

## Inhibitory receptor expression on neonatal immune cells

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### Summary

Neonates are born with quantitative and qualitative defects in both adaptive and innate immune responses. The immune system is regulated by several mechanisms, including the signalling of inhibitory receptors. Increased expression of inhibitory receptors may result in a higher threshold for activation and suppressed function of neonatal cells. The aim of this study was to determine whether the expression of seven inhibitory receptors is increased on neonatal immune cells compared to adult immune cells. In a healthy birth cohort, we examined the expression of seven inhibitory immune receptors on neonatal neutrophils, monocytes, natural killer (NK) cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The expression of leucocyte-associated immunoglobulin (Ig)-like receptor-1 (LAIR-1), signal inhibitory receptor on leucocytes-1 (SIRL-1), CD31, signal-regulatory protein alpha (SIRP $\alpha$ ), Siglec-9, CD200R, immune receptor expressed on myeloid cells-1 (IREM-1) and the membrane-bound ligand CD200 was studied by flow cytometry on leucocytes in cord blood ( $n = 14$ ), neonatal venous blood ( $n = 24$ ) and adult venous blood ( $n = 22$ ). Expression of LAIR-1, CD31 and CD200 was increased consistently across all neonatal T cell subsets. Neonatal monocytes exhibited decreased expression of LAIR-1 and IREM-1 compared to adults. Furthermore, cord blood and neonatal venous blood samples contained a distinct LAIR-1-positive neutrophil population, which was not detected in adult blood. We demonstrated distinct expression of inhibitory receptors on neonatal peripheral blood immune cells in a healthy birth cohort. This is the first evidence that inhibitory receptors play a role in regulation of the neonatal immune system. Consistently increased inhibitory receptor expression on T cells may be an important mechanism in preventing the development of allergy and autoimmunity.

**Keywords:** immune regulation, macrophages/monocytes, neonatal, neutrophils, T-cells

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### Introduction

Neonates are susceptible to severe bacterial and viral infections [1]. Susceptibility to infection appears to be due to immature innate and adaptive immune responses [2–4]. Monocytes and antigen-presenting cells (APCs) show a reduction in T helper type 1 (Th1) cell-polarizing proinflammatory cytokine secretion [5], being impaired in the production of tumour necrosis factor (TNF)- $\alpha$  [6–8] and type 1 interferons (IFNs) [9,10]. Neonatal neutrophils are functionally impaired, showing defects in chemotaxis, adhesion, transmigration and anti-microbial activity [7,11]. Neonatal lymphocytes are largely naive and dampened in

their ability to respond to antigen stimulation [2]. The diminished proinflammatory function of the neonatal immune system may be crucial to protect them from dangerous, hyper-reactive immune responses during the transition from the sterile environment *in utero* to the antigen-rich world outside [12,13]. However, it leaves neonates more vulnerable to bacterial and viral infections and to the development of severe sepsis [11].

Both innate and adaptive immune responses are regulated by many factors, one of which is the signalling of inhibitory immune receptors [14–16]. Inhibitory immune receptors, most of which contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), can antagonize cell-activating

signals [17], thereby raising the threshold for immune cell activation [18]. The capacity of inhibitory immune receptors to prevent activation of cells depends on the relative strength of the activating and inhibitory signal given to the cell. This is influenced by the density of the receptor on the cell surface, as well as by the availability of the ligand.

To examine the expression of inhibitory immune receptors in neonates, we initially selected a panel of inhibitory receptors known to be expressed on innate myeloid cells. Several of these are also expressed on multiple lymphoid cell types. Activation of the ITIM-based inhibitory receptor leucocyte-associated immunoglobulin (Ig)-like receptor-1 (LAIR-1) by its natural ligand, collagen or by cross-linking antibodies has been shown to inhibit immune cell function *in vivo* [19–21]. Signal inhibitory receptor on leucocytes-1 (SIRL-1), another ITIM-based inhibitory receptor, is expressed at high levels on monocytes and neutrophils, and it has been demonstrated that higher SIRL-1 expression on monocytes correlates with a lower production of proinflammatory cytokines [22]. CD31, expressed at high levels on myeloid cells, is known to inhibit proinflammatory cytokine production in macrophages and dampen the immune response following endotoxin exposure [23]. Signal-regulatory protein alpha (SIRP $\alpha$ ) is also expressed in high levels on myeloid cells, and is an important regulator of monocyte and neutrophil phagocytosis and endothelial transmigration [24,25]. Siglec-9 is a prominent inhibitory receptor on neutrophils and has been demonstrated to dampen neutrophil activation and bactericidal abilities [26]. Interactions between CD200R, found on both myeloid cells and T and B lymphocytes, and its membrane-bound ligand CD200, expressed on endothelial cells, neurones and lymphocytes, are important in controlling immune-cell infiltration and response in the lungs during infection [27,28]. Immune receptor expressed on myeloid cells-1 (IREM-1) negatively regulates the activity of myeloid cells and has been identified recently as important in the prevention of myeloid cell tissue infiltration [29,30].

We hypothesized that the expression of inhibitory immune receptors on both neonatal innate and adaptive immune cells is increased compared to those of adults. In this study, we compared the expression pattern of these seven inhibitory immune receptors and one membrane-bound ligand on monocytes, neutrophils, natural killer cells, T cells and B cells isolated from cord blood, blood from neonates at the age of 1 month and adult blood. We demonstrated that the expression of inhibitory receptors on cord blood and on neonatal immune cells is distinct from adults.

## Materials and methods

### Study population

This was a cross-sectional study [31]. Cord blood samples were obtained from healthy term infants born in the University Medical Centre (UMC) Utrecht either by uncomplicated

spontaneous vaginal delivery or primary caesarean section. Neonatal blood samples came from healthy infants aged between 3 and 8 weeks. Adult blood samples were donated by healthy volunteers. Written informed consent was obtained from all participants or their caregivers. The study protocol was approved by the Medical Ethical Committee of the UMC Utrecht.

### Blood samples and flow cytometry

Blood samples were used as whole blood. Erythrocytes were lysed using fluorescence activated cell sorter (FACS) lysing solution (BD Biosciences, San Jose, CA, USA). All samples were processed and analysed within 6 h of the time that blood was drawn. Six-colour flow cytometry was used to analyse the eight inhibitory molecules on various cell subtypes. T cells were stained with CD3-eFluor450 (eBioscience, San Diego, CA, USA), CD4-phycoerythrin cyanin 7 (PECy7) (eBioscience), CD8-peridinin chlorophyll (PerCP)Cy5,5 (BioLegend, San Diego, CA, USA) and CD45RO-APC (BioLegend). B cells were defined as CD3-negative and stained with CD19-PerCP (BioLegend); for practical purposes, B cell subsets were not distinguished. Natural killer (NK) cells were defined as CD3-negative and stained with CD56-APC (BD Biosciences). Monocytes were stained with CD14-APCCy7 (BD Biosciences) and CD16-Pacific Blue (BD Biosciences). Granulocytes were stained with CD16-Pacific Blue (BD Biosciences), CD62L-PECy5 (BD Biosciences) and CD11b-APC (BD Biosciences). Inhibitory receptors were stained with LAIR-1-PE (BD Biosciences), SIRL-FITC (as described previously [22]), CD31-PE (BD Biosciences), SIRP $\alpha$ -PE (clone SE5A5; BioLegend, and clone 15–414; Acris Antibodies GmbH, Herford, Germany), Siglec-FITC (BD Biosciences), CD200R-PE (MorphoSys UK Ltd t/a AbD Serotec, Oxford, UK) and IREM-1-PE (BioLegend). CD200 was stained with CD200-FITC (MorphoSys UK Ltd t/a AbD Serotec). Mouse IgG1 $\kappa$ -PE (BD Biosciences), IgG1 $\kappa$ -fluorescein isothiocyanate (FITC) (BD Biosciences) and IgG2a-FITC (Zebra Bioscience BV, Enschede, The Netherlands) were used as isotype controls. Cells were incubated with antibody in the dark at room temperature for 30 min.

Cells were analysed using a LSRII flow cytometer (BD Biosciences) and at least 10 000 events were collected per specified gate. LAIR<sup>+</sup> neutrophils were sorted from whole blood samples using a FACSria cell sorter (BD Biosciences). Lymphocytes, monocytes and granulocytes were gated depending on forward- and side-scatter characteristics. Data were analysed using the software program FACSDiva (BD Biosciences).

A receptor was considered to be expressed on a certain cell type if the median mean fluorescence intensity (MFI) was twice that of isotype control. The percentage of positive expression was calculated based on isotype controls. During the 18-month research period, regular maintenance to the flow cytometer resulted in a small shift of MFI. Correction

was performed based on the ratio between the medians of the two data sets. Combined results are reported only if patterns in both data sets were similar.

### Statistical analyses

All data were analysed using the SPSS program version 15.0. Statistical differences in mean MFIs or percentage expression between cord, neonatal and adult blood were determined by one-way analysis of variance (ANOVA) with *post-hoc* analysis (Bonferroni test for multiple comparisons). Means in MFIs or percentage expression of inhibitory receptors of CD16<sup>+</sup> and CD16<sup>-</sup> monocytes were compared using the paired Student's *t*-test.

### Results

Concentrations of leucocyte subsets were analysed in 14 cord blood samples, 24 venous blood samples from 1-month-old neonates and 22 adult venous blood samples (Table 1). The concentrations of various T cell subsets varied, as expected, between neonates and adults [2]. Monocytes can be divided into the 'classical' CD14<sup>hi</sup>CD16<sup>-</sup> and 'proinflammatory' CD14<sup>+</sup>CD16<sup>+</sup> subtypes [32,33]; there was no difference in the concentrations of these subpopulations between cord blood, 1-month-old neonates and adults [34,35]. Expression of LAIR-1, CD31 and Siglec-9 was found to be increased on CD14<sup>+</sup>CD16<sup>+</sup> monocytes compared to the total monocyte population and the expression of SIRL-1 and SIRP $\alpha$  was decreased on CD14<sup>+</sup>CD16<sup>+</sup> monocytes compared to total monocytes (Fig. S1). In contrast with previously reported

findings, we found no difference in the expression of CD200R between these monocyte subtypes [36].

Differences in the expression of inhibitory molecules between cord blood, 1-month-old neonates and adults were analysed in terms of both percentage expression and MFI. Distinct profiles of surface expression of inhibitory immune receptors were found. A complete overview of the findings of this study is shown in Table S1; these data are summarized in Table 2. We confirmed that the expression of Siglec-9 is increased on cord blood neutrophils compared to adults (MFI 296 *versus* 204,  $P < 0.01$ , Fig. 1a) [37]. Interestingly, the difference is no longer significant at the age of 1 month. While adult neutrophils do not express LAIR-1, cord blood and neonatal venous blood samples contained a small but distinct LAIR-1-positive neutrophil population (1.40% of total neutrophils,  $P < 0.01$ ) (Fig. 1b). Because immature neutrophils have been described previously to express LAIR-1 [38], LAIR-1-expressing neutrophils were stained and sorted into two independent experiments. It was determined that 72–75% of the LAIR-1<sup>+</sup> cells were mature polymorphonuclear neutrophils (PMNs) (data not shown). In contrast to the enhanced expression of Siglec-9 and LAIR-1 on cord blood neutrophils, expression of IREM-1 was down-regulated on neonatal monocytes. IREM-1 was expressed on only 27.0% of cord blood monocytes, while it was expressed on 67.9% of neonatal and 65.6% of adult monocytes ( $P < 0.001$ ). Interestingly, this difference is smaller within the CD14<sup>+</sup>CD16<sup>+</sup> monocyte population (Fig. 1c).

Besides their expression on myeloid cells, three of these receptors are also expressed on lymphoid cells. The expression of LAIR-1 was increased across all neonatal lymphocyte subsets (Fig. 2). We found a similar pattern of increased expression on neonatal lymphocytes for CD31 and CD200 (Table S1). In contrast, CD200R expression was decreased significantly on B cells from cord blood and neonates compared to adults (Table S1).

**Table 1.** Relative cell counts of the lymphocyte and monocyte subsets in each of the study populations.

	Cord blood	1-month-old neonate	Adult
Total patients	14	24	22
Lymphocytes			
T cells	69.2	72.3	74.5
CD4 <sup>+</sup>	74.2 (1.7) <sup>†</sup>	73.6 (1.9) <sup>†</sup>	65.4 (2.6)
CD45RO <sup>-</sup>	60.6 (4.8) <sup>‡</sup>	79.7 (2.8) <sup>†</sup>	46.7 (4.5)
CD45RO <sup>+</sup>	39.3 (4.8) <sup>‡</sup>	20.3 (2.8) <sup>†</sup>	53.2 (4.5)
CD8 <sup>+</sup>	25.8 (1.7) <sup>†</sup>	26.4 (1.9) <sup>†</sup>	34.6 (2.6)
CD45RO <sup>-</sup>	70.6 (3.9)	84.2 (2.7) <sup>†</sup>	58.7 (4.9)
CD45RO <sup>+</sup>	29.3 (3.9)	15.7 (2.7) <sup>†</sup>	41.3 (4.9)
B cells	16.2 (1.7)	20.0 (1.9) <sup>†</sup>	12.4 (1.3)
NK cells	14.6 (2.2) <sup>‡</sup>	7.6 (1.0) <sup>†</sup>	13.1 (1.3)
Monocytes			
CD14 <sup>hi</sup> CD16 <sup>-</sup>	88.2 (1.8)	90.4 (1.0)	88.3 (2.3)
CD14 <sup>+</sup> CD16 <sup>+</sup>	11.8 (1.8)	9.6 (1.0)	11.7 (2.3)

Mean relative cell counts are shown with standard error of the mean in parentheses. *P*-values represent the outcome of one-way analysis of variance (ANOVA). <sup>†</sup>Mean relative cell count differs from that of adult venous blood ( $P < 0.05$ , outcome of one-way ANOVA). <sup>‡</sup>Mean relative cell count differs from that of neonatal venous blood ( $P < 0.05$ , outcome of one-way ANOVA). NK, natural killer.

### Discussion

Neonates are more vulnerable to severe infections [38] as a result of immature innate and adaptive immune responses [2,7,11]. The immune system is regulated by a fine balance of activating and inhibitory receptor signalling [17,18,39,40]. Previous studies have demonstrated that inhibitory immune receptors are important regulators of innate immune responses to infection [41,42]. In this descriptive study, we demonstrated distinct neonatal expression of inhibitory receptors on both adaptive and innate immune cells. We confirmed previously published findings that the surface expression of Siglec-9 is increased on cord blood neutrophils [37], and extend this observation to the neonatal age. We showed that the expression of several other inhibitory immune receptors is also distinct on neonatal monocytes, neutrophils and natural killer cells. Additionally, we showed that across all T cell subsets,

**Table 2.** Summary of the study results.

	LAIR-1	SIRL-1	CD31	SIRP $\alpha$	Siglec-9	CD200	CD200R	IREM-1
<b>Cord blood</b>								
Neutrophils	↑	=	=	=	↑**		=	=
Monocytes CD16 <sup>-</sup>	=	=	=	=	=		=	↓***
Monocytes CD16 <sup>+</sup>	=	=	=	=	=		=	↓*
NK cells	=		=					
CD4 <sup>+</sup> CD45RO <sup>-</sup> T cells	↑*		↑***			↑***		
CD4 <sup>+</sup> CD45RO <sup>+</sup> T cells	↑***		↑**			↑***		
CD8 <sup>+</sup> CD45RO <sup>-</sup> T cells	↑**		↑***					
CD8 <sup>+</sup> CD45RO <sup>+</sup> T cells	↑***		↑***			↑***		
B cells	↑***		↑**			=	↓**	
<b>Neonatal venous blood</b>								
Neutrophils	↑**	=	=	=	=		=	=
Monocytes CD16 <sup>-</sup>	↓*	=	=	=	=		=	=
Monocytes CD16 <sup>+</sup>	=	=	=	=	=		=	=
NK cells	=		↓***					
CD4 <sup>+</sup> CD45RO <sup>-</sup> T cells	↑*		↑*			=		
CD4 <sup>+</sup> CD45RO <sup>+</sup> T cells	↑***		=			↑***		
CD8 <sup>+</sup> CD45RO <sup>-</sup> T cells	↑**		↑***					
CD8 <sup>+</sup> CD45RO <sup>+</sup> T cells	↑***		↑***			↑**		
B cells	↑***		=			↓*	↓***	

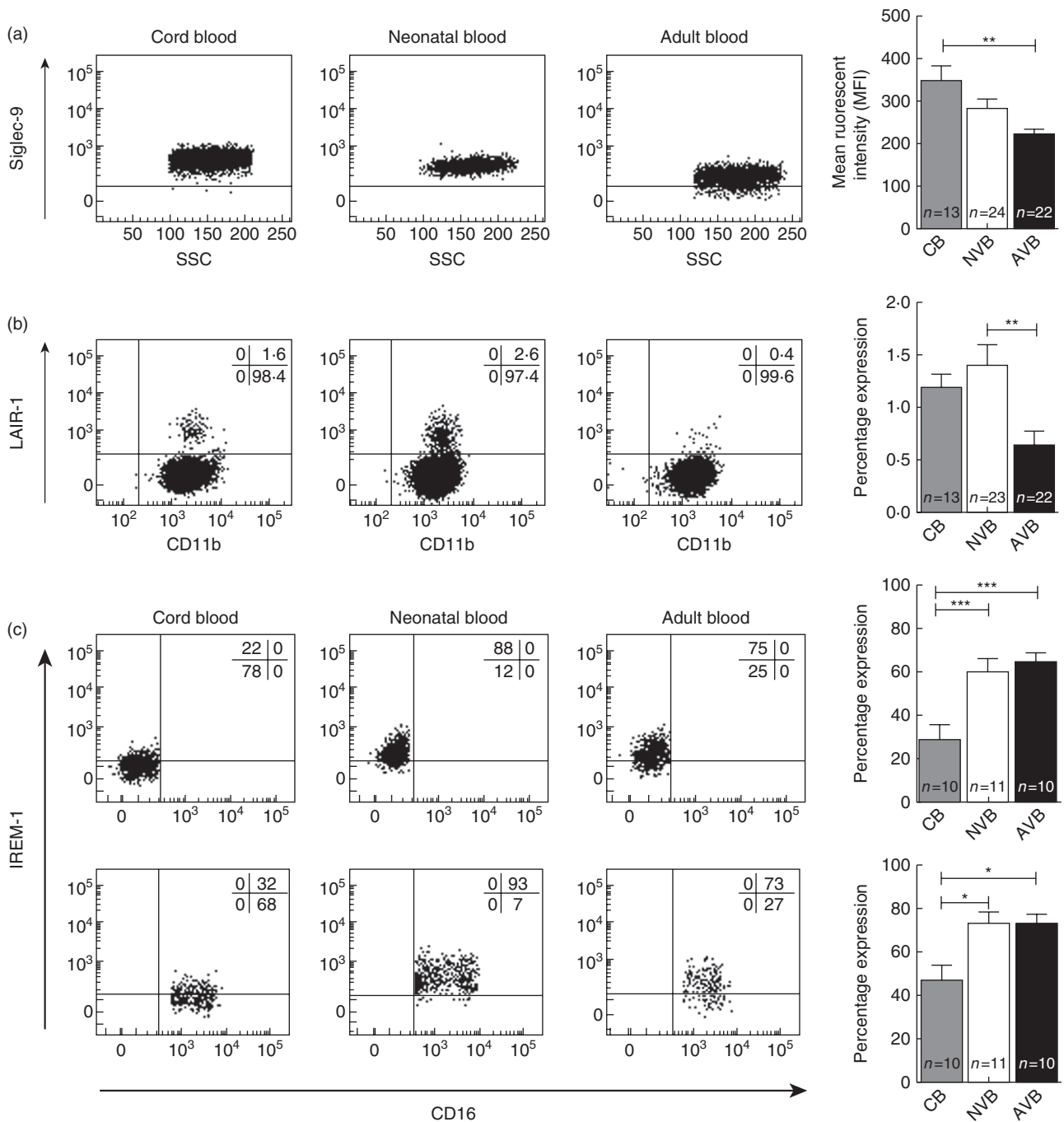
P-values are the outcome of one-way analysis of variance (ANOVA) with *post-hoc* Bonferroni correction (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Shows the expression of each inhibitory receptor per cell type as increased, decreased or similar compared to adult cells. Where no symbol is shown, a receptor was not expressed on that cell type. LAIR-1, leucocyte-associated immunoglobulin (Ig)-like receptor-1; SIRL-1, signal inhibitory receptor on leucocytes-1; SIRP $\alpha$ , signal-regulatory protein alpha; IREM-1, immune receptor expressed on myeloid cells-1; NK, natural killer.

neonatal T cells express higher levels of inhibitory receptors than adults.

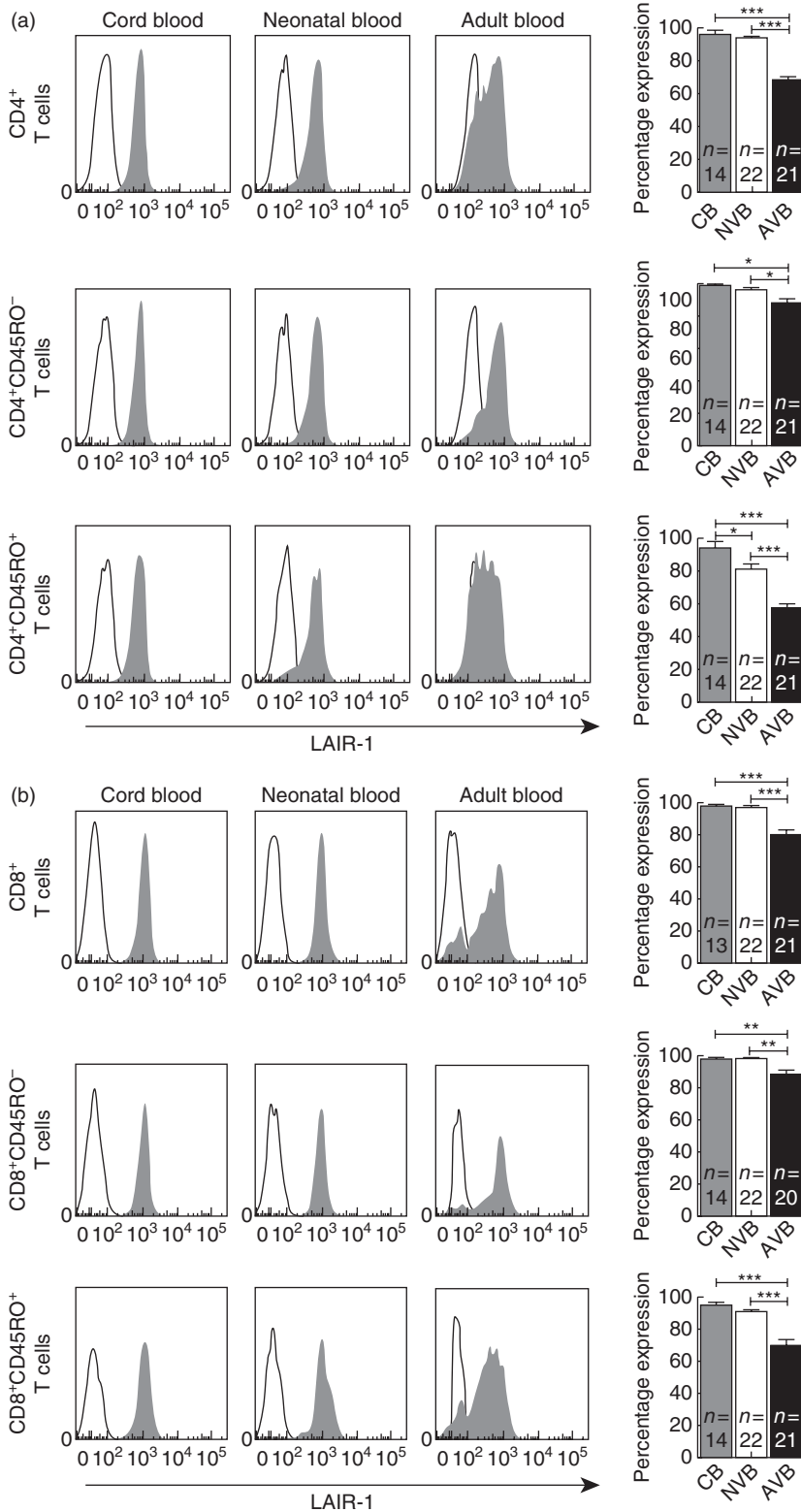
This study focused on innate immune cells because these cells are most important in protecting neonates during their first month of life. We chose seven inhibitory receptors for the current study. First, we selected well-characterized receptors that have been shown to be important in regulating innate immune cell function. Secondly, we also chose to study those receptors in which we have a long-standing interest [22,27,40]. Although the majority of inhibitory receptors on each innate immune cell type are expressed at similar levels in adults and neonates, we also observed some distinct differences. We found that IREM-1 was decreased on neonatal monocytes and that Siglec-9 and LAIR-1 were increased on neonatal neutrophils [37]. A previous study has demonstrated that Siglec-9 is increased on cord blood neutrophils, which is relevant, as the sialylated capsular polysaccharide of group B streptococcus uses Siglec-9 to dampen the bactericidal neutrophil response [26]. Group B streptococcus is the major pathogen during neonatal age, but not thereafter [43]. We were able to confirm increased Siglec-9 expression in neonates and show that this difference largely disappears during the first month of life. This is consistent with other observations that neonatal neutrophils obtain a mature phenotype during the first few weeks after birth [44]. Adult PMNs in peripheral blood do not express LAIR-1 [45]. We observed that cord blood and neonatal venous blood samples contain small populations of neutrophils expressing

high levels of LAIR-1. Interestingly, these were not immature neutrophils, which are known to express LAIR-1 [45], but mature PMNs. More functional studies are required to understand fully the implications of the distinct neonatal expression profile of inhibitory receptors on innate immune cells.

In addition to the innate immune response, this study adds to our understanding of the function of the neonatal adaptive immune system. During the first weeks of life the adaptive immune system is immature; this is due partly to the fact that neonates have yet to develop large memory and effector lymphocyte populations, but even following adequate antigen stimulation neonatal T lymphocyte responses are often diminished in magnitude [2]. We confirmed our previous finding that LAIR-1 expression is increased on naive T cells compared with memory and effector cells [46], and demonstrate for the first time that, compared to adults, LAIR-1 expression is increased on both neonatal naive T cells as well as neonatal effector T cells. Interestingly, LAIR-1 expression is highest on cord blood T cells, showing that while not reaching the level on adult cells, LAIR-1 levels decrease during the first 4 weeks of life. We also found that the expression of the inhibitory receptors CD31 as well as the expression of CD200, the membrane-bound ligand of CD200R, is generally increased on all subsets of neonatal T cells. Therefore, we theorize that the consistent increase of inhibitory receptors on neonatal T cells plays a role in dampening the adaptive immune response *in utero*



**Fig. 1.** Neonatal neutrophils and monocytes express different levels of inhibitory receptors. (a) The expression of Siglec-9 is increased on cord blood neutrophils and this difference begins to disappear during the first weeks of life. Representative fluorescence activated cell sorter (FACS) dot-plots and a graphic representation of all data is shown. CB: cord blood; NVB: neonatal venous blood; AVB: adult venous blood. (b) Neonatal and cord blood samples contain a distinct leucocyte-associated immunoglobulin (Ig)-like receptor-1 (LAIR-1)-positive neutrophil population not seen in adult blood. The level of CD11b, a neutrophil activation marker, is the similar between LAIR-1<sup>+</sup> and LAIR-1<sup>-</sup> neutrophils. Representative FACS dot-plots and a summary of all data is shown. (c) The expression of immune receptor expressed on myeloid cells-1 (IREM-1) is decreased on cord blood but not neonatal monocytes compared to adult monocytes. Interestingly, this difference is smaller within the CD14<sup>+</sup>CD16<sup>+</sup> monocyte population. Representative FACS dot-plots and graphic representation of all the data are shown. *P*-values are the outcome of one-way analysis of variance (ANOVA), with \**P* < 0.05; \*\**P* < 0.01 and \*\*\**P* < 0.001.



**Fig. 2.** Leucocyte-associated immunoglobulin (Ig)-like receptor-1 (LAIR-1) expression is higher on neonatal T cells. (a) LAIR-1 expression is increased on both neonatal CD45RO<sup>-</sup> and CD45RO<sup>+</sup> CD4<sup>+</sup> T cells. Representative fluorescence activated cell sorter (FACS) histograms and graphic representation of all data are shown. CB, cord blood; NVB, neonatal venous blood; AVB, adult venous blood. (b) A similar increase in LAIR-1 expression is seen on all CD8<sup>+</sup> T cell subsets. Representative FACS histograms and data summaries are shown. *P*-values are the outcome of one-way analysis of variance (ANOVA), with \**P* < 0.05; \*\**P* < 0.01 and \*\*\**P* < 0.001.

and during the first weeks of life, leaving neonates susceptible to infection with intracellular pathogens.

The potential strengths and weaknesses of this study require discussion. The major strength of this study is

that we had access to unique samples, namely, venous blood from a population healthy 1-month-old neonates. Examining both immune cells from cord blood as well as from healthy neonates has two advantages. First, it eliminates the

possibility that specific observations are restricted to cord blood, which has a low pH [47] and is drawn under stressful conditions. Secondly, it provides insight into the rapid changes that the normal immune system undergoes during this critical neonatal age. A weakness of this study is that multiple comparisons were performed. When comparing between our three populations, Bonferroni correction was performed to correct for multiple comparisons. No attempt was made to correct for the fact that we tested multiple receptors on multiple cell types. However, we note that of 79 statistical analyses performed, 45 were significant and many were highly significant ( $P < 0.001$ ). Finally, we tested only a limited number of seven inhibitory receptors, while more than 40 different inhibitory receptors exist [14,48].

We conclude that neonatal innate and adaptive immune cells exhibit a pattern of inhibitory receptor expression that is distinct from adults. We also demonstrate that there is a consistent increase of the inhibitory molecules LAIR-1, CD31 and CD200 on both naive and memory T cells in neonates. This suggests a novel mechanism by which neonates regulate their immune response to novel food and aeroallergens during the first few weeks of life.

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## Disclosure

None of the authors have any affiliations with, or financial involvement in, any organization with a direct financial interest in the subject of the research discussed in this paper.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Inhibitory receptor expression on monocyte subtypes. Surface expression of inhibitory receptors was compared between total monocytes and CD14<sup>+</sup>CD16<sup>+</sup> proinflammatory monocytes in 21 healthy adults. *P*-values shown are the outcomes of paired-sample *t*-tests, with \**P* < 0.05; \*\**P* < 0.01 and \*\*\**P* < 0.001.

**Table S1.** Expression of seven inhibitory receptors and one membrane-bound ligand in neonates and adults. Summary of median mean fluorescence intensities (MFIs) (a) and percentage of positive expression (b) found for every inhibitory receptor per cell type and sample type. CB, cord blood; NVB, neonatal venous blood; AVB, adult venous blood. Where no value is shown, a receptor was not expressed on that cell type (mean MFI less than twice that of isotype control).

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