$ T cells likely contribute to the surveillance of these pathogens. To obtain insight into local T cell immunity to respiratory viruses in humans, we determined the specificity, phenotype, and function of lung–residing CD8$^+$ T cells and peripheral blood CD8$^+$ T cells in a paired analysis. The lung contained markedly higher frequencies of influenza (FLU)–specific and respiratory syncytial virus (RSV)–specific CD8$^+$ T cells when compared with the circulation. This contrasted with an equal distribution of cytomegalovirus- and Epstein-Bar virus–specific CD8$^+$ T cells. Noticeably, a substantial fraction of the lung–residing FLU– and RSV–specific CD8$^+$ T cells had progressed to a relatively late differentiation phenotype, reflected by low expression of CD28 and CD27. Lung–derived FLU–specific CD8$^+$ T cells had low activation requirements, as expansion of these cells could be initiated by cognate peptide in the absence of helper cell–derived signals. Thus, the human lung contains high numbers of differentiated FLU– and RSV–specific memory CD8$^+$ T cells that can readily expand upon reexposure to virus. Resident lung T cells may provide immediate immunological protection against pulmonary virus infections.

CORRESPONDENCE
Godelieve J. de Bree:
g.j.debree@amc.uva.nl

Abbreviations used: CFSE, carboxyfluorescein diacetate succinimidyl ester; FLU, influenza; HPS, human pool serum; LMC, lung mononuclear cell; MFI, mean fluorescence intensity; RSV, respiratory syncytial virus.

The mucosal surface of the respiratory tract supplies a large area for the entrance of viruses. Still, although frequently challenged, the respiratory tract is normally kept free from overt infections. The human lung parenchyma and the airways harbor, next to cells of the innate immune system, high numbers of T lymphocytes that may contribute to the local defense against these viruses. Indeed, several studies have shown high numbers of T cells in the lungs and provided support for local T cell activation in bronchoalveolar lavage and sputum of healthy individuals, as well as in the context of such inflammatory pulmonary diseases as chronic obstructive pulmonary disease, asthma, and interstitial lung diseases (1–5). Compared with the circulating pools, increased expression of the activation antigen CD69 is found on both CD4$^+$ and CD8$^+$ T cells derived from the lungs (3). Moreover, the strong ability to produce the effector cytokine IFN-γ infers that local T cells are equipped to rapidly combat emerging pulmonary viral infections (6). However, at this moment, it is unclear to what ex-

tent T cells specific for respiratory viruses reside in the human lung and how they may contribute to local defense mechanisms.

Most data on the development of antiviral CD8$^+$ T cell responses and their role in the protection against viral infections have been retrieved from animal studies (7). After primary infection, naive T cells expand and differentiate into cytotoxic effector cells that are able to eliminate virus-infected cells. After viral clearance, the effector T cell pool contracts and a virus-specific memory T cell pool persists that can undergo rapid reactivation after reinfection. Concerning respiratory viruses, these memory cells persist both in secondary lymphoid organs and the lung (8, 9). Reinfection not only recruits virus-specific memory cells from the secondary lymphoid organs to the lungs, but it also induces the rapid expansion of local virus-specific memory cells (10). Therefore, protective immunity against respiratory viral infections may to a significant extent result from reactivation of CD8$^+$ memory T cells localized in the lungs.

Through MHC/peptide-tetramer technology, the buildup of human virus–specific CD8$^+$ T cell responses can be analyzed (11). Function-

The online version of this article contains supplemental material.

ally different types of memory T cells can be discerned with respect to the expression of the cell surface molecules IL-7Rα, CCR7, CD28, and CD27, as well as molecules involved in cytotoxicity, such as perforin and granzyme B (12). Marked differences have been detected between virus-specific cells reactive toward acute viruses, including the respiratory viruses influenza (FLU) and syncytial virus (RSV), and CD8+ T cells recognizing herpesviruses, such as CMV. Circulating CD8+ T cells specific for acute viruses, as FLU and RSV, maintain high CD27, CD28, and IL-7Rα expression (13, 14), do not contain cytotoxic molecules, and have been typified as early memory T cells (15). On the other side of the spectrum, most circulating CMV-specific T cells have a late differentiation phenotype characterized by down-regulation of CD28, CD27, and to a large extent IL-7Rα, and constitutive cytotoxicity ability (12, 15, 16). These data suggest that different viruses elicit distinct functional subsets of CD8+ T cells that have evolved to generate protective immunity to these pathogens.

In this study, we had the unique opportunity to investigate the presence and function of virus-specific cells in the human lung to acquire knowledge on the contribution of local T cell immunity in protecting humans from respiratory viruses.

RESULTS
Lung–residing CD4+ and CD8+ T cells show an activated phenotype
To determine the activation state of total lung CD4+ and CD8+ T cells, the expression of the activation marker HLA-DR was analyzed on lung and circulating T cells. HLA-DR
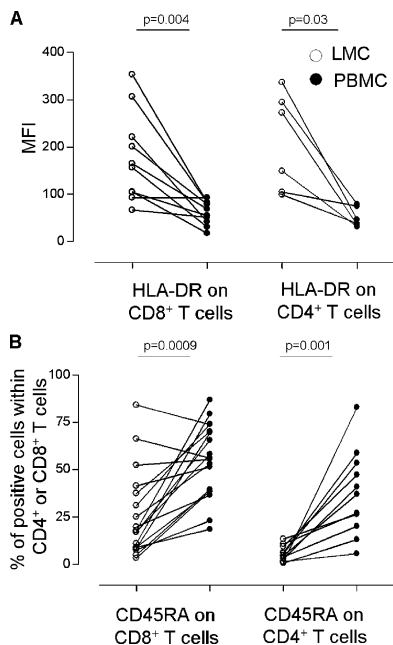
expression was significantly higher on CD8+ and CD4+ T cells in the lung than in the peripheral blood (HLA-DR on CD8+ cells, P = 0.004; HLA-DR on CD4+ cells, P = 0.03; Fig. 1 A). In addition to HLA-DR, the conversion of CD45RA to its isoform CD45R0 has also been associated with antigen-induced T cell activation (17). In accordance with prior contact with antigen, lung-residing CD4+ and CD8+ T cells displayed a significantly lower expression of CD45RA when compared with circulating T cells (CD45RA on CD8+ cells, P = 0.0009; CD45RA on CD4+ cells, P = 0.001; Fig. 1 B).

Lung–derived CD4+ and CD8+ T cells have a differentiated memory phenotype
Because the respiratory tract is continuously challenged by invading respiratory pathogens, the appearance of activated CD4+ and CD8+ T cells in the lung raises the question of whether these cells are part of a local memory T cell pool and may possibly serve to control respiratory viral infections. If so, it might be expected that the local T cell population contains high frequencies of antigen-experienced cells but only low numbers of naive T cells. Indeed, few naive CD8+ T cells, characterized by the CD45RA+
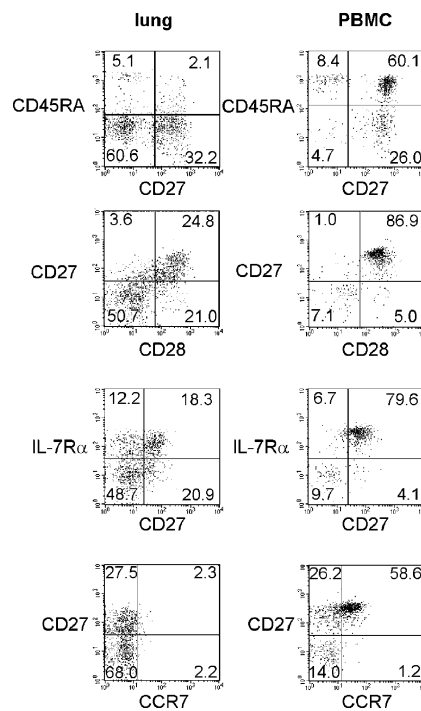
Figure 2. Lung–residing CD8+ T cells show a more differentiated phenotype compared with circulating CD8+ T cells. Dot plots of CD8+ T cells from PBMCs and LMCs of one representative donor. CD45RA-PE fluorescence versus CD27-FITC fluorescence, CD27-PE fluorescence versus CD28-FITC fluorescence, anti–IL-7Rα–PE fluorescence versus CD27-FITC fluorescence, and CD27-PE fluorescence versus anti–CCR7-PE fluorescence of lymphocytes gated on CD8+ T lymphocytes. The dot plots on the left represent LMCs, and the dot plots on the right represent PBMCs.

Figure 1. Lung–residing CD4+ and CD8+ T cells are activated. Expression of HLA-DR on CD8+ and CD4+ T cells (A) and expression of CD45RA on CD8+ and CD4+ T cells (B) on LMCs and PBMCs. Wilcoxon matched-pairs signed-rank test.

CD27^{bright} phenotype, were found in the lung (Fig. 2, top-right quadrant; reference 18). Similar data were obtained for the CD4^{+} T cell compartment (not depicted). To further dissect the antigen–primed population, the expression of additional differentiation markers on both CD8^{+} (example shown in Fig. 2 A) and CD4^{+} T cells was analyzed. Lung CD8^{+} T cells had a significantly lower expression of CD27, CD28, and IL-7Rα (Figs. 2 and 3A) and had lost expression of CCR7 (Fig. 2, bottom) compared with matched peripheral blood CD8^{+} T cells. Also in the CD4^{+} T cell fraction, the expression of CD27 and CD28 was significantly lower in the lung than in the circulation (Fig. 3 B). These latter findings indicate that the populations of CD4^{+} and CD8^{+} T cells in the lung contain cells with a relatively late memory differentiation phenotype (15).

Studies on circulating T cells have implied that the loss of CD27 during T cell differentiation coincides with the stable acquisition of cytotoxic effector molecules (18). We determined the granzyme B content of CD8^{+} T cells in the lung and peripheral blood in a subgroup of donors ($n = 5$). Lung and circulating CD8^{+} T cells contained comparable frequencies of granzyme B–expressing lymphocytes (percentage of granzyme B^{+} CD8^{+} T cells in the lung: median 9.9, range 1.5–40.6 vs. in PBMCs: median 9.8, range 0.3–31.6). However, the granzyme B expression in the CD27^{−} subset was markedly lower in the lung-derived CD8^{+} T cells (mean fluorescence intensity [MFI]: 128.9 median, range 33.8–343) than in circulating CD27^{−} CD8^{+} T cells (MFI: 350 median, range 66.2–1104). An example of a representative donor is shown in Fig. 4. These data suggest that although pulmonary CD8^{+} T cells have the phenotypic characteristics of more

differentiated CD27^{−} CD28^{−} cells, they did not yet acquire complete cytotoxic potential.

The expression of IL-7Rα is down-regulated on virus-specific T cells during acute antiviral responses (19, 20). Additionally, we recently showed that in healthy individuals latently infected with herpesviruses, many T cells specific for these viruses lack IL-7Rα expression (16). To discriminate between these two entities, we determined the activation status of lung IL7Rα^{−} CD8^{+} T cells in two donors. We found that a substantial part of the IL-7Rα^{−} cells expressed HLA-DR (the percentages of HLA-DR^{+} cells of total CD8^{+} IL-7Rα^{−} cells were 62 and 72%), indicating that to a considerable extent, local immune activation is responsible for the presence of IL-7Rα^{−} cells.

Relative enrichment of differentiated FLU– and RSV–specific memory CD8^{+} T cells in the lung

To evaluate the specificities of lung-residing T cells, the frequency of CMV-, EBV-, FLU-, and RSV-specific CD8^{+} T cells was determined by HLA/peptide tetramers (Table I). A significantly higher percentage of FLU tetramer^{+} CD8^{+} T cells in the lung (percentage of tetramer^{+} cells within CD8^{+} cells: median 1.2, range 0.2–10.0) compared with their equivalents in the peripheral blood (median 0.06, range 0–0.3; P = 0.002) was found. Also, the percentage of RSV tetramer^{+} CD8^{+} T cells tended to be higher in the lung (median 0.3, range 0.09–1.9) when compared with the pe-

Figure 4. Lung–derived CD27^{−} CD8^{+} T cells have a lower granzyme B content than circulating CD27^{−} CD8^{+} T cells. (A) Histograms of CD8 expression (LMC on the left and PBMC on the right). The horizontal line indicates the gate for CD8[+bright] lymphocytes (top). Histogram of CD27 expression gated on CD8[+bright] cells (bottom). The horizontal line indicates the gate for CD27^{+} and CD27^{−} cells. (B) Histogram of granzyme B expression gated on CD8^{+} CD27^{+} cells (left) and CD8^{+} CD27^{−} cells (right). The solid line represents LMC, and the dashed line represents PBMCs. The numbers indicate the MFI for granzyme B. An example of one representative experiment is shown.
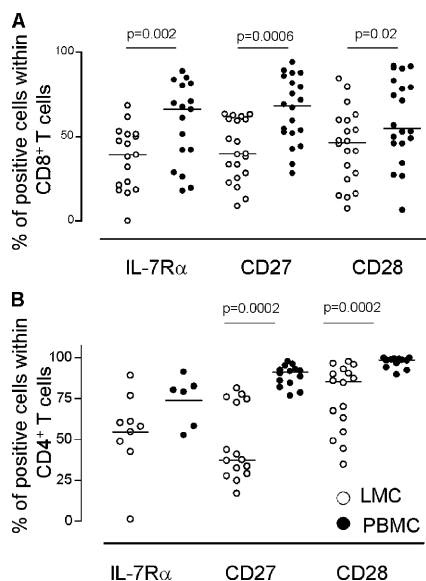
Figure 3. Lung–residing CD8^{+} T cells show a more differentiated phenotype compared with circulating CD8^{+} T cells. Scatter plot showing the expression of IL-7Rα, CD27, and CD28 on CD8^{+} T cells (A) and CD4^{+} T cells (B) on LMCs and PBMCs. Wilcoxon matched-pairs signed-rank test.
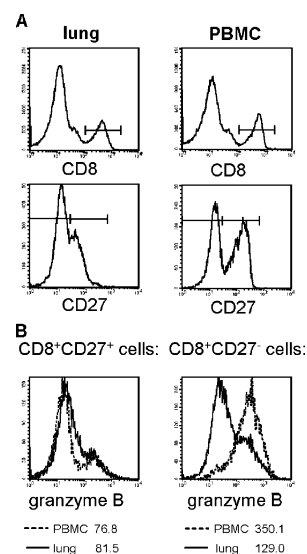
Table I. MHC class I tetramers

HLA–type specificity	Virus	Sequence	Reacting donors
A*0101	RSV	YLEKESIYY	5/8[a]
A*0101	FLU	CTELKLSDY	5/8
A*0201	FLU	GILGFVFTL	5/12
A*0201	CMV	NLVPTMVATV	2/12
B7	CMV	TPRVTGGGAM	2/4
B7	EBV	RPPIFIRRL	2/4
B8	EBV	RAKFKQLL	2/4

[a]Number of donors reacting with the tetramer/number of donors with HLA-type matching the tetramer.

ripheral blood (median 0.02, range 0–0.04; P = 0.12). In contrast, frequencies of EBV- (lung: median 1.0, range 0.2–11.0) and CMV-specific T cells (lung: median 2.4, range 0.07–4.3) did not differ from those in the circulation (EBV: median 0.9, range 0.2–2.5; CMV: median 1.9, range 0.8–12.4; P = 0.6 and P = 0.6, respectively; Fig. 5). Because the lung T cell pool contains, in contrast to the circulating T cell pool, very few naive cells, this might distort the observed accumulation of FLU- and RSV-specific cells in the lung. Therefore, we corrected the percentages of tetramer^{+} cells in PBMCs and LMC for the fraction of naive (CD45RA^{+} CD27^{bright}) cells in PBMCs and lung mononuclear cells (LMCs). However, this correction did not change the significance levels of the previous observations (Table II).

Based on their functional properties and CD27^{+} CD28^{+} CCR7^{+} phenotype, circulating FLU- and RSV-specific CD8^{+} T cells have recently been characterized as memory T cells with an early differentiation phenotype (13, 14). In a subgroup of donors, the characteristics of lung- and PBMC-derived FLU- (n = 9), RSV- (n = 5), CMV- (n = 4), and EBV- (n = 4) specific cells were analyzed by tetramer staining (example shown for two donors for FLU and EBV in Fig. 6). Lung-residing FLU-specific T cells had a low expression of CD28 but had retained a relatively high expression of both CD27 and IL-7Rα (Fig. 7). In contrast, in all donors eligible to analysis, RSV-reactive CD8^{+} T cells in the lung had low expression of both CD28 and CD27 (Fig. 7). In addition, we determined in three donors whether lung-derived FLU- and RSV-specific CD8^{+} cells contained granzyme B (shown for two donors in Fig. 6 E). In all three donors, the tetramer^{+} cells showed a very low granzyme B
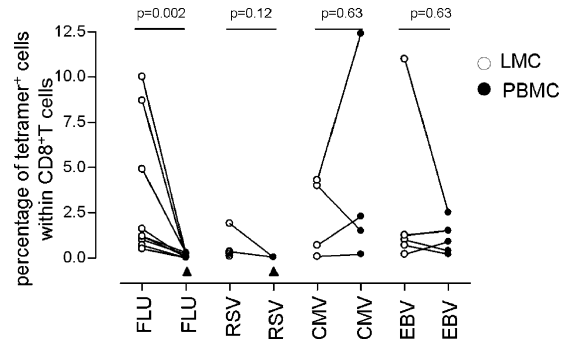

Figure 5. Paired analysis of the frequency of FLU–, RSV–, CMV–, and EBV–specific CD8^{+} T cells in the lung and PBMCs. The frequencies of virus-specific cells are expressed as the percentage of tetramer^{+} cells within CD8^{+} T cells (y axis). In 4 of the 10 donors analyzed with FLU tetramers and 3 out of the 5 donors analyzed with RSV tetramers, tetramer^{+} cells could not be detected in the PBMC. Wilcoxon matched-pairs signed-rank test. The differences in percentages of RSV-, CMV-, and EBV-specific CD8^{+} T cells within the lung and PBMCs were not statistically significant.

expression (median percentage of granzyme B^{+} cells gated on tetramer^{+} cells was 3.6, range 3.5–12.5). Taken together, these data indicate that despite the loss of CD28 and CD27, FLU- and RSV-specific cells are noncytotoxic memory cells. Furthermore, simultaneous analysis of the cell cycle marker Ki-67 and FLU tetramers showed that all tetramer^{+} cells were Ki-67^{−}, indicating that local FLU-specific cells are not actively replicating (not depicted). In contrast, the distinctions between local and circulating FLU- and RSV-specific T cells and CMV- and EBV-specific CD8^{+} T cells showed a similar expression of CD27 and CD28 on LMCs and PBMCs (Figs. 6 and 7).

Lung–residing FLU–specific CD8^{+} T cells do not need additional stimulation by common γ chain cytokines to expand

A central feature of virus-specific memory CD8^{+} T cells is their ability to expand upon reencountering antigen. We studied whether lung-derived virus-specific CD8^{+} T cells differed from circulating virus-specific T cells with respect to their proliferative requirements. To this end, we labeled LMCs or PBMCs of the same donor with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured them in the presence of peptide or with peptide in combination

Table II. Percentages of nonnaive tetramer^{+} cells in the lung and PBMCs

virus	% of tetramer^{+} cells within nonnaive CD8^{+} cells in lung[a]	% of tetramer^{+} cells within nonnaive CD8^{+} cells in PBMCs	p–value[b]
FLU	1.20 (0.3–11.6)[c]	0.16 (0–0.34)	0.002
RSV	0.35 (0.12–2.0)	0 (0–0.07)	0.06
EBV	0.9 (0.3–11.3)	0.98 (0.22–2.42)	0.6
CMV	2.4 (0.07–5.0)	2.05 (0.2–12.9)	1.0

[a]Percentage of nonnaive cells is calculated by correcting the percentage of total CD8^{+} cells for the percentage of naive (CD45RA^{+} CD27^{bright}) cells.
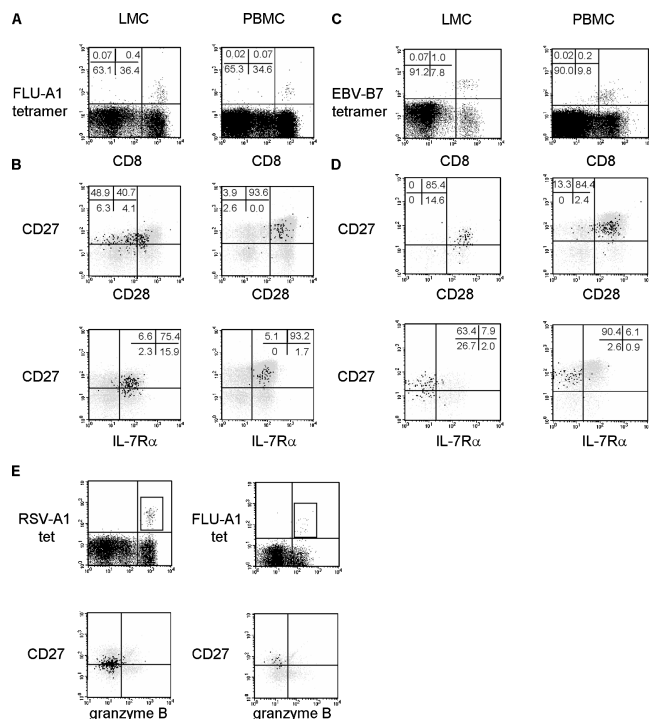[b]Wilcoxon matched-pairs signed-rank test.
[c]Median (range).

Figure 6. FLU tetramer⁺ cells in the lung show a lower expression of CD27 and CD28 and a similar IL-7Rα expression compared with circulating FLU tetramer⁺ cells, whereas the CD27, CD28, and IL-7Rα expression on EBV tetramer⁺ cells in the lung resembles that of tetramer⁺ cells in the blood. Representative dot plots of a donor stained with the FLU-A1 tetramer (A and B) and a donor stained with the EBV-B7 tetramer (C and D). Dot plot of CD8–PerCP-Cy5.5 fluorescence versus FLU-A1–tetramer-APC (A) or EBV-B7–tetramer-APC (C) gated on total lymphocytes in the LMCs (left) and PBMCs (right; B and D). (E) RSV- and FLU-specific cells in the lung do not contain granzyme B. Dot plots of LMCs of two donors reacting with the RSV-A1 tetramer (dot plots on the left) or FLU-A2 tetramer (dot plots on the right) are shown. The top dot plots show CD8 versus RSV or FLU tetramer staining. The quadrant represents the gate for tetramer⁺ cells. The bottom dot plots show granzyme B versus CD27 expression. CD8⁺ T cells are shown in gray, and tetramer⁺ CD8⁺ T cells are shown in black. The numbers indicate percentages within tetramer⁺ cells in the corresponding quadrants.
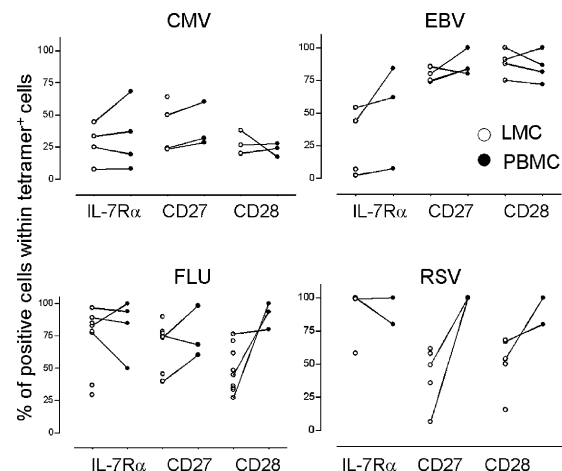

Figure 7. FLU and RSV tetramer⁺ CD8⁺ T cells in the lung show a lower expression of CD28 and CD27 compared with their circulating counterparts, but retain a high IL-7Rα expression. Scatter plots showing the expression of IL-7Rα, CD27, and CD28 on CMV tetramer⁺ CD8⁺ T cells (top left), EBV tetramer⁺ CD8⁺ T cells (top right), FLU tetramer⁺ CD8⁺ T cells (bottom left), and RSV tetramer⁺ CD8⁺ T cells (bottom right).

with IL-2, IL-7, or IL-15. After 6 d, the mean number of cell divisions of divided tetramer⁺ cells was calculated. Lung-derived FLU-specific cells appeared to expand better in vitro by stimulation with FLU peptide alone (mean number of cell divisions for LMCs: median 1.8, range 1.3–2.2; PBMCs: median 0.5, range 0–1.0), whereas PBMC-derived FLU-specific cells needed the addition of IL-2, IL-7, or IL-15 to induce proliferation of FLU-specific cells (Fig. 8 and Table III). The proliferative requirements of EBV- and CMV-specific CD8⁺ T cells derived from the lung were, however, comparable with EBV- and CMV-specific CD8⁺ T cells derived from PBMCs (Table III).

We tested whether the higher proliferative capacity of lung-derived FLU-specific cells was an intrinsic T cell property or alternatively related to differences in the composition

of antigen–presenting cells between lung and blood. From two donors (Fig. 8 A, donors 1 and 2), total CD8⁺ T cells of paired PBMC and LMC samples were sorted and labeled with CFSE. Autologous PBMCs were loaded with FLU peptide, irradiated, and used as stimulators for those purified CD8⁺ T cells (shown for one representative donor in Fig. 8 C). After 7 d, the mean number of cell divisions of divided tetramer⁺ cells was calculated as described in Materials and methods (see below). The mean number of cell divisions of lung-derived FLU-specific cells was higher in both donors (2.8 and 2.0 in the lung and 1.0 and 1.0 in PBMCs, respectively) than those of PBMC-derived FLU-specific cells. These experiments show that the higher proliferative capacity of FLU-specific lung-derived CD8⁺ T cells is a property of these cells.

DISCUSSION

In this study, we show that the human lung contains a large pool of FLU- and RSV-specific CD8⁺ T cells. These local CD8⁺ T cells specific for respiratory viruses have progressed to a differentiated memory state that is characterized by a partial loss of CD27 and CD28, but preserved IL-7Rα expression. Antigen-specific reactivation in vitro of lung-derived FLU-specific CD8⁺ T cells resulted in a potent proliferative response without a strict need for additional help from common γ chain cytokines.

The respiratory tract is the primary target for entry of respiratory viruses. Although data on healthy individuals are lacking, studies in patients at risk for developing respiratory virus infections have demonstrated that the airways harbor a broad spectrum of viruses, such as rhinoviruses and RSV (21, 22). Because these viruses hardly induce clinical symptoms under physiological conditions, the lung immune system must be
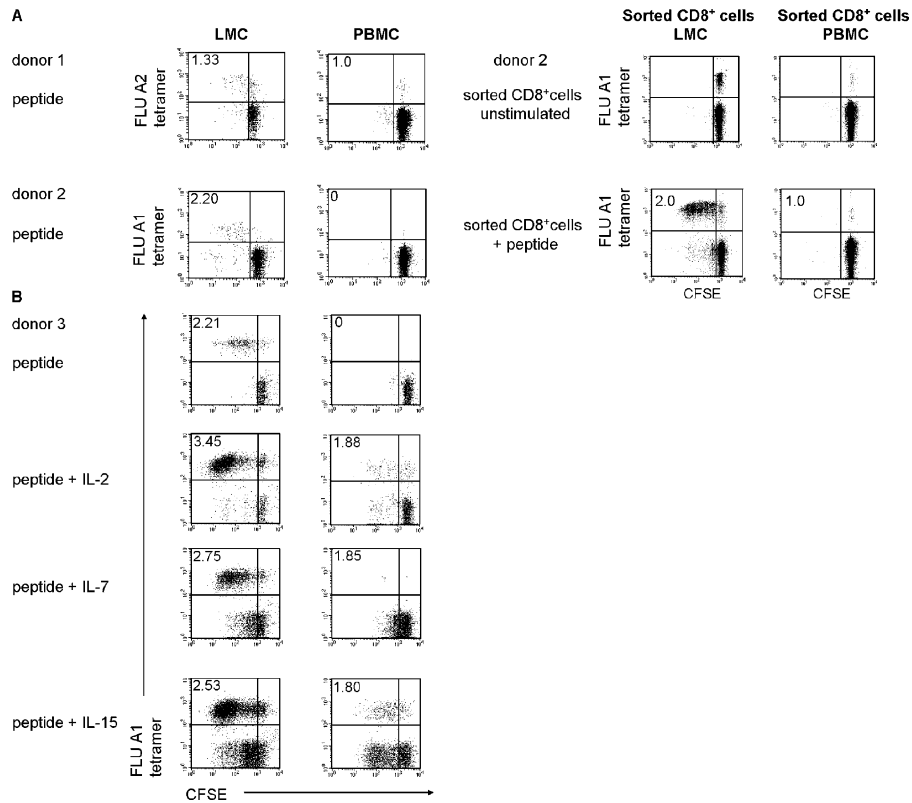
Figure 8. Lung–residing FLU tetramer⁺ CD8⁺ T cells proliferate better than circulating FLU tetramer⁺ CD8⁺ T cells. Total PBMCs and LMCs of three donors were cultured for 6 d with FLU-A1 peptide alone (A and top panel in B), or with peptide in combination with IL-2, IL-7, or IL-15 (shown for one donor in B). Dot plots show FLU-A1–tetramer versus CFSE gated on total CD8⁺ T cells. Dot plots of one representative experiment are show in the top right panels. Dot plot shows FLU tetramer versus CFSE. Top dot plots show sorted CD8⁺ T cells cultured in the presence of autologous, irradiated PBMCs. Bottom dot plots show sorted CD8⁺ T cells stimulated with irradiated PBMCs loaded with FLU peptide. Numbers indicate the mean number of divisions of divided tetramer⁺ cells (see also Materials and methods).

well adapted to eliminate and restrain infections, probably involving CD4⁺ and CD8⁺ T cells as key players. Several studies have indicated that local T cells exhibit activation characteristics (2, 3, 5). In line with other studies (23), we found that the lung has an enrichment of CD45RA⁻ CD4 T cells, indicating that the CD4⁺ T cell pool in the lung consists mainly of memory-type cells. To discriminate between naive CD8⁺ T cells and memory CD8⁺ T cells, single CD45RA analysis is unsuitable because not only do naive CD8⁺ T cells express CD45RA (18), but effector-type CD8⁺ T cells do as well. In fact, this might explain the broader range in CD45RA expression on lung-derived CD8⁺ cells when compared with the CD45RA expression on lung CD4⁺ T cells (Fig. 1 B). Naive CD8⁺ T cells can, however, be accurately defined as CD45RA⁺ CD27[bright+] (18). Lymphocytes in the lung showed a markedly lower fraction of naive CD8⁺ CD45RA⁺ CD27[bright+] cells than circulating CD8⁺ T cells. Additionally, all lung-residing T cells lacked the chemokine receptor CCR7 in accordance with the notion that predominantly nonnaive T cells using other chemokine receptors migrate to the lung.

Table III. Median of mean numbers of cell divisions of divided tetramer⁺ cells after in vitro stimulation

	FLU lung	FLU PBMCs	EBV CMV lung	EBV CMV PBMCs
peptide	1.8 (1.3–2.2)[a]	0.5 (0–1.0)[a]	1.3 (1.2–1.4)	1.1 (1.0–1.2)
IL-2 + peptide	3.0 (2.7–4.6)	3.2 (1.9–5.0)[b]	3.6 (2.4–3.7)	4.3 (4.2–9)
IL-7 + peptide	2.5 (2.1–3.4)	2.8 (1.9–3.7)	2.5 (1.4–2.7)	1.9 (1.5–4.5)
IL-15 + peptide	2.7 (2.5–3.0)	3.0 (1.8–3.7)	2.3 (2.2–3.0)	4.0 (3.0–6.8)

Mean number of divisions of divided cells (see also Materials and methods) of CD8⁺ tetramer⁺ cells after culturing for 6 d with peptide alone or in combination with IL-2, IL-7, or IL-15. Three donors that responded with the CMV (2) or EBV (1) tetramer, and four donors that responded with FLU tetramers were analyzed.
[a]P = 0.25; Wilcoxon matched-pairs signed-rank test.
[b]Median (range).

All T cell populations analyzed were derived from tissue specimens from patients undergoing surgery for a localized solitary peripheral lung carcinoma. Because the lung tissue used for analysis in this study was taken at a far distance from the carcinoma, we consider it unlikely that the malignancy had an impact on the activation state of virus-specific cells. We found that the total CD8$^+$ T cell pool contained a large fraction of recently activated HLA-DR$^+$ cells with a low expression of IL-7Rα. Because IL-7Rα is down-modulated upon TCR triggering, it can be assumed that the low IL-7Rα expression on activated CD8$^+$ T cells in the lung might be caused by recent viral exposition (16, 20). Viruses that preferentially infect the respiratory tract are candidates for the emergence of activated virus-specific lung T cells. A significant accumulation of FLU- and, to a lesser extent, RSV-specific CD8$^+$ T cells in the lung was found. However, these FLU- and RSV-specific cells retained a high IL-7Rα expression and did not acquire granzyme B (Fig. 6, E and 7), which indicates that these viruses presumably do not contribute to the activation of the large virus-specific CD8$^+$ T cell pool in the lung. The limited availability of tetramers for a restricted number of viruses narrows the analysis of CD8$^+$ T cells specific for other respiratory viruses. Therefore, we could not evaluate the fraction of CD8$^+$ T cells specific for such other common respiratory viruses as rhinoviruses or adenoviruses that may possibly contribute to the accumulation of activated CD8$^+$ T cells in the lung.

Prior human studies have focused on the properties of virus-specific CD8$^+$ T cells specific for latent and cleared viruses (7, 12, 15) in the circulation. It is, however, conceivable that the compartment in which virus-specific cells reside leaves an impact on their differentiation characteristics. The low CD28 and CD27 expression on total lung CD8$^+$ T cells indicates that these cells have progressed further along the presumed differentiation pathway (12, 15) than the circulating CD8$^+$ T cell pool. The differentiation process of CD8$^+$ T cells to effector cells is characterized by loss of CD27 and acquisition of cytotoxic effector molecules as granzyme B. Recent studies have shown that the emergence of cytotoxic CD8$^+$ T cells in the circulation is highly associated with CMV latency, suggesting that interaction with CMV antigen drives the formation of cytotoxic cells in the circulation (24, 25). In the lung, we found markedly lower granzyme B content in the CD8$^+$ CD27$^-$ T cell fraction when compared with the CD8$^+$ CD27$^-$ fraction in the circulation. Although it remains speculative which viruses drive the differentiation of CD27$^-$ CD8$^+$ T cells in the lung, it cannot be excluded that CMV is unique in that it elicits T cells with high cytotoxic capacity. The limited granzyme B amount in CD27$^-$ cells may suggest that in the lung, where there is frequent antigenic exposure, other factors, such as the antigenic environment and cytokine milieu, may suppress the evolvement of cytotoxic capacity in fully differentiated virus-specific T cells to some extent.

Loss of CD28 and CD27 has been related to prolonged antigenic stimulation (26, 27). During differentiation, virus-specific CD8$^+$ T cells have been shown to first lose CD28 from their cell surface, followed by loss of CD27 upon further differentiation (28). The low expression of CD28 on FLU-specific CD8$^+$ cells (Fig. 7) indicates that FLU-specific cells in the lung have reached an intermediate differentiation state. In addition to low CD28 expression levels, RSV-specific cells in the lung also have low CD27 expression levels, suggesting that RSV-specific cells are relatively further differentiated than FLU-specific cells. The only known mechanism that induces a CD27$^-$ phenotype is antigen-induced interaction between CD27 and its ligand CD70 (29, 30). In this respect, the observation that CD27 expression on lung T cells is markedly lower on RSV than on FLU-specific T cells might imply that humans are intermittently but relatively frequently exposed to RSV. CD70 is induced by the presence of antigen and can be modulated by cytokines, such as IL-2 and IL-15 (28–30). The presence of a large population of activated CD4$^+$ memory cells in the lung (Fig. 1) may provide a source of IL-2. In addition, a study by Muro et al. (31) showed high IL-15 mRNA expression in the lung of healthy individuals even under inflammatory conditions. Overall, our data indicate that the appearance of virus-specific CD8$^+$ T cells in the lung compartment might be shaped on the one hand by the presence or frequent contact with its cognate antigen and on the other hand by the local cytokine milieu.

An important feature of memory cells is the ability to persist in the absence of antigen. After elimination of the virus, a large fraction of virus-specific effector cells undergoes apoptosis and a smaller fraction of memory cells is maintained. In the search for factors that may determine which memory cells are maintained, the expression of the IL-7Rα on virus-specific cells is associated with properties of long-lived memory cells (20). In humans, the expression of IL-7Rα is down-modulated upon T cell receptor triggering (16). We found that CMV-specific cells in the lung did not differ in IL-7Rα expression from circulating CMV-specific cells. EBV-specific cells tended to have a lower expression in the lung when compared with peripheral blood. In contrast, FLU- and RSV-specific cells retained a high IL-7Rα expression in the lung. Currently, there are no data available on the regulation and role of IL-7Rα on tissue-residing virus-specific T cells. Therefore, it remains speculative as to whether the IL-7Rα is also down-modulated in tissues upon antigen-specific activation as has been described in relation to circulating virus-specific T cells (16). When we extrapolate these findings to the high expression of IL-7Rα on FLU- and RSV-specific cells in the lung, we might hypothesize that these cells are, but not continuously, activated by their cognate antigen and may rely on IL-7 for their maintenance in the absence of antigen.

An essential property of memory cells is their ability to rapidly expand upon antigen challenge. It has been shown that next to antigen, in vitro cytotoxic as well as memory T cells need additional factors to proliferate (32, 33). Lung-derived FLU-specific cells proliferated better than circulating FLU-specific cells in response to stimulation with peptide alone. In studies in mice, the proliferative capacity in re-

sponse to in vitro peptide stimulation has been shown to depend on the persistence of the antigen (34). It was found that memory cells that were continuously exposed to antigen proliferated less well in response to viral peptide in vitro than memory cells specific for a cleared virus. As mentioned earlier, the high expression level of IL-7Rα on FLU–specific cells suggests that lung–derived FLU–specific cells are not persistently in contact with FLU. Together with the observation that local FLU–specific cells are partly CD27$^-$, it might be expected that they are activated by viral epitopes intermittently. Frequent subclinical reexposure to FLU (or RSV) may result in a differentiated state of the memory cells and may lower the threshold for antigen–induced expansion.

A central issue in understanding local T cell immunity is whether virus–specific CD8$^+$ T cells reside in the lung and are locally reactivated upon infection, or whether circulating virus–specific CD8$^+$ T cells are recruited from the circulation. Direct measurements on the contribution of either mechanism are impossible to perform in humans. However, extensive studies in mice have shown that defense against a secondary pulmonary FLU infection is achieved mainly by reactivation of local FLU–specific CD8$^+$ T cells, and to a lesser extent by recruitment of circulating virus–specific cells (8, 9). The large percentage of differentiated FLU- and RSV–specific CD8$^+$ cells in the human lung, in contrast to very low frequencies in the circulation, combined with the observation that the lung contains ample numbers of activated (i.e., DR$^+$) cells suggests that in the human lung, recurring respiratory infection is also guarded by the local resident T cell pool.

In sum, our data show that the characteristics of FLU- and RSV–specific T cells in the human lung differ from those in the blood. The lung contains pools of differentiated respiratory virus–specific CD8$^+$ T cells that are potentially on standby to provide immediate defense against respiratory infections. Because the primary aim of immune boosting by vaccination is the effective expansion of the virus–specific memory T cell compartment, the data in this study point out that it may be worthwhile to consider vaccination strategies for respiratory viruses that target T cells in the lung.

MATERIALS AND METHODS

Donors. 22 subjects (median age 70, range 47–77) undergoing lobectomy for a localized solitary peripheral lung carcinoma were included. All patients were free of symptoms of upper respiratory tract infection and did not receive antibiotics 2 wk before inclusion in the study. 5 out of 22 patients had a history of cardiopulmonary disease. Of those patients, two had diabetes. None of the patients suffered from renal failure. None of the patients in this study received systemic corticosteroids or other immunosuppressive therapy at the time of inclusion in the study or in the past. Furthermore, none of the patients received chemotherapeutic treatment. All of the 22 subjects were (ex-) smokers. Of them, six smoked on the moment of analysis. These six subjects did not show differences in responses in relation to the nonsmoking individuals in this study. All participants aged >65 yr ($n = 15$) had been immunized with the yearly FLU vaccine. Additionally, we determined whether there were differences in the frequencies of FLU–specific cells between vaccinated and nonvaccinated individuals. This was not the case (PBMC: P =

0.9; lung: P = 0.9; Mann-Whitney). Patients were recruited from the Academic Medical Center, Amsterdam, and the Hilversum Hospital, Hilversum, Netherlands. All participants gave written informed consent before inclusion in the study, which was approved by the Medical Ethics Committee of the Academic Medical Center of the University of Amsterdam.

Isolation of PBMCs and HLA typing. Heparinized venous blood samples were obtained 1 d before surgery. PBMCs were isolated by standard density gradient techniques and cryopreserved until analysis. HLA typing for HLA-A1, HLA-A2, HLA-B7, and HLA-B8 was performed by PCR (Table I).

Isolation of mononuclear cells from lung tissue. LMCs were isolated from lung tissue as described by Holt et al. (35). In brief, 1×1 cm tissue specimens were sliced to 1 mm and incubated for 20 min in RPMI containing 20 mM Hepes, 15% (wt/vol) FCS, and 50 U/ml DNase (Sigma-Aldrich) while shaking at 37°C. A second incubation step, 60 min while shaking at 37°C, was performed in the same medium supplemented with 300 U/ml collagenase. A cell suspension of lung tissue was obtained by grinding the tissue through a flow-through chamber. Erythrocyte counts were confirmed to be <5% of erythrocyte counts in the paired blood sample. Mononuclear cells were isolated from the lung homogenate by standard density gradient techniques and cryopreserved until analysis. The yield of 1 cm^3 of lung tissue was generally 10×10^6 mononuclear cells.

Tetrameric complexes. The following HLA–peptide tetrameric complexes were provided by K. Tesselaar and D. van Baarle (Sanquin, Amsterdam, Netherlands): HLA-A2 tetramer loaded with the CMV pp65-derived NLVPMVATV peptide, HLA-B7 tetramer loaded with the CMV pp65-derived TPRVTGGGAM peptide, HLA-B7 tetramer loaded with EBV EBNA3A-derived RPPIFIRRL peptide, HLA-B8 tetramer loaded with EBV BZLF1-derived RAKFKQLL peptide, HLA-A2 tetramer loaded with FLU M1-derived GILGFVFTL peptide, and HLA-A1 tetramer loaded with RSV M-derived YLEKESIYY peptide. HLA-A1 tetramer loaded with FLU NP-derived CTELKLSDY peptide was obtained from Proimmune. All tetramers used were allophycocyanin-conjugated. In Results, the different tetramers are named after the virus and the HLA type, e.g., CMV A2 tetramer.

Flow cytometric analysis. PBMC or LMC (0.5×10^6 cells) were incubated with tetrameric complexes and different combinations of the following antibodies: CD27-FITC (homemade clone 3A12), HLA-DR-FITC (Becton Dickinson), CD69-FITC (Becton Dickinson), CD45RA-PE (Sanquin), CD28-PE (BD Biosciences), anti–CCR7-PE (Becton Dickinson), anti–IL-7Rα-PE (Immunotech), CD38-PE (Becton Dickinson), CD8–PerCP-Cy5.5 (Becton Dickinson), and CD4–PerCP-Cy5.5 (Becton Dickinson). Cells were labeled according to the manufacturers' instructions, washed with PBS containing 0.01% (wt/vol) NaN$_3$ and 0.5% (wt/vol) bovine serum albumin (PBA).

Intracellular Ki-67 and granzyme B staining was performed by incubating 0.5×10^6 PBMCs or LMCs with tetrameric complexes and combinations of antibodies CD27-FITC (homemade clone 3A12) and CD8-PerCP (Becton Dickinson), washed once, fixed with 50 μl buffered formaldehyde aceton solution, and then permeabilized by washing with 0.1% saponin and 50 mM D-glucose. Cells were then incubated with anti–Ki-67–FITC (DakoCytomation) or anti–granzyme-B–PE (Sanquin) antibodies. Cells were analyzed using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

CFSE labeling. PBMCs and LMCs were labeled with 0.5 μM CFSE (Invitrogen) in PBS for 10 min, with shaking at 37°C. Cells were washed and resuspended in IMDM containing 10% human pool serum and antibiotics.

Activation of total LMC- and PBMC–derived T cells. CFSE-labeled cells were cultured in culture medium (IMDM containing 10% [wt/vol] human pool serum and antibiotics) for 6 d in a 48-well plate at a concentration

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of 0.5 × 10^6 cells/ml per well. Cells were stimulated with the specific CMV, FLU, RSV, or EBV peptide alone, or in combination with CMV, FLU, or RSV antigen (all 10 μl/ml; Microbix Biosystems Inc.), or either 50 U/ml IL-2 (Biotest Ag), 10 ng/ml IL-7 (Strathmann), or 10 ng/ml IL-15 (R&D Systems). Peptides were added at a final concentration of 1.25 μg/ml. Flow cytometric analysis was performed after 6 d. The mean number of divisions of the divided cells was calculated as follows. First, based on the CFSE histogram gated on total $CD8^+$ cells (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20051365/DC1), markers were set on each cell division. Next, the marker settings were copied to the CFSE histogram gated on $tetramer^+$ cells of the same culture (Fig. S1 B), and the cell numbers in each division were determined (n) and used for calculation with the following formula: $[\Sigma_{n \geq 1}(n \, P_n/2^n)]/[\Sigma_{n \geq 1}(P_n/2^n)]$ (n is the number of cell divisions, and P is the number of cells in each division) (16).

To obtain pure $CD8^+$ T cells, LMCs and PBMCs were sorted in a $CD8^+$ fraction using a FACSAria (BD Biosciences). Sorted $CD8^+$ cells were labeled with CFSE and cocultured with irradiated autologous PBMCs that were loaded with FLU A2 peptide for 2 h at 37°C. Irradiated autologous cells were added to purified $CD8^+$ T cells in a ratio of 50,000–20,000 per well and cultured for 7 d in a 96-well plate. Flow cytometric analysis was performed, and the mean number of divisions of the divided cells was calculated as described above.

Statistical analysis. The comparisons made in this study imply the assessment of differences between lung and peripheral blood within patients, which typically results in a matched-pairs situation. The statistical test used is the nonparametric Wilcoxon matched-pairs signed-rank test. The tests were performed with GraphPad Prism software, version 4. The significance level was set at 0.05.

Online supplemental material. Fig. S1 shows an example of a CFSE histogram that is used to determine the number of cells in each division. It is available at http://www.jem.org/cgi/content/full/jem.20051365/DC1.

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