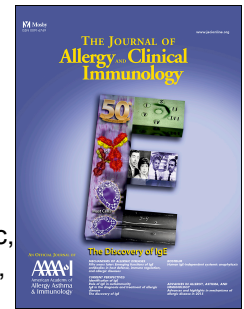


Accepted Manuscript

LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis

Ruben J. Geerdink, MSc, Marije P. Hennis, PhD MD, Geertje H.A. Westerlaken, BSc, Alferso C. Abrahams, MD, Kim I. Albers, MD, Jona Walk, MD, Esther Wesselink, MD, Riny Janssen, PhD, Louis Bont, PhD MD, Linde Meyaard, PhD



PII: S0091-6749(17)31585-3

DOI: [10.1016/j.jaci.2017.08.031](https://doi.org/10.1016/j.jaci.2017.08.031)

Reference: YMAI 13050

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 31 March 2017

Revised Date: 27 July 2017

Accepted Date: 4 August 2017

Please cite this article as: Geerdink RJ, Hennis MP, Westerlaken GHA, Abrahams AC, Albers KI, Walk J, Wesselink E, Janssen R, Bont L, Meyaard L, LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.08.031.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis

Ruben J. Geerdink, MSc (1), Marije P. Hennus, PhD MD (2), Geertje H.A. Westerlaken, BSc (1), Alferso C. Abrahams, MD (3), Kim I. Albers, MD (1), Jona Walk, MD (1), Esther Wesselink, MD (1), Riny Janssen, PhD (4), Louis Bont*, PhD MD (1,5), Linde Meyaard*, PhD (1)

(1) Laboratory for Translational Immunology, Department of Immunology, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

(2) Department of Pediatric Intensive Care, Wilhelmina Children Hospital, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

(3) Department of Nephrology and Hypertension, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands

(4) National Institute for Public Health and the Environment, 3721 MA Bilthoven, The Netherlands

(5) Laboratory for Translational Immunology, Department of Pediatrics, Wilhelmina Children Hospital, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

*These authors contributed equally to this study

Correspondence to: Linde Meyaard, University Medical Centre Utrecht, room KC02.063.1, Lundlaan 6, 3584 EA Utrecht, The Netherlands. Tel.: +31 88 75 542 69; fax: +31 88 7554305; email: l.meyaard@umcutrecht.nl

Sources of support: This work was supported by the Netherlands Organization for Scientific Research Graduate Program grant 022.004.018 to R.J. Geerdink. L. Meyaard is supported by Vici grant 91815608 from the Netherlands Organization for Scientific Research. L. Meyaard and L. Bont have received funding for an investigator-initiated study to validate LAIR-1 as a therapeutic target in an experimental animal model of respiratory syncytial virus infection.

Capsule summary:

The inhibitory immune receptor LAIR-1 is expressed on airway neutrophils and regulates extracellular trap formation during severe RSV bronchiolitis. LAIR-1 ligation represents a promising therapeutic strategy to prevent neutrophil-induced lung injury.

Key words: Respiratory syncytial virus, immune regulation, inhibitory immune receptors, immune injury, NETs

Abbreviations:

BAL	bronchoalveolar lavage
cIg	isotype-matched control antibody
FCS	fetal calf serum
fMLF	formyl-methionyl-leucyl phenylalanine
LAIR-1	leukocyte-associated immunoglobulin-like receptor-1
MPO	myeloid peroxidase
NET	neutrophil extracellular trap
PAF	platelet-activating factor
RSV	respiratory syncytial virus
Siglec-9	sialic acid-binding immunoglobulin-like lectin-9
SIRL-1	signal inhibitory receptor on leukocytes-1
SIRP α	signal-regulatory protein α

LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis

To the Editor:

Respiratory syncytial virus (RSV) infection-induced bronchiolitis is the second most important cause of infant death, following malaria. An estimated 253,000 children die each year of RSV-related disease.¹ We have previously shown that severe RSV bronchiolitis leads to recurrent episodes of wheezing long after the infection is cleared.²

Rapid and efficient clearance of viral respiratory infections requires a potent immune response. However, the intricate lung tissue architecture that facilitates gas exchange is fragile and, in addition to direct virus-induced pathology, excessive inflammation can cause damage that disrupts this vital function. The immune response must therefore be tightly regulated to balance pathogen eradication with immune-induced collateral tissue damage. Inhibitory immune receptors, or immune checkpoints, play a crucial role in preventing the latter. The inflammatory response in the airways of RSV bronchiolitis patients is characterized by extensive neutrophil infiltration: neutrophils comprise more than 80% of infiltrated leukocytes. We have recently reviewed how airway-infiltrated neutrophils may contribute to the control of viral replication, while concurrently inducing immune injury due to the promiscuous cytotoxicity of antimicrobial armaments.³

We therefore set out to identify inhibitory receptors that regulate the inflammatory neutrophil response during viral respiratory infection. In infants mechanically ventilated for RSV bronchiolitis, we determined the expression of six inhibitory receptors (four shown) on airway-infiltrated neutrophils obtained by bronchoalveolar lavage (BAL) and circulating neutrophils in peripheral blood by flow cytometry (Fig 1A-C). Of these receptors, only leukocyte-associated immunoglobulin-like receptor (LAIR)-1 was undetectable on blood neutrophils but highly expressed on the airway-infiltrated neutrophils (Fig 1B). Similarly, neutrophils in nasal lavages of adult acute rhinitis patients expressed LAIR-1, but their blood counterparts did not (Fig E1). Airway-infiltrated neutrophils of children intubated for reasons unrelated to RSV also express

LAIR-1 (Fig E2). Thus, we find that LAIR-1 is expressed on airway-infiltrated neutrophils, but absent on circulating neutrophils, during upper or lower viral respiratory tract infection. While expression of the inhibitory collagen receptor LAIR-1 on mononuclear immune cells is well documented, its expression on mature neutrophils *in vivo* has not previously been reported.⁴

Further analysis revealed that LAIR-1 expressing, airway-infiltrated neutrophils were highly activated, as indicated by increased CD11b expression and shedding of CD62L, when compared to blood neutrophils (Fig 1C). To study the role of activation versus tissue migration in the induction of LAIR-1 expression, we analyzed peripheral blood neutrophils of four pediatric patients with severe, blood culture-positive sepsis by Gram-negative (*H. influenza*) or Gram-positive bacteria (*S. capitis*, *S. pneumoniae*) for LAIR-1 surface expression at several time points. Circulating neutrophils in all four sepsis patients expressed high levels of LAIR-1, which decreased with clinical improvement (Fig 1D). This was not explained by the recruitment of immature neutrophils from bone marrow (Fig E3). Thus, activation per se – in absence of migration into tissue – was sufficient to induce LAIR-1 expression on neutrophils *in vivo*.

LAIR-1 expression was induced *in vitro* on healthy isolated peripheral blood neutrophils by combined stimulation with formyl-methionyl-leucyl phenylalanine (fMLF) and platelet-activating factor (PAF) (Fig 1E). The rapid (≤ 10 min) induction of LAIR-1 surface expression argued against *de novo* protein production. Indeed, confocal microscopy revealed that LAIR-1 is present in the granules of resting neutrophils (Fig 1F). LAIR-1 expression is similarly induced on primed neutrophils exposed to the conditioned culture medium of RSV-infected pulmonary epithelial cells (Fig E4).

To investigate the functional relevance of LAIR-1 surface expression on activated neutrophils, we studied neutrophil extracellular trap (NET) formation. Although antiviral properties of NETs have been reported, NETs can also damage the lungs during viral infection and cause airway obstruction.³ Neutrophils isolated from the blood of RSV bronchiolitis patients

showed little to no NET formation – measured as DNA release using fluorescent microscopy – in the absence of *ex vivo* stimulation (Fig 2A). In contrast, airway-infiltrated neutrophils readily formed NETs during 90 minutes of *ex vivo* culture (Fig 2A-B). Co-staining of DNA with myeloid peroxidase (MPO) confirmed the presence of NETs (Fig 2C). Correspondingly, a previous study found that RSV particles and fusion protein induce NET formation.⁵ Ligation of LAIR-1 with agonistic antibodies significantly reduced NET formation by airway neutrophils during *ex vivo* culture compared to isotype-matched control antibodies (Fig 2B). In contrast, antibody-mediated ligation of SIRT-1 – which suppresses NET formation by peripheral blood neutrophils⁶ – only modestly reduced NET formation. This corresponds to the lower surface expression of SIRT-1 on airway-infiltrated compared to circulating neutrophils – the mirror image of LAIR-1 (Fig 2D). Thus, LAIR-1 ligation inhibits NET formation by airway-infiltrated neutrophils in response to RSV infection. This is in line with a prior study where silencing of LAIR-1 in *ex vivo*-differentiated mouse neutrophils enhanced NET formation.⁷

Recently, two novel RSV antivirals were successfully tested in clinical trials with experimentally infected adults.⁸ Treating naturally infected infants with similar timing – within 12 hours of detecting RSV in nasal washes – may prove difficult. In fact, RSV loads are already declining when patients are admitted into intensive care.⁹ Parallels might be drawn with oseltamivir, which needs to be administered within 36-48 hours of the onset of symptoms to be effective against influenza. Despite declining viral loads, large numbers of neutrophils persist in the airways of RSV bronchiolitis patients.⁹ Ligation of LAIR-1 may offer a potential immunotherapeutic strategy to limit neutrophil-induced immune injury.

We acknowledge several potential limitations. First, the total patient sample size is limited. Nonetheless, all patients showed the same pattern of inhibitory receptor expression and ligation of LAIR-1 restricted NET formation in all cases. Second, our patient cohort does not include a control population of non-ventilated children with mild RSV infection, because we cannot sample the

airways of non-ventilated infants. Third, although antibody-mediated ligation of LAIR-1 reduces NET formation by airway-derived neutrophils *ex vivo*, it is unclear whether LAIR-1 ligation reduces NET formation *in vivo* or how much NET formation needs to be reduced *in vivo* to decrease disease severity. Finally, LAIR-1-mediated regulation of neutrophil functions other than NET formation were not studied.

In conclusion, we provide the first evidence that airway neutrophils of infants with RSV bronchiolitis are regulated by the immune inhibitory receptor LAIR-1. We propose that suppressing neutrophil activity via LAIR-1 ligation under the umbrella of newly available RSV antivirals may provide a novel strategy to treat one of the most important infections during infancy.

Ruben J. Geerdink, MSc (1)
 Marije P. Hennis, PhD MD (2)
 Geertje H.A. Westerlaken, BSc (1)
 Alferto C. Abrahams, MD (3)
 Kim I. Albers, MD (1)
 Jona Walk, MD (1)
 Esther Wesselink, MD (1)
 Riny Janssen, PhD (4)
 Louis Bont*, PhD MD (1,5)
 Linde Meyaard*, PhD (1)

(1) Laboratory for Translational Immunology, Department of Immunology, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

(2) Department of Pediatric Intensive Care, Wilhelmina Children Hospital, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

(3) Department of Nephrology and Hypertension, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands

(4) National Institute for Public Health and the Environment, 3721 MA Bilthoven, The Netherlands

(5) Laboratory for Translational Immunology, Department of Pediatrics, Wilhelmina Children Hospital, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

*These authors contributed equally to this study

References

1. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012;380(9859):2095-128.
2. Blanken MO, Rovers MM, Molenaar JM, Winkler-Seinstra PL, Meijer A, Kimpen JL, et al. Respiratory syncytial virus and recurrent wheeze in healthy preterm infants. *The New England journal of medicine*. 2013;368(19):1791-9.
3. Geerdink RJ, Pillay J, Meyaard L, Bont L. Neutrophils in respiratory syncytial virus infection: A target for asthma prevention. *The Journal of allergy and clinical immunology*. 2015;136(4):838-47.
4. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). *J Leukoc Biol*. 2008;83(4):799-803.
5. Funchal GA, Jaeger N, Czepielewski RS, Machado MS, Muraro SP, Stein RT, et al. Respiratory syncytial virus fusion protein promotes TLR-4-dependent neutrophil extracellular trap formation by human neutrophils. *PLoS One*. 2015;10(4):e0124082.
6. Van Avondt K, van der Linden M, Naccache PH, Egan DA, Meyaard L. Signal Inhibitory Receptor on Leukocytes-1 Limits the Formation of Neutrophil Extracellular Traps, but Preserves Intracellular Bacterial Killing. *J Immunol*. 2016;196(9):3686-94.
7. Sangaletti S, Tripodo C, Vitali C, Portararo P, Guarnotta C, Casalini P, et al. Defective stromal remodeling and neutrophil extracellular traps in lymphoid tissues favor the transition from autoimmunity to lymphoma. *Cancer Discov*. 2014;4(1):110-29.
8. DeVincenzo JP, McClure MW, Symons JA, Fathi H, Westland C, Chanda S, et al. Activity of Oral ALS-008176 in a Respiratory Syncytial Virus Challenge Study. *The New England journal of medicine*. 2015;373(21):2048-58.
9. Lukens MV, van de Pol AC, Coenjaerts FE, Jansen NJ, Kamp VM, Kimpen JL, et al. A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants. *J Virol*. 2010;84(5):2374-83.

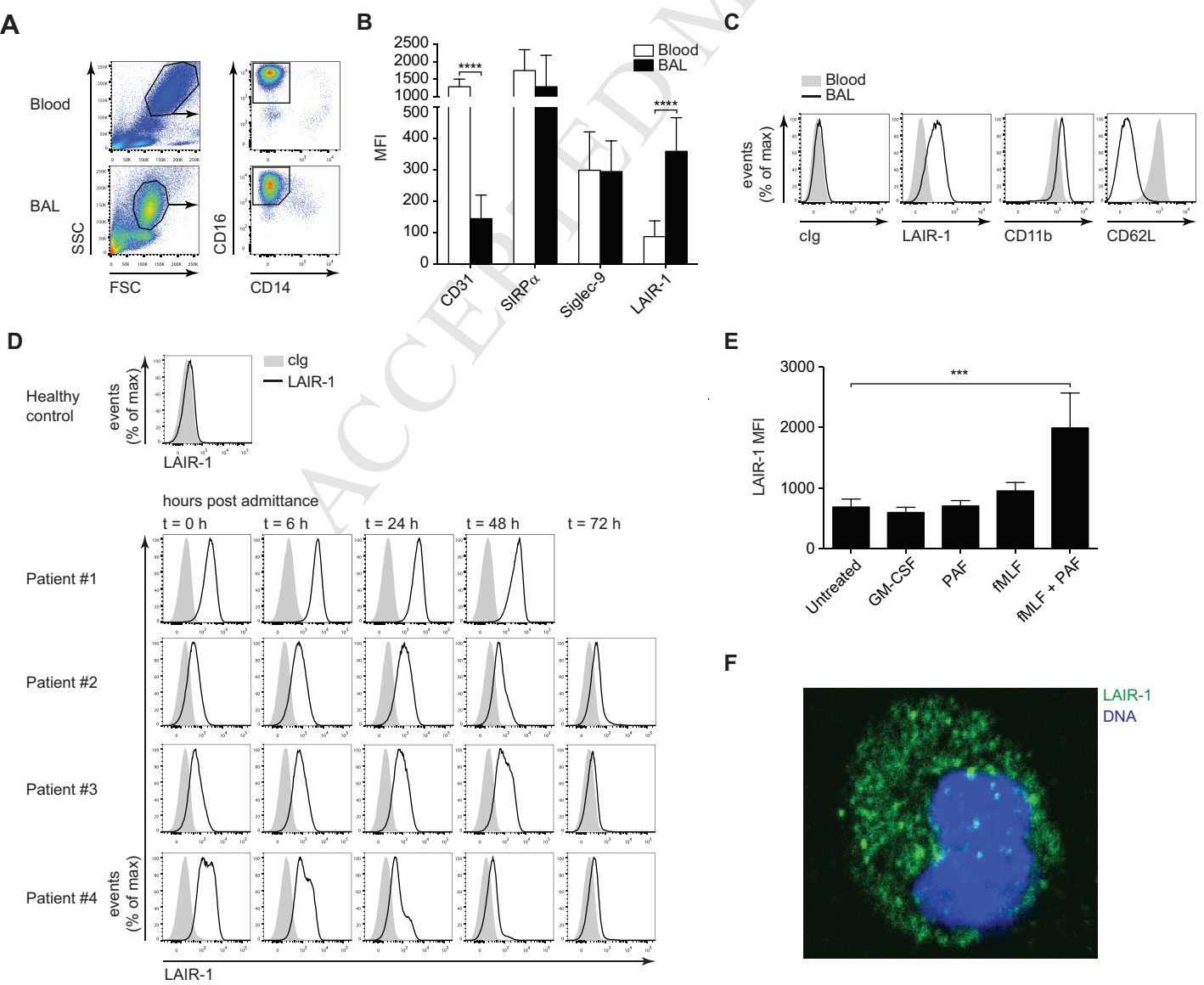
Figure Legends

Figure 1. *In vivo* activated human neutrophils express LAIR-1.

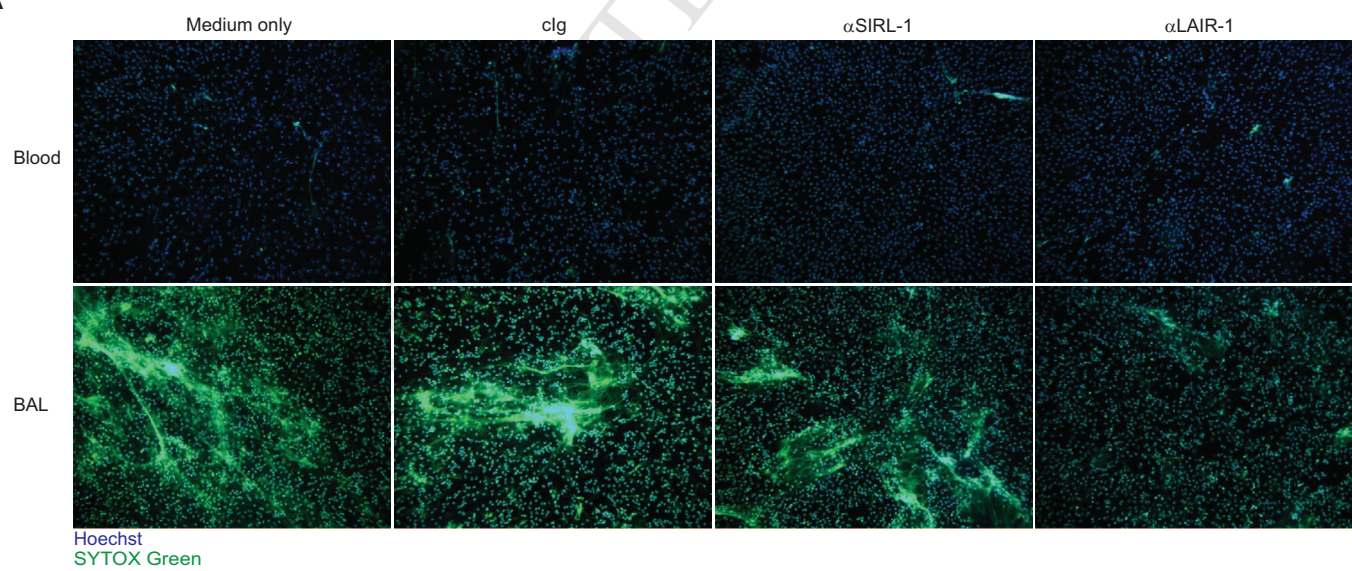
Representative example of the gating strategy for neutrophils from blood and BAL of RSV bronchiolitis patients (n = 9) (A), the quantified expression of inhibitory receptors as MFI (n = 9) (B), and a representative example of activation markers (C) on these neutrophils (n = 9). (D) LAIR-1 expression on peripheral blood neutrophils of sepsis patients (n = 4) and a representative healthy control child. (E) Surface expression of LAIR-1 after *in vitro* stimulation (n = 3). (F) Representative image of intracellular LAIR-1 expression by resting neutrophils (n = 3). *** p < 0.001. BAL, bronchoalveolar lavage; clg, isotype-matched control antibody; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; MFI, median fluorescence intensity.

Figure 2. LAIR-1 suppresses NET formation by airway-infiltrated neutrophils obtained from RSV bronchiolitis patients.

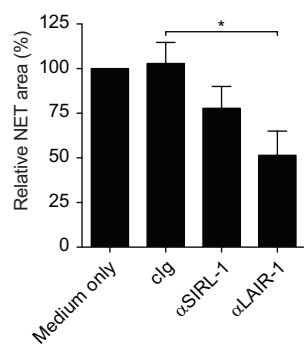
(A) Representative example of SYTOX Green-visualized NETs produced by neutrophils from blood and BAL of RSV bronchiolitis patients (n = 6) incubated *in vitro* with an isotype-matched control antibody (clg), anti-SIRL-1 antibody, anti-LAIR-1 antibody, or medium only. (B) Quantified NET area normalized to medium only for each patient (n = 6). (C) Co-staining of Hoechst 33342- and SYTOX Green-positive NET DNA with MPO (n = 6). (D) Representative example of surface expression of LAIR-1 and SIRL-1 on neutrophils from blood and BAL of RSV bronchiolitis patients (n = 9). * p < 0.05. BAL, bronchoalveolar lavage; clg, isotype-matched control antibody; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; MFI, median fluorescence intensity; MPO, myeloperoxidase; SIRL-1, signal inhibitory receptor on leukocytes-1.



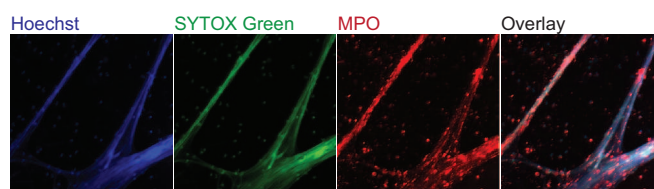
A



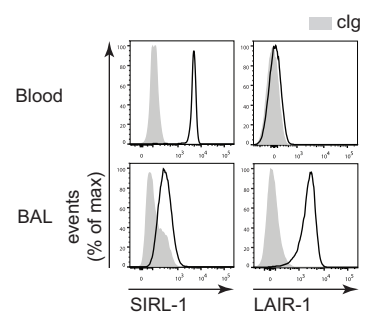
B



C



D



Online supplement

Methods

Patients

The RRR study was initiated to study the expression of LAIR-1 on blood and airway specimens during acute respiratory infection (protocol 10-247). We included a cohort of previously healthy term or otherwise healthy preterm children gestational age >32 weeks, aged less than one year who needed mechanical ventilation for RSV bronchiolitis. Airway-infiltrated cells were obtained from infants with PCR-proven RSV bronchiolitis by tracheobronchial aspiration within 48-72 hours after intensive care unit admission. For the current study, we recruited three control populations. First, to study whether LAIR-1 expression on neutrophils was specific to RSV bronchiolitis, we also recruited adults with simple rhinorrhea (EXIRA study, protocol 11-264). Second, to distinguish whether neutrophil migration or *in vivo* immune activation was sufficient for LAIR-1 expression, we studied children mechanically ventilated for severe culture-proven sepsis (KIRS study, protocol 13-032). Third, neutrophils were isolated from peripheral blood of healthy volunteers for other *in vitro* experiments. The ethical review board approved all studies and participants or their parents (legal guardians) provided written informed consent.

Flow cytometry

Flow cytometry was performed on red blood cell-depleted single-cell suspensions incubated with fluorochrome-conjugated antibodies using a BD Canto II or LSR Fortessa; cells were sorted with a BD FACS Aria III. Acquisitions were analyzed using FlowJo software (version 10.0.7, Treestar).

Neutrophil isolation

Human neutrophils were isolated from heparinized venous blood of patients or healthy donors by density gradient centrifugation with Ficoll (Amersham Biosciences) followed by lysis of red blood cells with ammonium chloride buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA).

In vitro neutrophil stimulation

To study the *in vitro* induction of LAIR-1 expression, neutrophils from healthy donors were isolated and stimulated at 37 °C with granulocyte-macrophage colony-stimulating factor (GM-CSF, 1 µM) for 30 min, platelet-activating factor (PAF, 1 µM) for 10 min, formyl-methionyl-leucyl phenylalanine (fMLF, 1 µM) for 10 min, or PAF (1 µM) and fMLF (1 µM) for 10 min. In other experiments, neutrophils were primed for 15 min with GM-CSF (1 µM) and stimulated with conditioned culture medium of RSV-infected cells or conditioned culture medium of non-infected cells as control. For this, A549 cells were infected with RSV-A2 at a multiplicity of infection of 1. Culture medium was collected at 2 days post infection and stored at -80 °C until used. After stimulation, LAIR-1 surface expression was determined by flow cytometry.

Intracellular LAIR-1 expression

For detection of intracellular LAIR-1, neutrophils were fixed with 1% paraformaldehyde (PFA) in PBS, permeabilized with 0.1% saponin (Sigma) in PBS and stained for LAIR-1 expression with the 8A8 monoclonal antibody (mouse IgG1, own production), which was detected with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Life Technologies). After staining, the cells were plated on 0.001% (v/v) poly-L-lysine-coated coverslips. The coverslips were embedded with Fluoromount-G (Southern Biotech), supplemented with 1 µM Hoechst 33342 (Life Technologies) and analyzed by confocal microscopy (Zeiss LSM710) at 630-fold magnification (Plan-Apochromat 63x/1.40NA objective) with ZEN imaging software (Zeiss).

NET formation by BAL neutrophils

NET formation by airway and blood neutrophils of RSV bronchiolitis was analyzed by fluorescence microscopy as previously described.¹ Cells obtained from RSV bronchiolitis patients by BAL and neutrophils isolated from heparinized venous blood of patients were treated with anti-LAIR-1 antibody (10 µg/mL, clone 8A8, mouse IgG1, own production), anti-SIRL-1 antibody (10 µg/mL, clone 1A5, mouse IgG1, own production), an isotype-matched control antibody (10 µg/mL, clone MOPC-21, mouse IgG1, BD Biosciences), or culture medium only at 4°C for 30 min. Anti-LAIR-1, anti-SIRL-1, and isotype-matched control antibodies were cross-linked with secondary F(ab')₂ goat anti-mouse IgG fragments (20 µg/mL, SouthernBiotech) for 15 min at 4 °C. Cells were suspended in RPMI-1640 with 2% fetal calf serum (FCS), transferred onto glass coverslips coated with 0.001% (v/v) poly-L-lysine (Sigma), and incubated at 37 °C with 5% CO₂ for 90 min with 0.5 µM of SYTOX Green (Life Technologies) present for the last 15 min of incubation. Cells were fixed with 2% (v/v) PFA in PBS and stained with 1 µM of Hoechst 33342 (Life Technologies) in PBS. In some experiments, coverslips were incubated with polyclonal rabbit antibodies against myeloid peroxidase (MPO; Abcam) for 16 h at 4 °C, which were visualized with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibodies (Life Technologies). Coverslips were mounted with Fluoromount-G (SouthernBiotech). To assess the extent of NET formation, SYTOX Green fluorescence was measured with wide-field microscopy at 100-fold magnification (Pathway 855 Bioimaging System, BD) using a Hamamatsu Orca High Resolution 155 CCD camera. The system was controlled with BD AttoVision software (version 1.7/855). Quantification of NETs was performed using nine random fields-of-view per sample that were analyzed with Fiji software.² The same contrast and fluorescence thresholds were applied to the images from all conditions. SYTOX Green-positive pixel counts were divided by the total number of pixels of thresholded 8-bit images, and expressed as the percentage of image area covered by positive fluorescence staining. Additionally, SYTOX Green-permeable neutrophils that retained regular nuclear morphology were excluded from the NET quantification using ImageJ particle analysis. To examine MPO localization in NETs, coverslips were examined by confocal microscope (Zeiss LSM710) at 200-fold magnification (Plan-Apochromat 20x/0.8NA objective) using ZEN imaging software (Zeiss).

Statistics

Differences between inhibitory receptor expression on airway and blood neutrophils of RSV patients were analyzed using a paired 2-tailed Student's t-test. The statistical significance of differences between two groups of patients was calculated with the unpaired Mann-Whitney test, more than two groups were compared with the Kruskal-Wallis test. Statistical analyses were performed using Prism 6 (GraphPad) software. P values <0.05 were considered significant.

Supplemental references

1. Van Avondt K, Fritsch-Stork R, Derksen RH, Meyaard L. Ligation of signal inhibitory receptor on leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus. *PLoS One*. 2013;8(10):e78459.
2. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7):676-82.

Figure E1. Upper airway neutrophils of acute rhinitis patients express LAIR-1.

Blood and BAL cells obtained from rhinitis patients were stained for cell lineage and activation status, as well as a panel of inhibitory receptors, for analysis by flow cytometry. Neutrophils were gated based on their characteristic forward- and side-light scatter properties and further identified by negative CD14 and CCR3 staining, as well as positive CD16 staining. Blood and BAL neutrophils as gated in (A) are compared for their expression of the inhibitory receptors CD31, SIRL-1, SIRP α , Siglec-9, and LAIR-1 in (B). Data are presented as means $\pm$ SD of eight (B) patients. Staining for activation markers (CD11b and CD62L), LAIR-1, and isotype-matched control Ig on BAL versus blood neutrophils of a representative patient are depicted in (C). * p = 0.05. BAL, bronchoalveolar lavage; cIg, isotype-matched control antibody; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; SIRL-1, signal inhibitory receptor on leukocytes-1; Siglec-9, sialic acid-binding immunoglobulin-like lectin 9.

Figure E2. Airway-infiltrated neutrophils of ventilated, RSV-negative pediatric patients express LAIR-1.

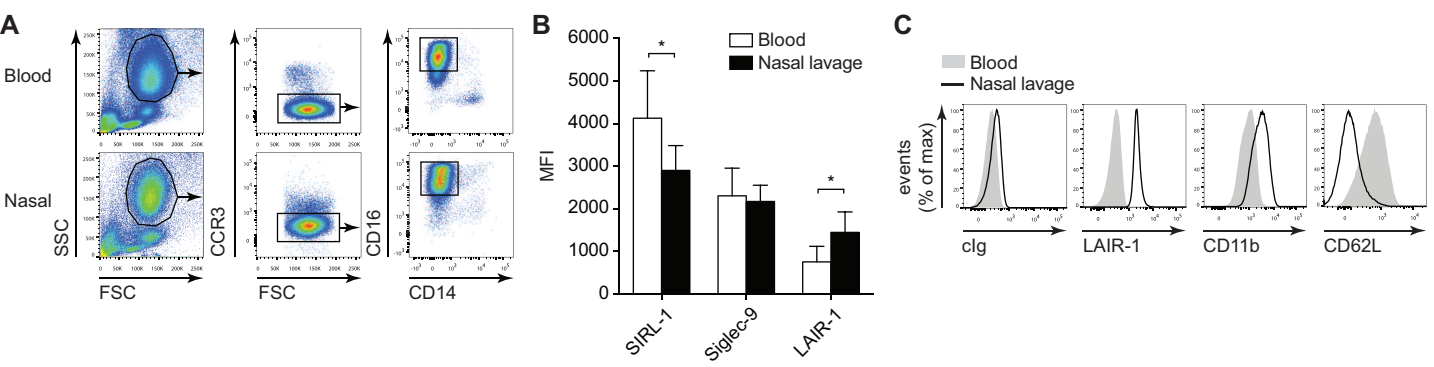
Patient #1: eleven months-old pneumonia patient requiring mechanical ventilation with positive sputum culture for *S. aureus*, *S. pneumonia*, and *M. catarrhalis*, but negative for RSV. Patient #2: one month-old patient requiring mechanical ventilation for central hypoventilation. Patient #3: sixteen months-old patient requiring mechanical ventilation for upper respiratory tract obstruction. Neutrophils in tracheal aspirates of patients were analyzed for LAIR-1 expression by flow cytometry. LAIR-1, leukocyte-associated immunoglobulin-like receptor-1.

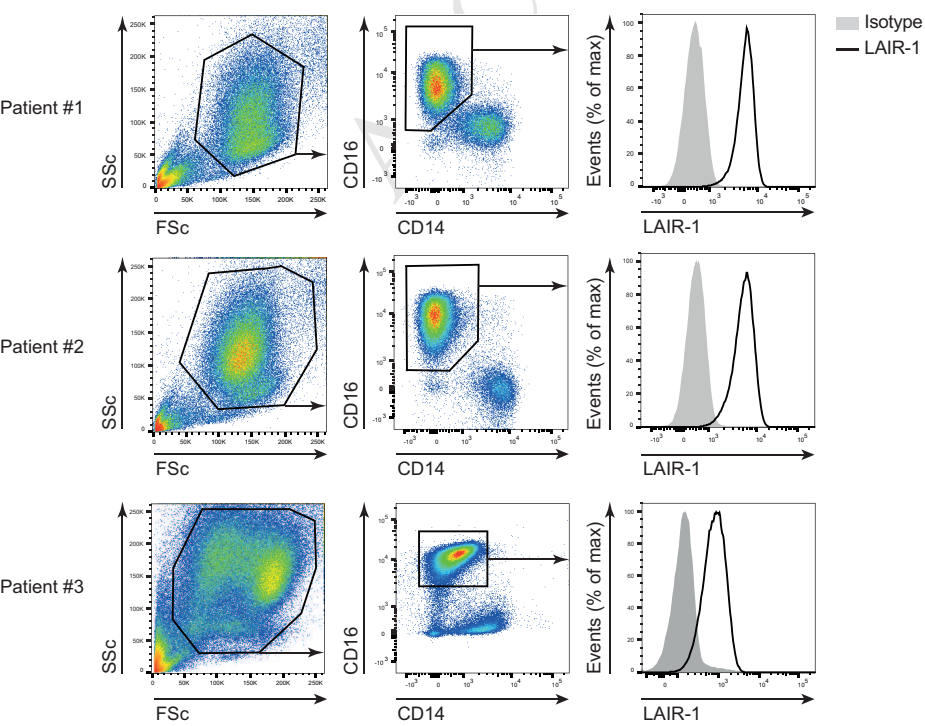
Figure E3. LAIR-1 expressing circulating neutrophils in sepsis patients are mature.

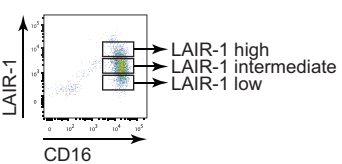
Neutrophils from peripheral blood of a sepsis patient (n = 1) were sorted based on phenotypical neutrophil markers and LAIR-1 expression. Low, intermediate and high LAIR-1 expressing neutrophils were sorted and subjected to Giemsa staining to examine nuclear morphology. LAIR-1, leukocyte-associated immunoglobulin-like receptor-1.

Figure E4. Conditioned culture medium of RSV-infected A549 cells induces LAIR-1 expression on GM-CSF-primed neutrophils.

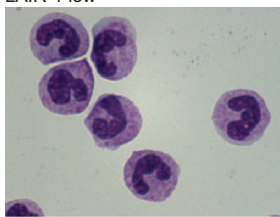
(A) A representative example of LAIR-1 expression on healthy donor neutrophils (n=5) induced by 30 min incubation with conditioned culture medium of RSV-infected A549 cells (RSV supernatant) or conditioned culture medium of uninfected A549 cells (medium) following prior 15 min priming with granulocyte-macrophage colony-stimulating factor (GM-CSF). (B) Quantified LAIR-1 median fluorescence for 5 healthy donors. (C) Confocal microscopy for LAIR-1 (green) and DNA (blue) of unstimulated versus RSV supernatant-stimulated neutrophils (n=3). LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; RSV, respiratory syncytial virus.



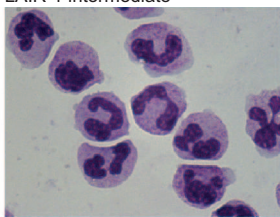




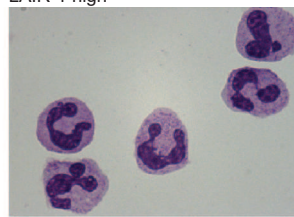
LAIR-1 low



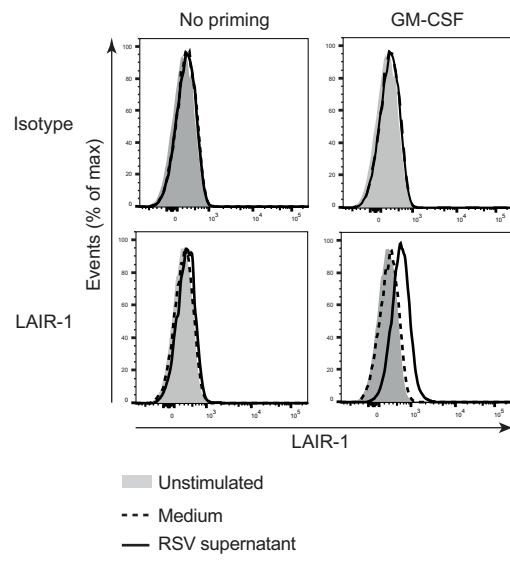
LAIR-1 intermediate



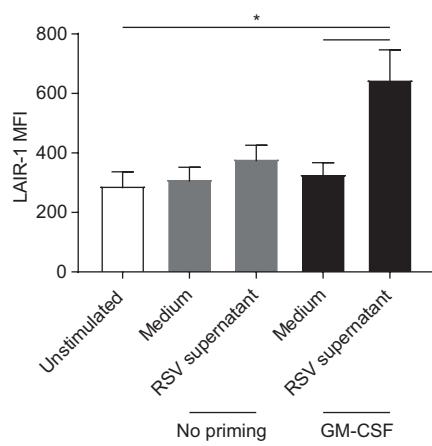
LAIR-1 high



A



B



C

