

Mucosal T cells recovered from mice after infection with respiratory syncytial virus display a memory/activation phenotype

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Pgp-1 expression was studied as a marker of memory/activation on systemic and mucosal T cells of BALB/c and C57BL/6 mice after infection with respiratory syncytial virus (RSV), using two-color dual fluorescence flow cytometry employing anti-L3T4 (CD4), anti-Ly2 (CD8), and anti-Pgp-1 (CD44) monoclonal antibodies. Pgp-1 was expressed in relatively low densities on T cells of C57BL/6 mice, allowing differentiation of a dual population of Pgp-1^{lo} and Pgp-1^{hi} T cells after antigenic stimulation *in vivo*. On the contrary, T cells of BALB/c mice were uniformly Pgp-1^{hi}, making this mouse strain less suitable for studies with this marker. In blood and spleen consistently more CD8⁺ than CD4⁺ T cells were Pgp-1^{hi}, while in BAL more CD4⁺ than CD8⁺ T cells were Pgp-1^{hi}. After primary but not after secondary infection, CD4⁺ Pgp-1^{hi} T cells increased significantly in the blood and spleen. After secondary infection both CD4⁺ Pgp-1^{hi} and CD8⁺ Pgp-1^{hi} T cells increased in the BAL. It is hypothesized that after primary infection systemic RSV-specific T cells acquire an activation/memory phenotype as characterized by an enhanced expression of Pgp-1, resulting in a faster and stronger influx of these cells in the lungs after secondary infection.

J. L. L. Kimpen¹, P. L. Ogra²

¹Beatrix Children's Hospital, University Hospital, Groningen, the Netherlands, ²Department of Pediatrics, University of Texas Medical Branch at Galveston, Texas, USA

JLL Kimpen, Beatrix Children's Hospital, University Hospital, Groningen, Oostersingel 59, 9713 EZ Groningen, The Netherlands.

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Viral clearance from the lungs after infection with RSV depends on a functional T cell system (1). Experimental and clinical evidence suggests that T cells play an important role in the pathogenesis of RSV-induced disease (2–4). RSV-specific T cells, recovered from the lungs of experimentally infected animals by bronchoalveolar lavage (BAL) can be considered representative of T cells attracted to the respiratory mucosa after local viral infection (5). Primary infection of laboratory animals with live RSV induces an influx into the bronchoalveolar lumen of predominantly cytotoxic CD8⁺ T lymphocytes (5, 6). After secondary infection CD4⁺ T cells are found in BAL in greater numbers in association with CD8⁺ T cells (7).

It is increasingly clear that cell surface glycoproteins act as accessory molecules during cell-cell interaction (8). Phagocyte glycoprotein 1 (Pgp-1) is a recently described, widely distributed, and species-specific membrane molecule possibly contributing to intercellular interactions as an adhesion molecule (9). In addition, Pgp-1 has been described as a valua-

ble marker for activated and/or memory T cells (10). Antigen-specific T cells are enriched in the Pgp-1^{hi} subset (10). The acquisition of surface Pgp-1 expression is a stable event in differentiation and is associated with enhanced functional characteristics (11, 12).

In previous reports T cell redistribution kinetics between systemic and mucosal tissues after primary and secondary infection of mice with RSV have been described (5, 7). In the present study Pgp-1 expression on mucosal and systemic T cells after infection of BALB/c and C57BL/6 mice with live RSV was evaluated.

Methods

Respiratory syncytial virus (Long strain) was cultured and purified as described previously (7). Ten to 12 week old specific-pathogen-free female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in isolator cages.

Table 1. Experimental protocol.

	primary infection	re-infection
control	no infection	-
D0	S	-
D0	10 ⁶ pfu IN	10 ⁶ pfu IN
D3	S	-
D7	S	-
control	-	no re-infection
D28	-	S
D28	-	10 ⁶ pfu IN
D31	-	S
D35	-	S

Each group consists of 2-5 animals (pfu: plaque-forming units, S: sacrifice, IN: intranasal inoculation)

Mice were infected and killed as described (5, 7), following the protocol outlined in Table 1. A time interval of 28 days following a primary infection was considered sufficient to elicit a memory immune response after rechallenge (3).

T cell subsets were enumerated in blood, spleen and bronchoalveolar lavage fluid (BAL) by two-color dual fluorescence flow cytometry employing phycoerythrin-conjugated monoclonal antibodies to L3T4 (CD4) and Ly2 (CD8) (Coulter Corporation, Hialeah, FL) and fluorescein isothiocyanate-conjugated monoclonal antibody to Pgp-1 (CD44) (PharMingen, San Diego, CA), as described (5, 7).

Statistics

Descriptive statistics were used where appropriate. Values in text and figures are expressed as mean $\pm$ SEM or SD as indicated. Two-tailed paired t-tests were employed to compare paired samples. One-way analysis of variance (ANOVA) was used to compare population means. If the significance level calculated with ANOVA was less than or equal to 0.05, two-tailed t-tests were employed to compare cell means. P-values less than or equal to 0.05 were considered significant. All calculations were performed with StatPac Gold Statistical Analysis Package (Walonic Associates, Inc.) on an IBM-PC.

Results

Peripheral blood and spleen lymphocytes of BALB/c and C57BL/6 mice were studied for baseline Pgp-1 expression. Flowcytometry analysis of T cells of BALB/c mice shows a uniform lymphocyte population with intermediate density of Pgp-1 expression, while T cells of C57BL/6 mice display a dual population of Pgp-1^{lo} and Pgp-1^{hi} cells (Fig. 1). Since these data suggest that C57BL/6 mice are more suitable than BALB/c mice for the study of

Pgp-1 expression after viral infection, all subsequent studies were carried out employing C57BL/6 mice.

Total white blood cell and lymphocyte counts were measured in BAL, blood and spleen, both after primary and secondary infection with RSV. The total white blood cell count in BAL increased from $8.2 \pm 3.1 \times 10^4$ before infection to $111.1 \pm 20.0 \times 10^4$ on Day 7 after infection ($P < 0.01$), while no significant changes in total white blood cell counts could be detected in the blood or spleen after primary infection of C57BL/6 mice with live RSV. On Day 7 after infection $36.3 \pm 1.9\%$ of the cells in the BAL were lymphocytes, compared with $9.1 \pm 1.6\%$ before infection ($P < 0.001$). It can be calculated that the lymphocyte count in the BAL increased 60-fold in the week following primary infection as compared to a 9-fold increase of other cell types, which were not further identified. After secondary infection, the maximum cell count in BAL rose to only $32.8 \pm 3.1 \times 10^4$ cells versus $9.8 \pm 0.9 \times 10^4$ before re-infection ($P = 0.0011$). The percent lymphocytes in BAL increased from $8.8 \pm 0.7\%$ before re-infection to $33.6 \pm 4.0\%$ of the cells on Day 3 after re-infection, decreasing again towards Day 7 ($17.1 \pm 4.7\%$) ($P = 0.007$). In the blood the percent lymphocytes decreased from $49 \pm 1.1\%$ of total white blood cells before reinfection to $26.5 \pm 1.4\%$ on Day 7 after re-infection ($P = 0.001$). A similar change was seen in the spleen, where differential lymphocyte count decreased from $65.6 \pm 2.8\%$ before re-in-

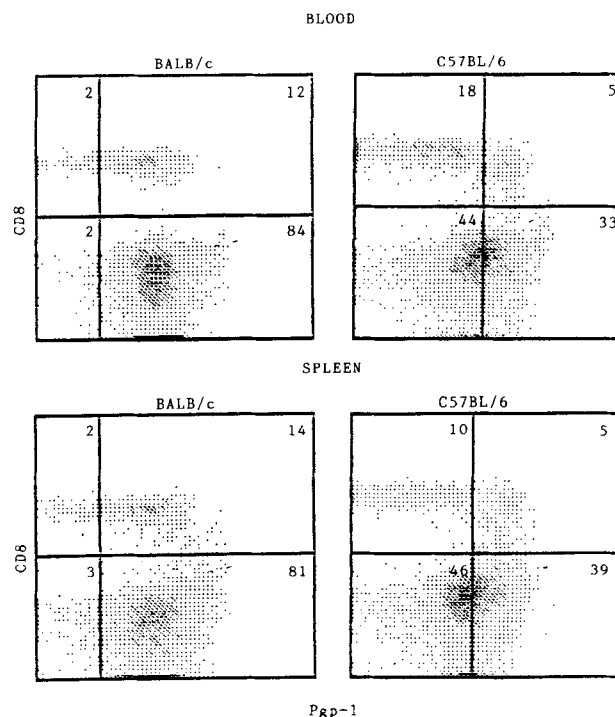


Fig. 1. Pgp-1 expression on peripheral blood and splenic CD8⁺ T cells of BALB/c mice and C57BL/6 mice. Numbers indicate percentages of cells in the respective quadrants.

Table 2. Pgp-1 expression on CD4⁺ and CD8⁺ cells in systemic and mucosal tissues after primary and re-infection with live RES. Values are expressed as mean $\pm$ SD.

	Day 0 (n=3)	Day 3 (n=2)	Day 7 (n=5)	P-value
Primary infection				
% Pgp-1 ^{hi} CD4 ⁺ T cells				
blood	12.1 $\pm$ 1.6	14.3 $\pm$ 2.0	25.2 $\pm$ 8.0	NS
spleen	20.3 $\pm$ 2.4	23.6 $\pm$ 3.1	39.8 $\pm$ 5.2	0.002
BAL	93.1 $\pm$ 4.5	95.9 $\pm$ 2.5	95.4 $\pm$ 3.6	NS
% Pgp-1 ^{hi} CD8 ⁺ T cells				
blood	24.2 $\pm$ 5.3	23.1 $\pm$ 7.1	30.2 $\pm$ 9.0	NS
spleen	40.1 $\pm$ 6.1	29.3 $\pm$ 5.9	45.3 $\pm$ 6.4	NS
BAL	no data	83.5 $\pm$ 6.5	91.0 $\pm$ 4.9	NS
	Day 28 (n=3)	Day 31 (n=3)	Day 35 (n=3)	P-value
Re-infection				
% Pgp-1 ^{hi} CD4 ⁺ T cells				
blood	9.9 $\pm$ 0.4	17.1 $\pm$ 2.2	12.8 $\pm$ 1.7	0.03
spleen	22.2 $\pm$ 4.0	25.8 $\pm$ 1.8	25.3 $\pm$ 6.1	NS
BAL	86.3 $\pm$ 1.5	92.6 $\pm$ 3.8	94.7 $\pm$ 1.7	0.04
% Pgp-1 ^{hi} CD8 ⁺ T cells				
blood	17.0 $\pm$ 5.5	21.5 $\pm$ 3.7	19.5 $\pm$ 4.2	NS
spleen	27.4 $\pm$ 5.0	35.0 $\pm$ 4.7	34.0 $\pm$ 8.3	NS
BAL	80.1 $\pm$ 0.1	93.2 $\pm$ 4.4	93.7 $\pm$ 5.0	0.02

fection to $44.6 \pm 4.6\%$ on Day 7 after re-infection ($P = 0.004$). After re-infection the cell count in the BAL reached its maximum earlier (on Day 3 after re-infection), but the magnitude of cellular reaction

Table 3. Percent Pgp-1^{hi} T cells according to subset and source. Values are expressed as mean $\pm$ SEM.

	CD4 ⁺	CD8 ⁺	P-value
blood	16.7 $\pm$ 1.7	23.9 $\pm$ 1.9	0.0001
spleen	27.7 $\pm$ 2.0	36.6 $\pm$ 2.1	0.0001
BAL	93.1 $\pm$ 1.2	88.9 $\pm$ 1.8	0.0089

was less pronounced then after primary infection (maximum cell count in the BAL after primary infection was $111.1 \pm 20.0 \times 10^4$ versus $32.8 \pm 3.1 \times 10^4$ after re-infection, $P = 0.027$).

Table 2 shows the percent of Pgp-1^{hi} T cells in blood, spleen and BAL after primary and secondary infection with RSV. Fig. 2 shows a representative experiment. More CD4⁺ T cells in blood and spleen were Pgp-1^{hi} on Day 7 after primary and Day 3 after re-infection than before infection. Table 3 shows that in blood and spleen consistently more CD8⁺ T cells were Pgp-1^{hi} than CD4⁺ T cells. In the blood $23.9 \pm 1.9\%$ of CD8⁺ T cells were Pgp-1^{hi} versus $16.7 \pm 1.7\%$ of CD4⁺ T cells ($P < 0.0001$). In the spleen 36.6 ± 2.1 of CD8⁺ and 27.7 ± 2.0 of CD4⁺ T cells were Pgp-1^{hi} ($P < 0.001$). In the spleen around two times more T cells express Pgp-1^{hi} than in the blood, both for CD4⁺ and CD8⁺ T cells.

Table 3 and Fig. 2 show that the majority of the T cells in the BAL express high levels of Pgp-1. In BAL significantly more CD4⁺ than CD8⁺ T cells are Pgp-1^{hi} ($93.1 \pm 1.2\%$ versus $88.9 \pm 1.8\%$ respectively, $P = 0.009$). Probably this difference is not clinically relevant. During the first week after primary infection, the percent Pgp-1^{hi} T cells in BAL

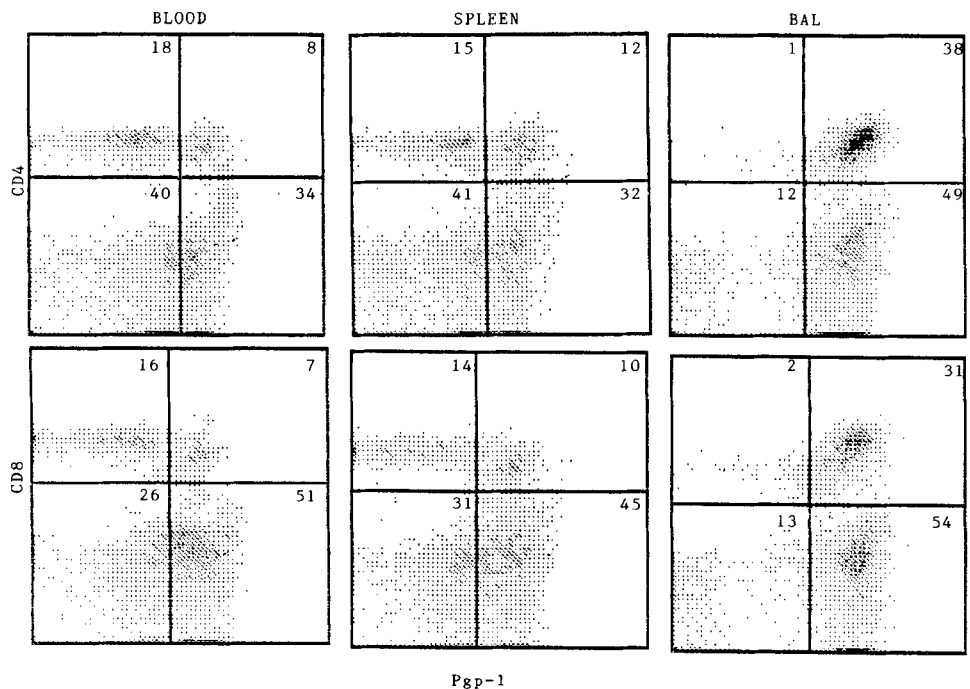


Fig. 2. Representative Pgp-1 expression on peripheral blood, splenic and BAL T cells, 7 days after primary infection of a representative C57BL/6 mouse with live RSV. Numbers indicate cell percentages of cells in the respective quadrants.

remains almost constant. However, after secondary infection, a significant increase of CD4⁺Pgp-1^{hi} and CD8⁺Pgp-1^{hi} T cells is noted. CD4⁺Pgp-1^{hi} T cells increase from $86.3 \pm 1.1\%$ before re-infection to $94.7 \pm 1.2\%$ on Day 7 after re-infection ($P = 0.039$). CD8⁺Pgp-1^{hi} T cells increase from 80.1 ± 0.1 before re-infection to 93.7 ± 3.5 on Day 7 after re-infection ($P = 0.019$).

Discussion

The present report describes Pgp-1 expression on T cells redistributed to mucosal sites after primary and secondary infection of C57BL/6 mice with RSV. C57BL/6 mice are comparable to BALB/c mice in regard to RSV-susceptibility (13), but differ from BALB/c in regard to the expression of Pgp-1 on the surface of their lymphocytes (14). This finding was confirmed in the present study. Although the magnitude of the T cell response after infection of C57BL/6 mice with live RSV was less compared to BALB/c mice (5, 7), a similar qualitative pattern can be recognized.

It was shown that the majority of T cells collected by bronchoalveolar lavage of mice infected with RSV are Pgp-1^{hi} while only 10–25% of peripheral blood T cells and 25–40% of spleen T cells display this phenotype. These findings suggest that lymphocytes attracted to the mucosa or produced locally by clonal expansion after viral infection are activated T cells or surveillance memory cells. This is supported by the findings of the present study. After primary infection Pgp-1 expression increased on the membrane of blood and spleen CD4⁺ T cells suggesting activation and memory induction. After re-infection a significant increase of Pgp-1^{hi} T cells in the BAL was noted. This difference in percentage Pgp-1^{hi} T cells seems not clinically relevant despite statistical significance. However, it can be calculated from the total lymphocyte movement that the number of Pgp-1^{hi} cells increases enormously after infection. Although even before infection the T cells in the BAL are mainly Pgp-1^{hi}, the number of lymphocytes in the BAL is very low. These cells are possibly surveillance memory T cells.

T cells in BAL of mice after infection with RSV are probably attracted to the respiratory mucosa after infection and subsequently activated as evident by enhanced expression of Pgp-1 (15). Alternatively, it is possible that RSV-specific Pgp-1^{hi} memory T cells home to the lungs where they undergo clonal expansion with the formation of a population of Pgp-1^{hi} effector cells. The present study does not allow to differentiate between these two hypotheses.

Pgp-1 is a glycoprotein of unknown function (10), however, a role for Pgp-1 as a lymphocyte homing receptor has been proposed (16). The difference in

Pgp-1 expression among T cells is quantitative rather than qualitative. Both CD4⁺ and CD8⁺ T cells have two well-defined subpopulations, Pgp-1^{lo} and Pgp-1^{hi} (17). Although the majority of CD4⁺ and CD8⁺ T cells of C57BL/6 mice are Pgp-1^{lo}, they become Pgp-1^{hi} after antigenic stimulation *in vitro* and *in vivo* (10, 18). This antigenic stimulation is accompanied by an increased capacity of Pgp-1^{hi} T cells to produce gamma-interferon and IL-3 as compared with Pgp-1^{lo} T cells (11, 12). Gamma-interferon is secreted by Th1 cells while IL-3 is secreted by both Th1 and Th2 cell populations. Unfortunately, these authors did not determine Th2-specific cytokines like IL-4 and IL-5, so that it is not possible to deduce from their studies whether a selective CD4⁺ T cell subset was activated or not. On the contrary, Openshaw et al. described changes in CD45 staining of BAL T cells of mice after sensitization of mice with the major surface glycoprotein G of RSV, suggesting a Th2 cell activation (19). IL-3 and IL-5 are potent activators of eosinophil function and survival (20–22). It has been shown that eosinophils are activated in the respiratory epithelium of patients with RSV bronchiolitis (23). The finding of Pgp-1^{hi} T cell influx in the airways after RSV infection correlates with these observations.

A detailed phenotypic and functional characterization of the T lymphocytes involved in RSV immunology is important to understand possible immunopathogenetic mechanisms (24). Moreover, this knowledge could be helpful for future vaccine design, since T cell epitopes on the virus should probably be incorporated in a candidate RSV vaccine. Finally, as the hypothesis that immune injury plays a critical role in RSV induced illness gains popularity, alternative therapeutic modalities which modulate these immunopathogenetic mechanisms should be considered in the treatment of infants with RSV bronchiolitis.

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