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Expression of chemokine receptor CX_3CR1 in infants with respiratory syncytial virus bronchiolitis

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Running title: CX_3CR1 in RSV bronchiolitis

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Abstract

Respiratory syncytial virus (RSV) glycoprotein G mimics fractalkine, a CX_3C chemokine, which mediates chemotaxis of leukocytes expressing its receptor, CX_3CR1. The aim of this study was to examine the relationship between RSV infection and expression of perforin and IFN-γ in CX_3CR1-expressing peripheral blood $CD8^+$ T cells. Samples were collected from infants with RSV bronchiolitis, both in the acute and convalescence phase (n=12), and from their age- and sex-matched healthy controls (n=15). Perforin expression and IFN-γ secretion in CX_3CR1^+ $CD8^+$ T cells were assessed by four-color flow cytometry. The NF-κB p50 and p65 subunit levels were also determined as markers of RSV-induced inflammation. Study results showed perforin and CX_3CR1 expression to be significantly lower in the convalescent phase of infected infants than in healthy controls. There was no significant difference in IFN-γ secretion and NF-κB binding activity between two time-points in RSV-infected infants, or when compared to healthy controls. Infants with prolonged wheezing had lower acute-phase CX_3CR1 levels in peripheral blood. These data indicate existence of an event persisting after acute RSV infection that is able to modulate effector functions of cytotoxic T cells, and also link disease severity with CX_3CR1 expression.

Key words*:* infants, respiratory syncytial virus, CX_3CR1, perforin, interferon-γ

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Introduction

For the last several decades, respiratory syncytial virus (RSV) carries the unhappy title of the leading cause of serious lower respiratory tract illness in infants (1). Although apparently only some of the infected infants develop bronchiolitis or pneumonia that require hospitalization, those numbers become alarming when one considers that 70% of all children acquire RSV infection before their first birthday, reinfections are almost obligatory (2), and there is no effective vaccine. For infants who recover from RSV bronchiolitis there seem to exist an increased risk for developing recurrent wheeze and asthma later in childhood (3).

The main effector cells that clear RSV from the body are suggested to be the $CD8^+$ cytotoxic T lymphocytes (CTLs). However, when inappropriately activated, these cells could also augment the disease (4). The principal CTL method for destroying the target cell is perforin-mediated cell lysis (5). Although perforin knockout mice can also clear the virus *via* FasL-mediated cell lysis, this is less efficient and could lead to enhanced RSV infection and disease (6). In *ex vivo* assays CTL cytotoxicity correlates with high perforin expression (7), so the CTL perforin level in RSV infection could reflect their ability to clear the virus effectively. Activated CTLs also produce interferon (IFN)-γ, a Th1-type cytokine with antiviral properties (8).

$Perforin^+$ CTLs have another marker on their surface (9) that could participate in the pathogenesis of the disease: CX_3CR1, receptor for chemokine fractalkine (CX_3CL1). Fractalkine is secreted by human endothelial cells activated by proinflammatory signals (TNF-α, IL-1, lipopolysaccharide, CD40 ligand, IFN-γ) and its mRNA is found in various organs, including the lungs (10, 11). It mediates the capture, adhesion and activation of CX_3CR1-expressing cells from the bloodstream and promotes their further migration (9, 12).

It also inhibits the anti-CD3 stimulated IFN-γ production by CX_3CR1^+ CTLs. Recently it has been suggested that the signaling upon fractalkine binding to its receptor is conducted partially *via* activation of nuclear factor-κB (NF-κB) family of transcription factors (13, 14). Various stimuli (infection, inflammation, stress) can cause detachment of NF-κB homo- and heterodimeric subunits from their inhibitory proteins and their translocation into the nucleus. Most common NF-κB dimers are p65 with p50 or p52. Their binding to DNA initiates transcription of different genes for proinflammatory cytokines, chemokines and adhesion molecules (reviewed in 15). Also, it seems that NF-κB induction is required for IFN-γ production in T cells and differentiation of Th1 type immune response (16).

In HIV, the frequency of CX_3CR1^+ CTLs positively correlates with viral loads, and HIV-specific CD8 T cells have an increased expression of CX_3CR1 compared to T cells in healthy individuals (17). However, CX_3CR1 in RSV infection may not play just a general homing receptor role. It has been shown that CX_3CR1 recognizes the RSV G glycoprotein because of its structural similarity to fractalkine: G protein also has a CX_3C motif (18). When G glycoprotein is added to a culture of human PBMCs, it inhibits fractalkine binding to CX_3CR1 and induces migration of CX_3CR1^+ cells towards a G glycoprotein gradient (18). G protein is produced in transmembrane and soluble form; as soluble G protein is released from intact infected cells into the culture fluids (19), an interaction between G protein and T cells in in vivo conditions is theoretically possible.

To examine the relationship between RSV infection and expression of perforin and CX_3CR1 during RSV infection, we obtained peripheral blood samples of infants admitted to the hospital for RSV bronchiolitis, both in the acute and convalescence phase, and from their age-

and sex-matched healthy controls. Perforin, CX_3CR1 and IFN-γ protein levels were determined by flow cytometry. In addition, NF-κB binding activity was measured by ELISA.

Material and Methods

Patients and controls

Twelve infants (6 girls and 6 boys) aged 2 weeks to 9 months (mean 4.7 months) admitted to the participating hospitals with verified RSV infection were included in the study during the 2004 winter epidemic. Bronchiolitis was defined as wheezing, <95% O_2 saturation and lung hyperinflation but infiltrate-free chest radiograph if taken. Heparinized blood samples were obtained within 24 hours of the virus verification in nasopharyngeal secretion (within the first 7 days from the onset of symptoms), prior to which the patients had only received supportive therapy and bronchodilatators (nebulized racemic epinephrinene hydrochloride or salbutamol sulfate). Afterwards, two of the children received 10 mg of methylprednisolone sodium succinate i.m. once or twice a day for 3 days. Also, four other children who subsequently presented with either nasopharyngitis, otitis media or pneumonia, received antibiotics. The control group consisted of 15 infants (8 male and 7 female) aged 2 to 9 months (mean 5.5 months) without clinically evident allergic, immunologic, or hematologic disorders, infectious diseases, or undergoing corticosteroid therapy. The study received approval from Ethics Committees of all participating hospitals. Parents gave an informed consent for blood sampling.

Clinical and laboratory findings

White blood count, erythrocyte sedimentation rate, C-reactive protein and bacteriologic analyses were determined from samples obtained during routine hospital procedure, thus reducing the possibility of superimposed or concomitant bacterial infection. The symptoms (wheezing, minimal oxygen saturation, maximal respiratory rate, fever) were determined according to standard values of these parameters.

Viral diagnosis

RSV-infection was verified in nasopharyngeal secretion by rapid detection (direct fluorescent antibody, DFA, Institute Virion Ltd., Switzerland) and/or virus isolation in cell culture (HeLa, GMK, HEp-2 cells) (20).

Immunologic tests

Heparinized peripheral blood samples were obtained within the first 7 days (mean 4.9 days) from the disease onset, following confirmation of RSV infection, and then in convalescence, 4 to 6 weeks after disease onset. Peripheral blood mononuclear cells (PBMCs) were isolated on density gradient (Ficcol-Paque, Uppsala, Sweden) from the remaining blood after the whole blood staining. Because of the small amount of blood that could be obtained, not all subsequent tests were performed in every infant. For functional IFN-γ testing the PBMCs were stored in liquid nitrogen so the frozen cells from acute phase, convalescent or healthy infants were thawed and then analyzed at the same time. Briefly, $2x10^6$ PBMCs were transferred to a cryovial (2 mL, Costar Corning Inc., NY, USA) containing 900 µL of freezing media (10% dimethylsulfoxide, DMSO, Sigma-Aldrich Co., St. Louis, USA, in fetal calf serum, FCS). Cryovials were placed in Nalgene Mister Frosty® (Sigma-Aldrich), stored for at least 4 hours at -70 °C, and then transferred to liquid nitrogen.

Monoclonal antibodies

To determine human T cell surface antigens, the following murine anti-human antibodies were used: APC-conjugated anti-CD3, PE-Cy5-conjugated anti-CD8 (both from BD Biosciences, Heidelberg, Germany) and PE-conjugated anti-CX_3CR1 (MBL International Co., Woburn, USA). Intracellularly located perforin and IFN-γ were stained with FITC-conjugated

antibody (BD Biosciences). FITC-, PE-, APC-, and PE-Cy5-conjugated isotype controls were used in each experiment for determination of non-specific binding.

Whole blood staining

Fifty μL of heparinized peripheral blood were incubated in round-bottom polystyrene Falcon tubes (BD Biosciences) with 5 μL of mouse anti-human monoclonal antibodies to CX_3CR1, CD3, and CD8 for 30 minutes in the dark at room temperature (RT). Red blood cells were lysed with adding FACS Lysing Solution (BD Biosciences) for 10 minutes in the dark at RT, and then washed twice with staining buffer (1% FCS and 0.1% NaN_3 in Dulbecco's PBS). After washing, the cells were fixed for 20 minutes at 4 $^{\circ}$C with 4% formaldehyde in PBS. Cells were then permeabilized with 0.1% saponin in PBS prior to the addition of perforin monoclonal antibody, and then incubated for 20 minutes at 4 $^{\circ}$C. To remove the unbound antibody, the cells were washed once again in 0.1% saponin, then resuspended in staining buffer and immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, USA) using the CellQuest software.

Cell surface and intracellular IFN-γ staining

Thawed cells from 8 patients and 8 healthy controls were washed and resuspended in RPMI 1640 (G) with 10% human AB serum. Cells ($0.5x10^6$) were then incubated in sterile Falcon tubes with 10 μg/mL brefeldin A (BFA) only or in the presence of BFA, 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 0.75 μg/mL ionomycin (all from Sigma-Aldrich) for 5 hours at 37 $^{\circ}$C and 5% CO_2. After washing, cells were labeled with anti-CX_3CR1, anti-CD3 and anti-CD8 monoclonal antibodies and incubated for 30 minutes in the dark at RT. The samples were washed again and fixed for 20 minutes at 4 $^{\circ}$C with 4% formaldehyde in PBS. After fixation the cells were processed in the same manner as described above for the whole blood

staining, except for the monoclonal antibodies used for intracellular staining: here the cells were labeled with anti-IFN-γ and incubated for 30 minutes at 4 °C. In order to critically evaluate the results obtained, we also estimated the effect of the freezing process, and additionally PMA and ionomycin stimulation, on CX_3CR1 expression. Namely, it has been previously shown that PMA activation downregulates different molecules on T cell such as expression of CD4, and to a lesser extent CD8 (21). We also observed that freezing itself and especially PMA and ionomycin activation decreased expression of CX_3CR1, resulting in substantial reduction in the percentage of $CD8^+CX_3CR1^+$ CTLs (Fig.1).

Nuclear cell extract preparation and NF-κB DNA binding ELISA

The nuclei were extracted from $3x10^6$ of freshly isolated PBMCs using the nuclear extract kit (Active Motif, Rixensart, Belgium). Isolated nuclear proteins were kept at -80 °C prior to use. The binding of NF-κB p65 and p50 units to DNA was then measured using the TransAM™ NF-κB p65 and p50 transcription factor assay kits (Active Motif) and results were quantified by spectrophotometry. NF-κB p65 assay was performed on nuclear extracts from 10 patients and 8 healthy controls, and p50 assay on extracts from 10 patients and 10 controls. As positive control we used Jurkat cell nuclear extract optimized to give a strong signal when used at 2.5 μg/well. The assay specificity was confirmed using the wild-type consensus oligonucleotide as competitor for NF-κB binding, and mutated consensus oligonucleotide with no effect on NF-κB binding. All procedures were conducted according to the manufacturer's instructions.

Statistical analysis

Results of the flow cytometry analysis for perforin and CX_3CR1 were expressed as percentage of gated cells or as mean fluorescence intensity (MFI). Wilcoxon's test for paired data and Mann-Whitney *U*-test for unpaired data were used. Correlation was evaluated using Spearman's rank correlation coefficient (r_s). A *P*-value less than .05 was considered significant. Reported *P*-values in multiple comparisons were adjusted according to Hochberg's modification of Holm's procedure. Analyses were made using the Statistica 6.0 software (StatSoft Inc., Tulsa, USA).

Results

Flow cytometry analysis of perforin and CX_3CR1 expression on whole blood cells

To ensure that the measured perforin and CX_3CR1 were indeed expressed on cytotoxic T lymphocytes, we set a second gate based on co-expression of CD3 and CD8 in addition to correlated analysis of forward and right angle scatter used to establish the lymphocyte gate. Perforin and CX_3CR1 levels were then expressed as both percentage and MFI on such double gated cells. Representative dot plots of four-color immunofluorescence staining are shown in Fig. 2. There was no statistically significant difference in the percentage of perforin$^+$ and CX_3CR1^+ CTLs between patients and control, nor between acute and convalescent stage in infected infants (data not shown). Four-color immunofluorescence staining in all children studied showed that the majority of $CD3^+CD8^+$ CX_3CR1-expressing T cells were also perforin positive (Fig. 2E), Perforin MFI in convalescence was significantly lower when compared to healthy infants (P = .0018 by Mann-Whitney U-test) (Fig. 3A). Convalescents also had lower CX_3CR1 MFI than healthy controls (P = .012 by Mann-Whitney U-test) (Fig. 3B). No difference was found between acute phase perforin and CX_3CR1 MFI and healthy controls (P = .45 and .06 by Mann-Whitney U-test, respectively), as well as in between-group comparison of acute phase and convalescent perforin and CX_3CR1 MFI (P = .05 and .69 by Wilcoxon matched pairs test, respectively). When perforin MFI was measured in CTL population gated through additional CX_3CR1^+-gate, it was significantly lower in both acute and convalescent RSV-infected infants than in healthy controls (P = .0097 and .0005 by Mann-Whitney U-test, respectively; Fig. 3C). There was no relationship between the age of the individual patients in the control group and the measured levels of perforin and CX_3CR1 expression, so the differences observed between patients and controls were not simply the result of age-related changes in protein expression (data not shown).

Flow cytometry analysis of IFN-γ levels in cryopreserved cells

The next step was to investigate whether the ability of CX_3CR1^+ CTLs to secrete IFN-γ differed among acutely ill, convalescent infants, and healthy controls. PBMCs were gated in the same manner as on whole blood staining analysis. No difference was found in the percentage of $CX_3CR1^+IFN\text{-}\gamma^+$ cells in RSV-infected infants between two time-points tested or when compared to healthy controls (Fig. 4A). This should be interpreted with caution, however; as mentioned in Methods, freezing and activation process tends to downregulate CX_3CR1. We found no statistically significant difference in the percentage of IFN-γ cells (or IFN-γ MFI) in CTLs between acute phase- and convalescent patients, or when compared to healthy infants (Fig. 4B). Also, there was no significant variation in percentage of $CD8^+$ T cells between acute and convalescent group, nor compared to controls.

NF-κB p65 and p50 subunit DNA binding

There was no significant difference in NF-κB p65 or p50 subunit concentrations determined in PBMC nuclear extract between acute phase RSV-infected infants, convalescents or healthy controls (Table 1).

Correlation with clinical and laboratory parameters

Analysis of the data obtained from all RSV-infected infants yielded no significant correlation of the levels of perforin, IFN-γ and NF-κB with the clinical parameters examined (days of wheezing, days of O_2 supplementation, minimal O_2 saturation and maximal respiratory rate). However, as illustrated in Fig. 5A, Spearman's rank correlation analysis of the data obtained from RSV-infected infants in acute phase showed a significant inverse correlation between CX_3CR1 MFI and days of wheezing. Infants with prolonged wheezing also showed a slight

tendency to have lower percentage of $CX_3CR1^+IFN\text{-}\gamma^+$ CTLs in the acute phase, but this was not found significant (Fig. 5B). We didn't observe a different pattern of immune response in convalescence in children who received additional glucocorticoid or antibiotic therapy after the acute-phase blood sample was obtained when compared to those only on bronchodilatators and supportive treatment (data not shown).

Discussion

The principal finding of this study was that the levels of CX_3CR1 and perforin expression on CTLs were lower in the convalescent phase of the disease in RSV-infected infants than in healthy controls, even though CTL population itself remained alike. CX_3CR1/perforin$^{+/+}$ T cells are proposed to be cytotoxic effector cells, a terminally differentiated subset with cytotoxic activity (12). A cell armed with perforin and CX_3CR1 receptor is potentially able to migrate towards either fractalkine (9, 12) or RSV G protein (18) gradient into the affected respiratory system, and destroy the infected cells by means of perforin/granzyme-mediated cell lysis. Therefore, a perforin$^{+}CX_3CR1^{+}$ cytotoxic T cell population might have an important role in the host defense against RSV infection. However, lower perforin content and CX_3CR1 expression in convalescent CTLs (measured 4 to 6 weeks of the disease onset) was a somewhat unexpected result, especially as it is known that even severely ill infants shed the virus for a maximum of 3 weeks after the infection (22).

There are several possible explanations for this observation. Recent studies on animal models (23-25) and cell-lines (26) intriguingly suggested that RSV could persist in the lungs by means of low-grade replication (in mice up to a 100 days after the acute infection). Continuous and subtle release of G protein could attract CX_3CR1^{high} cells into the lungs without affecting the apparently healthy infant; hence the lower CX_3CR1 expression levels. Or, CX_3CR1 might be internalized, as other chemokine receptors are known to recycle after interacting with their ligands (27). If the virus is cleared, though, fractalkine remains as a possible modulator of CX_3CR1 expression in convalescence. The requirement for fractalkine release is inflammation. There is other evidence indicating an existence of an ongoing cellular immune response after RSV clearance, probably in the form of Th2-type immune imprinting. IL-4 mediated suppression of CD8 cytolitic activity has been suggested both in mice (28) and

humans (29). RVS-infected infants have an increased percentage of $CD4^+$, $CD23^+$ and $CD25^+$ lymphocytes at the 5-month follow-up compared to acute-phase infants and healthy controls (30). CD25, a receptor for interleukin-2, is commonly used as a marker of T cell activation. Soluble CD25 levels were also found to be increased in both acute and convalescent phase of RSV infected infants (31). Continued inflammation in the lungs could trigger fractalkine release and so recruit perforin$^+$$CX_3CR1^+$ CTLs (or induce receptor recycling) even without systemic manifestations.

Although we found no significant difference in CX_3CR1 levels between acutely ill and healthy infants, the correlation of clinical parameters with immunologic data indicated that infants with prolonged wheezing (and therefore a more severe disease) apparently had lower acute-phase CX_3CR1 expression in peripheral blood. This might be due to the pronounced sequestration of CX_3CR1^{high} CTLs in the lungs (or increased receptor recycling in periphery) in a more severe disease, which is accompanied by a stronger inflammatory response (increased fractalkine release) and possibly higher viral load (increased G protein concentration). A connection between CX_3CR1 and disease severity has been recently described in a murine model (32), where G glycoprotein reduced respiratory rates via G protein-CX_3CR1 interaction and induction of substance P (reduced respiratory rates and even apnea can occur during RSV bronchiolitis in very young infants).

Recycling could also be responsible for the observed fall in CX_3CR1 levels in cryopreserved cells (described previously) and after PMA and ionomycin stimulation (17). PMA-and ionomycin-activated CTLs showed no difference in the percentage of IFN-γ cells or IFN-γ protein levels between infected infants in either phase of disease and healthy children. This was not surprising as PMA and ionomycin stimulation used to enhance detection of pre-

programmed cytokine production induces IFN-γ expression in T cells that are not necessarily RSV-specific, as we (33) and others (34) observed earlier. Also, we found no difference in NF-κB activation between RSV-infected infants at either time-point and their healthy controls. As IFN-γ production in T cells depends largely on NF-κB (16), this is in accordance with our IFN-γ results. However, the NF-κB p65 and p50 subunit DNA binding was measured in PBMC nuclear extract, so the possible changes in NF-κB activation in CTLs may have been masked within a total PBMC population.

Low perforin content in CTLs during convalescence also has several potential causes. It could as well be a result of RSV persistence. Studies in other chronic viral infections (7) showed that most of the virus-specific CTLs express low levels of perforin and are not directly cytotoxic. Additionaly, perforin content varies in distinct differentiation stages of $CD8^+$ T lymphocytes. Only effector and effector memory CTLs express perforin, whereas naïve and central memory do not (35). Low perforin expression in convalescent-phase CTLs (Fig. 3B) could therefore mean that for some reason, naïve and/or central memory CTL population remained prevalent in peripheral blood. Intriguingly, if perforin expression is analyzed in CX_3CR1^+ CTL subpopulation (Fig. 3C), its lower levels in infected infants vs. controls are found not only in convalescence, but also in acute disease. Moreover, infected infants can clearly be divided in two groups; first one where perforin levels drop from acute-phase to convalescence, and second where perforin content increases. Previously, when we investigated TLR4 expression (receptor for RSV F glycoprotein) in infants with RSV bronchiolitis (36), we also noted that increased TLR4 expression in monocytes was observed only in one subgroup of acutely ill infants. Results of a recent study about influence of epidemiologic, socioeconomic and clinical factors on RSV disease severity suggest that high

variability of clinical and immunologic parameters seen in RSV probably originates from patients' genetic background (37).

It should be noted that all our experiments were conducted on peripheral blood samples. Therefore, these findings might not reflect the actual situation in the respiratory tract, where RSV replication and tissue pathology take place. Of course, lung samples in human RSV studies are usually not available, and its closest resemblance, bronchoalveolar lavage, could be taken from intubated infants with bronchiolitis during the acute disease, but not from clinically healthy convalescents or controls. To substantiate our results, additional studies are necessary, and currently under way. In order to clearly show that changes in CX_3CR1 expression were not secondary to non-specific effects of an acute viral respiratory infection in general, infants with non-RSV viral bronchiolitis should be investigated as well. Since all infants included in our study presented with bronchiolitis, the possible link between low CX_3CR1 levels and more severe disease presentation should be tested among children with milder forms of RSV infection such as upper respiratory tract infection. Also, perforin dynamics during RSV infection has not been, to our knowledge, previously investigated, and CTL differentiation markers should be included in more sophisticated flow cytometric analysis. However, if one takes high perforin and CX_3CR1 levels as attributes of effector CTLs' ability to migrate towards and lyse infected cells, their lower convalescence levels in peripheral blood indicate either a lung invasion of effector cell population, or downregulation of markers due to cotinuous immune reaction several weeks after the acute infection.

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Table 1. Analysis of nuclear factor-κB (NF-κB) p65 and NF-κB p50 subunits.

Concentration of NF-κB p65 and NF-κB p50 subunits per well in peripheral blood mononuclear cell nuclear extract of respiratory syncytial virus-infected infants during acute phase and convalescence, and of their healthy controls. N indicates number of samples analyzed. Mann-Whitney *U*-test was used for comparison with healthy control values, and *P*-value less than .05 was considered statistically significant.

	NF-κB (μg/well)							
	p65				**p50**			
	N	Median	Range	*P* to controls	N	Median	Range	*P* to controls
acute phase	10	**1.69**	1.02-2.50	0.213	10	**0.93**	0.6-2.16	0.067
convalescence	10	**1.59**	1.28-2.81	0.131	10	**0.70**	0.49-1.86	0.057
controls	8	**1.93**	1.58-2.75		10	**1.50**	0.77-2.91	

Fig. 1. Percentage of $CX_3CR1^+CD8^+$ T cells of healthy infants in whole-blood samples (whole blood), cryopreserved isolated peripheral blood mononuclear cells (frozen PBMCs), and PBMCs stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (I) (PMA+I PBMCs). Squares indicate medians, boxes percentile boundaries, and whiskers minimum and maximum values.

Fig. 2. Expression of perforin and CX_3CR1. Representative dot plots of forward and right angle scatter (A), surface isotype control staining (B), CD3 and CD8 co-expression plot gated through lymphocyte gate (C), intracellular isotype control staining (D) and perforin and CX_3CR1 co-expression plot gated through lymphocyte and CD3/CD8 gate (E).

Fig. 3. Expression of CX_3CR1 and perforin in infected infants and controls. Mean fluorescence intensity (MFI) of CX_3CR1 (A) and perforin (B) in $CD3^+CD8^+$ cytotoxic T cells, and perforin (C) in CX_3CR1^+ $CD3^+CD8^+$ cytotoxic T cells of respiratory syncytial virus (RSV) infected infants during acute phase (acute) and convalescence (convalescents) (n=12), and of their healthy controls (controls) (n=15). The values obtained in a single infant in acute and convalescent phase of disease are linked with a line. Each circle represents one tested infant. Horizontal lines represent median values. Mann-Whitney *U*-test was used for between-group comparison, and *P*-value less than .05 was considered statistically significant.

Fig. 4. Percentage of interferon-(IFN)-γ-secreting cells among CX_3CR1^+ $CD3^+CD8^+$ T cells (A) and $CD3^+CD8^+$ T cells (B) stimulated with PMA and ionomycin (I) of respiratory syncytial virus-infected infants during acute phase (acute) and convalescence (convalescents) (n=8), and of their healthy controls (controls) (n=8). Each circle represents one tested infant. Horizontal lines represent median values.

Fig. 5. Negative correlation between the duration of wheezing (days) and CX_3CR1 mean fluorescence intensity (MFI) on $CD3^+CD8^+$ T cells in acute-phase RSV-infected infants (n=12) (A). No significant correlation was found between the duration of wheezing and percentage of interferon-(IFN)-γ-secreting, CX_3CR1^+ cytotoxic T cells (n=8) (B). Each rectangle represents one tested infant. The relationship between variables was assessed by Spearman rank order coefficient (r_s).

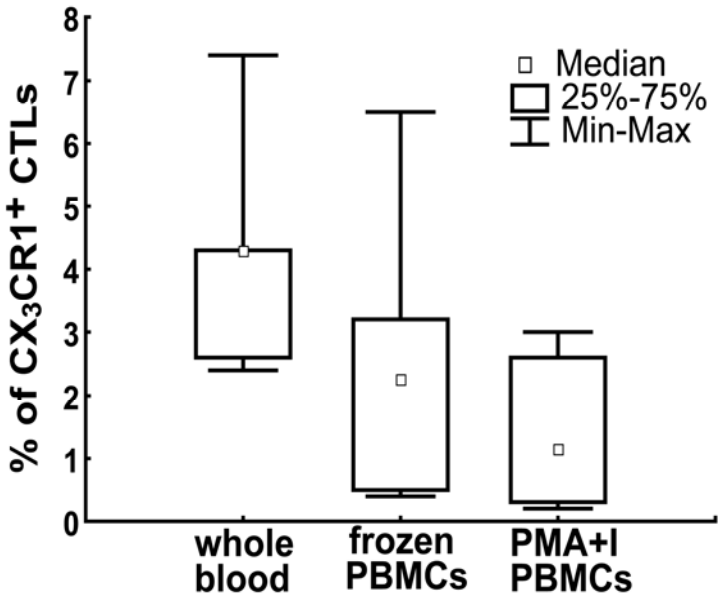
% of CX3CR1+ CTLs
8
7
6
5
4
3
2
1
0
Median
25%-75%
Min-Max
whole blood
frozen PBMCs
PMA+I PBMCs

Fig. 1.

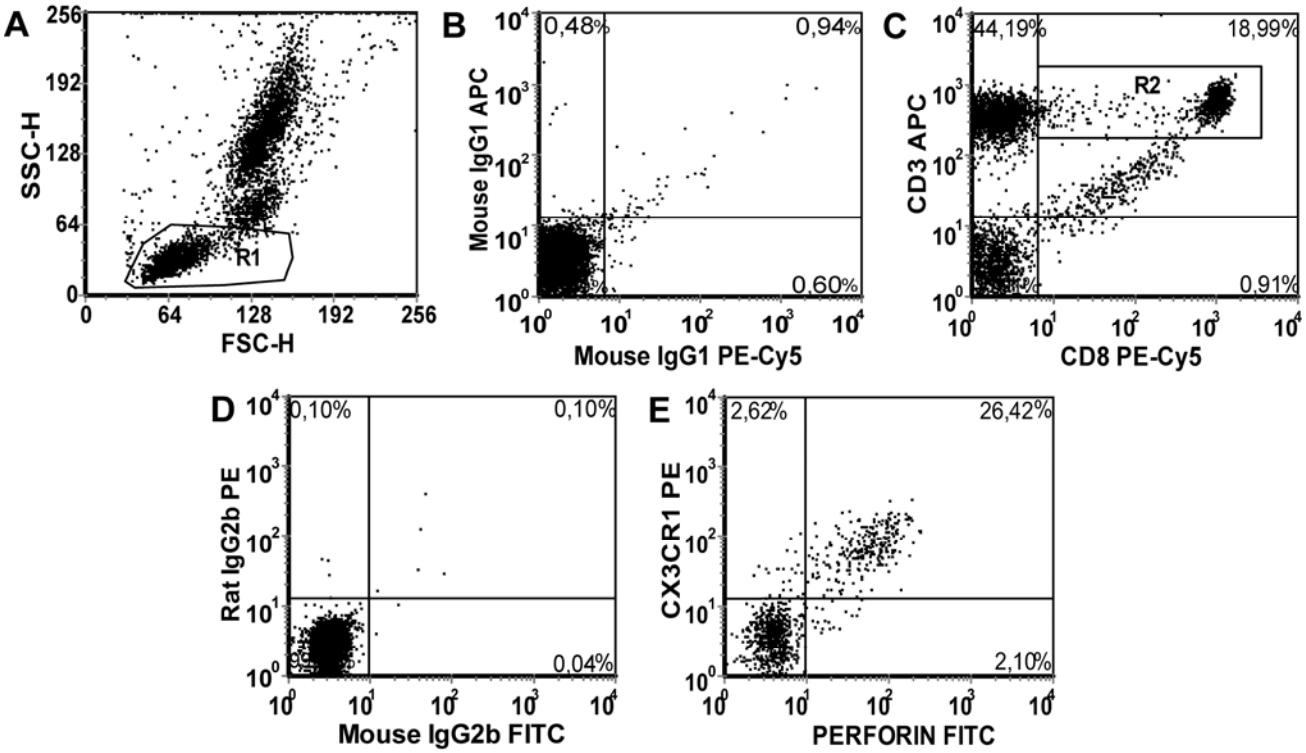
A
SSC-H
FSC-H
R1
B
Mouse IgG1 APC
Mouse IgG1 PE-Cy5
0,48%
0,94%
0,60%
C
CD3 APC
CD8 PE-Cy5
44,19%
18,99%
R2
0,91%
D
Rat IgG2b PE
Mouse IgG2b FITC
0,10%
0,10%
0,04%
E
CX3CR1 PE
PERFORIN FITC
2,62%
26,42%
2,10%

Fig. 2.

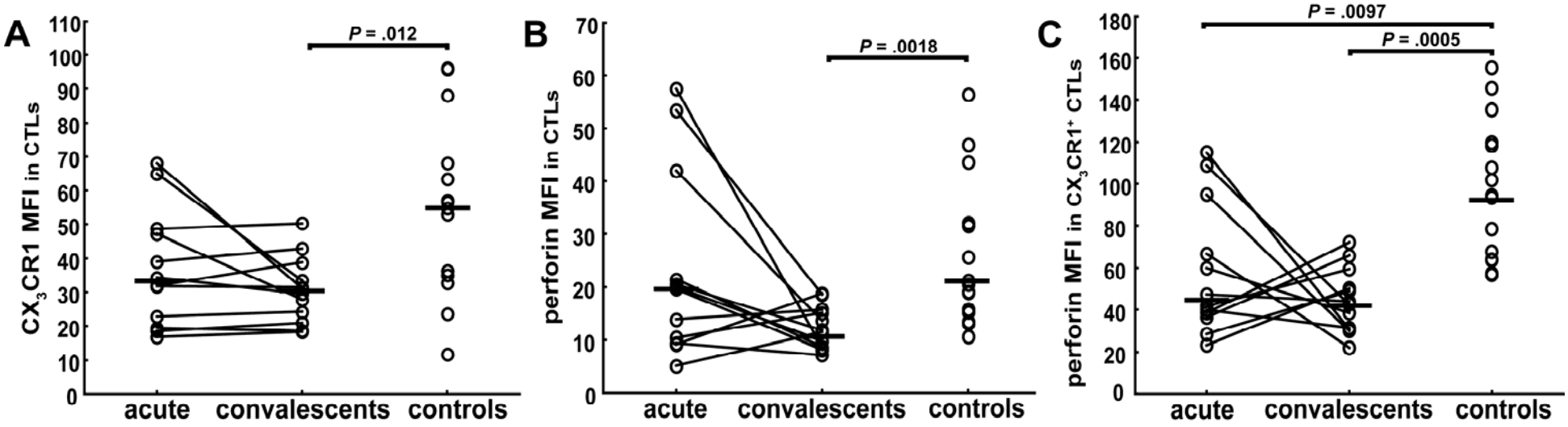
A
CX3CR1 MFI in CTLs
P = .012
acute
convalescents
controls
B
perforin MFI in CTLs
P = .0018
acute
convalescents
controls
C
perforin MFI in CX3CR1+ CTLs
P = .0097
P = .0005
acute
convalescents
controls

Fig. 3.

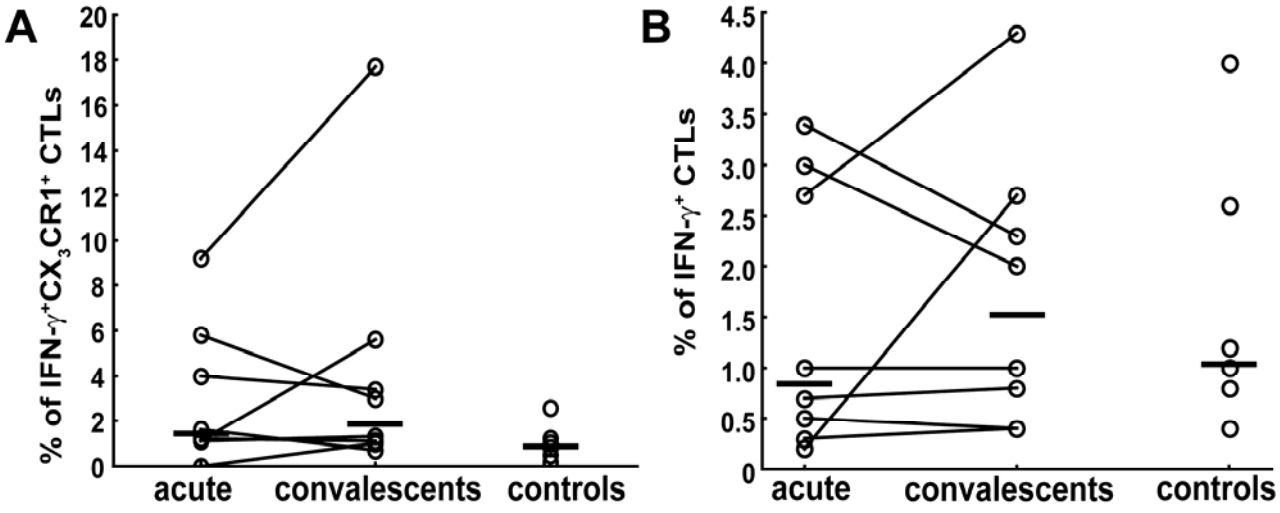
A
% of IFN-γ+CX3CR1+ CTLs
20
18
16
14
12
10
8
6
4
2
0
acute
convalescents
controls
B
% of IFN-γ+ CTLs
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
acute
convalescents
controls

Fig. 4.

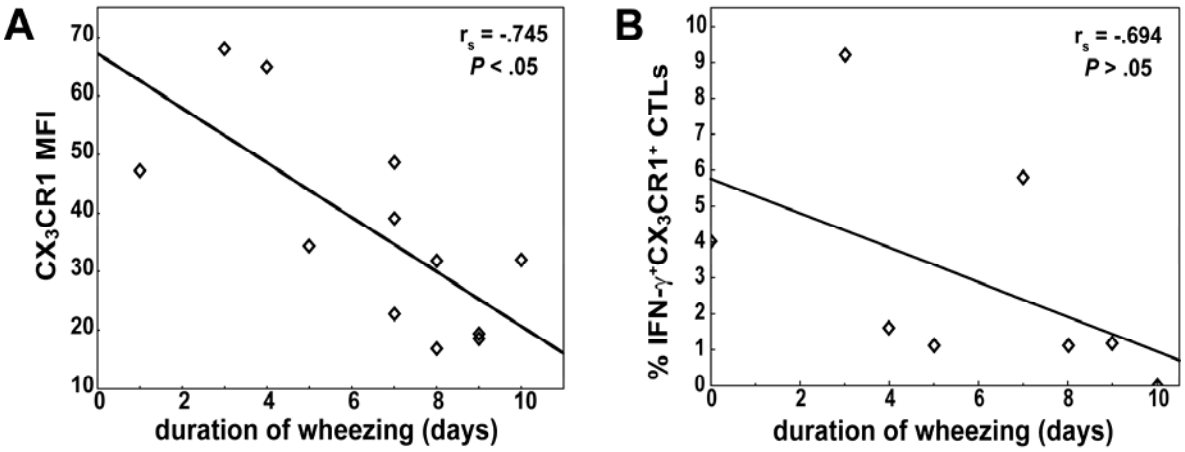
A
r_s = -.745
P < .05
CX_3CR1 MFI
duration of wheezing (days)
B
r_s = -.694
P > .05
% IFN-$\gamma^+CX_3CR1^+$ CTLs
duration of wheezing (days)

Fig. 5.