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Supplemental Information

Respiratory Syncytial Virus Infects Regulatory B Cells in Human Neonates via Chemokine Receptor CX3CR1 and Promotes Lung Disease Severity

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Figure S1

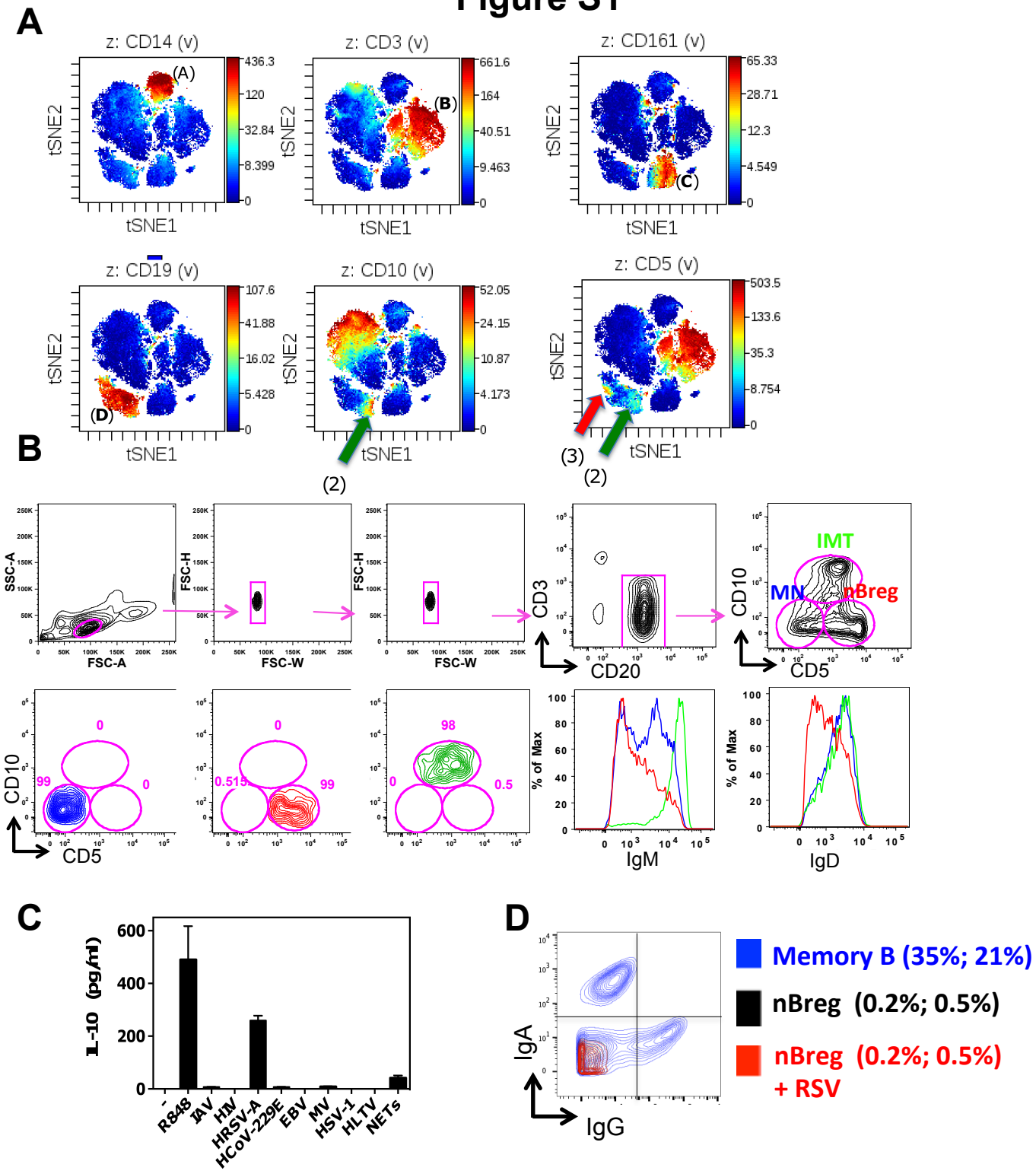
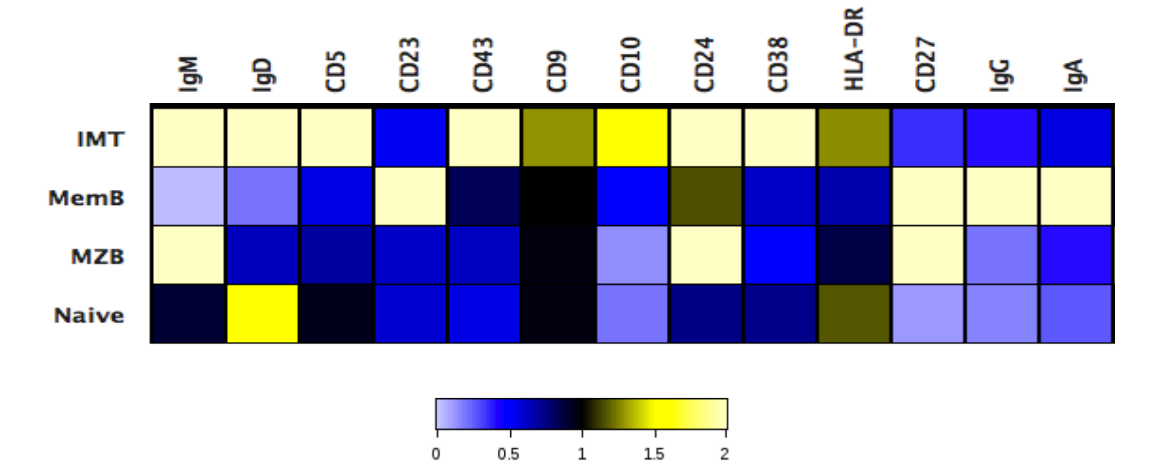


Fig. S1 (related to Fig. 1): viSNE analysis of CBMC

(A) Cord blood cells were analyzed as in Fig. 1A using viSNE which defines based on indicated lineage markers : (A) monocytes, (B) T cells , (C) NK cells, (D) B cells. B cell phenotypes 1, 2 and 3 can be clearly visualized independently of other blood cell types with (2) as $CD10^{pos}CD5^{lo}$ (green arrow) and (3) as $CD5^{hi}CD10^{neg}$ (red arrow). (B) Gating strategy for FACS sorting of neonatal B cells MN , IMT and nBreg, purity check and subsets IgM/IgD expression. (C) 10^5 cord blood nBregs were stimulated with the indicated stimuli. IL-10 was detected in the supernatants at 48 h (means of three donors +/-SD). (D) IgG and IgA detection on nBregs activated or not with RSV in comparison to adult Memory B cells. In parenthesis, frequencies of Ig isotype is indicated (IgA%; IgG%).

Figure S2



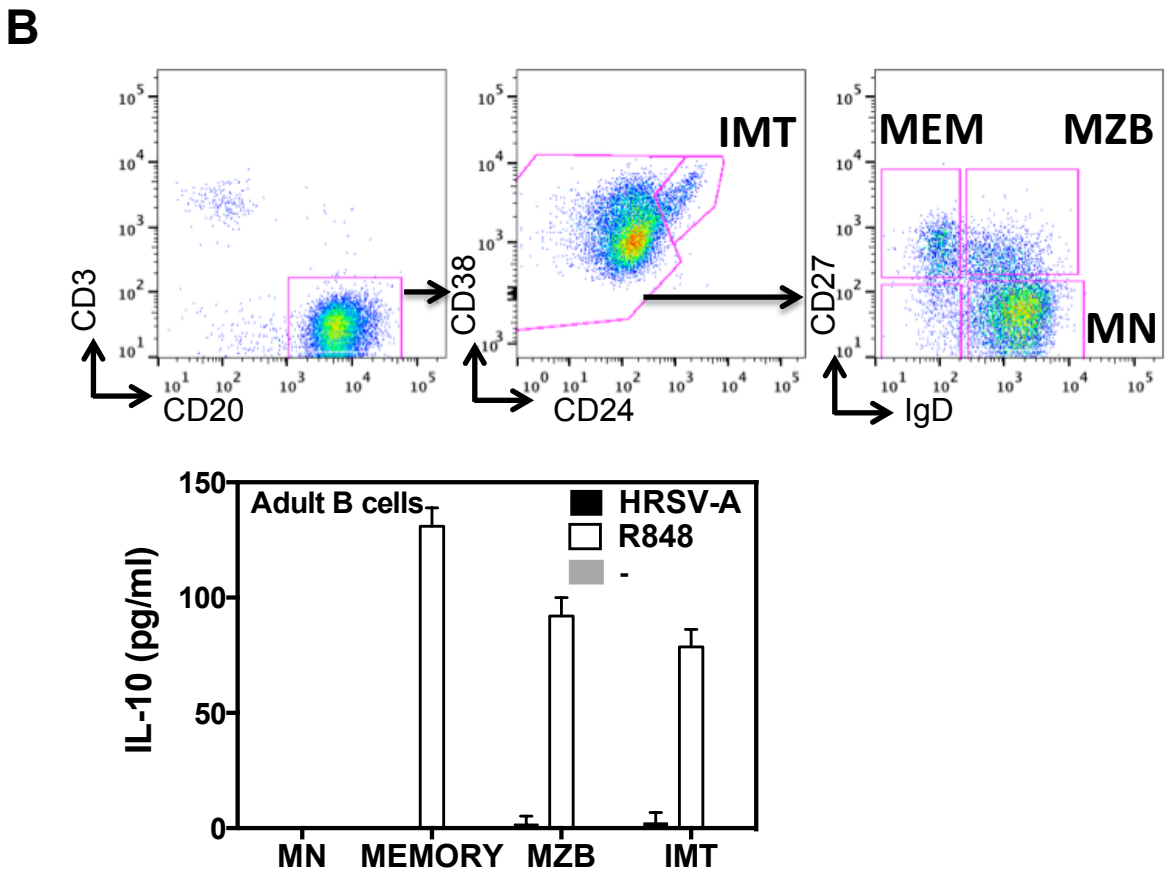


Fig. S2 (related to Fig. 1): IL-10 response of B cells to viruses
(A) CyTOF of adult B cell subsets for the expression of the indicated markers, data were normalized to the the total population of B cells.
(B) 10^5 adult blood B subsets were purified by FACS with indicated gating strategy. Mature Naïve (MN), memory B cells (MEM), marginal zone B cells (MZB) were pre-gated on CD24loCD38+/lo mature B cells, and (IMT) Immature transitional correspond to $CD24^{hi}CD24^{hi}$ B cells. Sorted B cells were then stimulated or not with HRSV-A or R848 and IL-10 was detected at 48h by ELISA. Results are expressed as the means of triplicates+/-SD and are representative of two experiments.

Figure S3

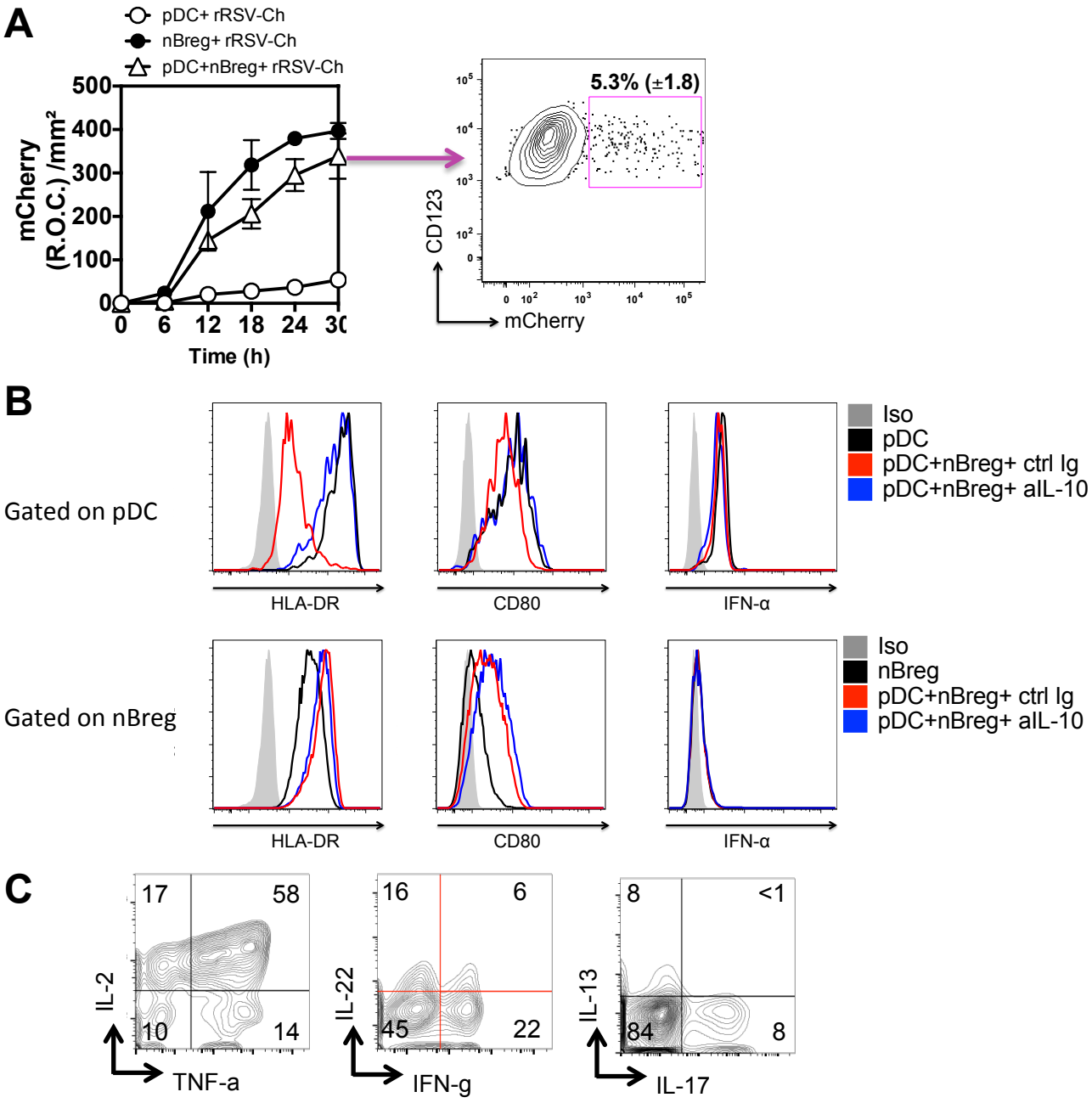


Fig. S3 (related to Fig. 2) :
(A-B) pDC and nBreg cells were sorted from cord blood and cultured with rRSV-Ch (MOI=2.5) alone or in co-culture for 30h-48h in the presence of anti-IL10 (a-IL10) or an isotype control Ig. (A) Left panel represents pDC infection as compared to nBreg cells cultured alone measured by live microscopy. Right panel FACS plot shows the frequency of RSV infected pDC when cultured alone. Results are means of triplicates and are representative of three experiments. (B) Histograms show the expression of HLA-DR, CD80 or intracellular IFN-α after 48h of stimulation with HRSV-A. Iso corresponds to isotype control staining, and ctrl Ig to anti-IL10 isotype control. (C) CD4 naive T cells were culture in TH17 conditions, and serve as positive control of Fig. 2 for intracellular staining of cytokines indicated on the X and Y axes.

Figure S4

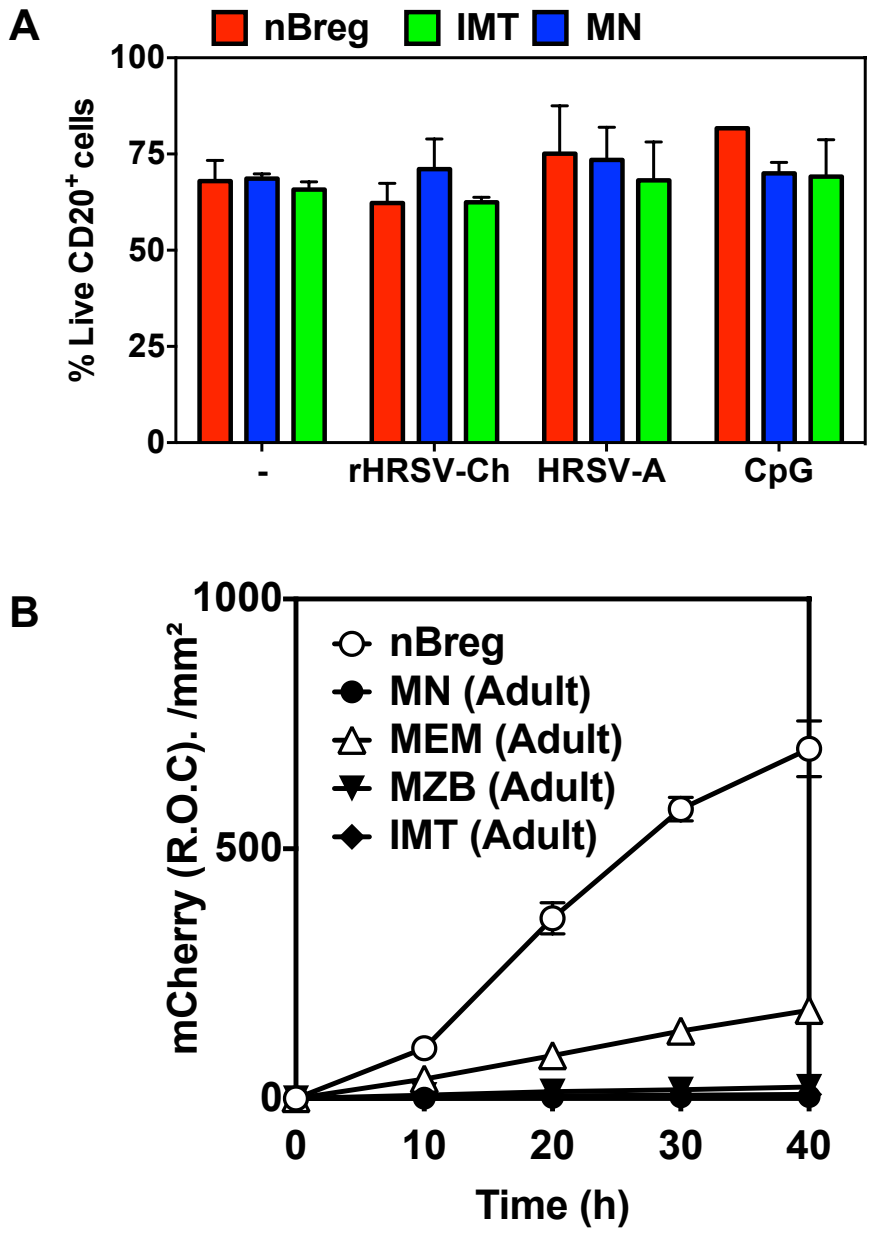


Fig. S4 (relate to Fig. 3)

(A) 10^5 B cell subsets were FACS sorted as nBreg, MN or IMT and stimulated by rHRSV-Ch, HRSV-A or CpG for 48 h. Live/dead cells were analyzed by FACS following DAPI staining. Live cells were negative for DAPI, and results are mean +/-SD of 3 experiments. (B) 10^5 nBregs and adult B cells were sorted as indicated in FigS2B and exposed to rHRSV-Ch (MOI=2.5). Viral infection was assessed mCherry expression using fluorescent live microscopy.

Figure S5

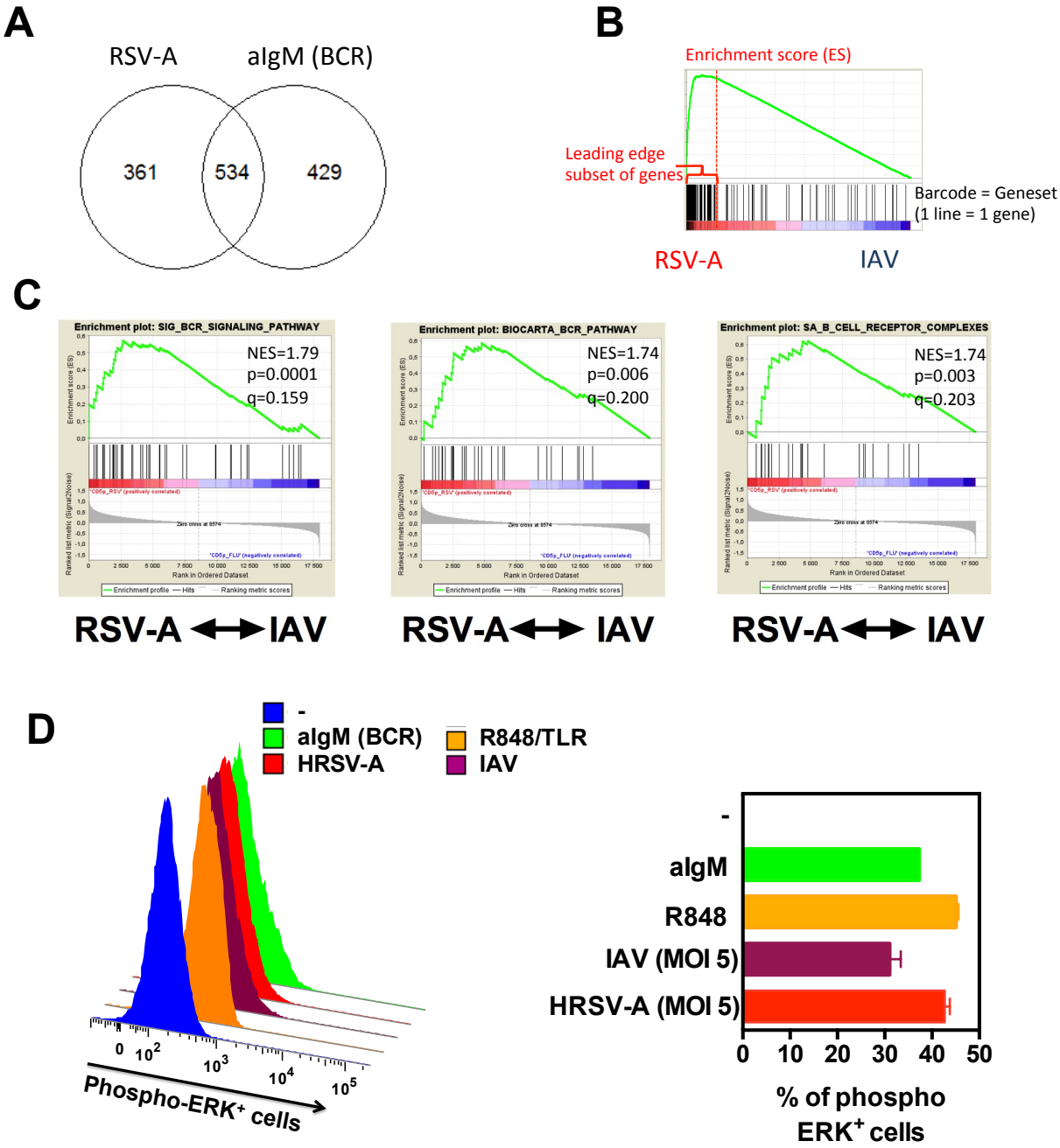


Fig. S5 (related to Fig. 4): Pathway analysis of RSV stimulated nBregs. (A-C) Cord blood nBregs were FACS-sorted as CD19+CD5+CD10- B cells, and they were either left unstimulated (-) or stimulated for 6 h with HRSV-A, IAV or anti-IgM. Gene expression profiles were compared by microarray analysis for 3 independent donors. (A) Venn diagram for the number of common and specific genes activated in nBregs for aIgM (BCR) and RSV. (B-C) GSEA analysis. (B) Description of GSEA analysis plot. (C) GSEA comparison of IAV and RSV activated nBregs for BCR receptor, signaling and molecular pathways. (D) nBregs were activated as indicated for 30 min. and ERK phosphorylation was assessed by FACS. FACS plots and mean of triplicates+/-SD are shown.

Figure S6

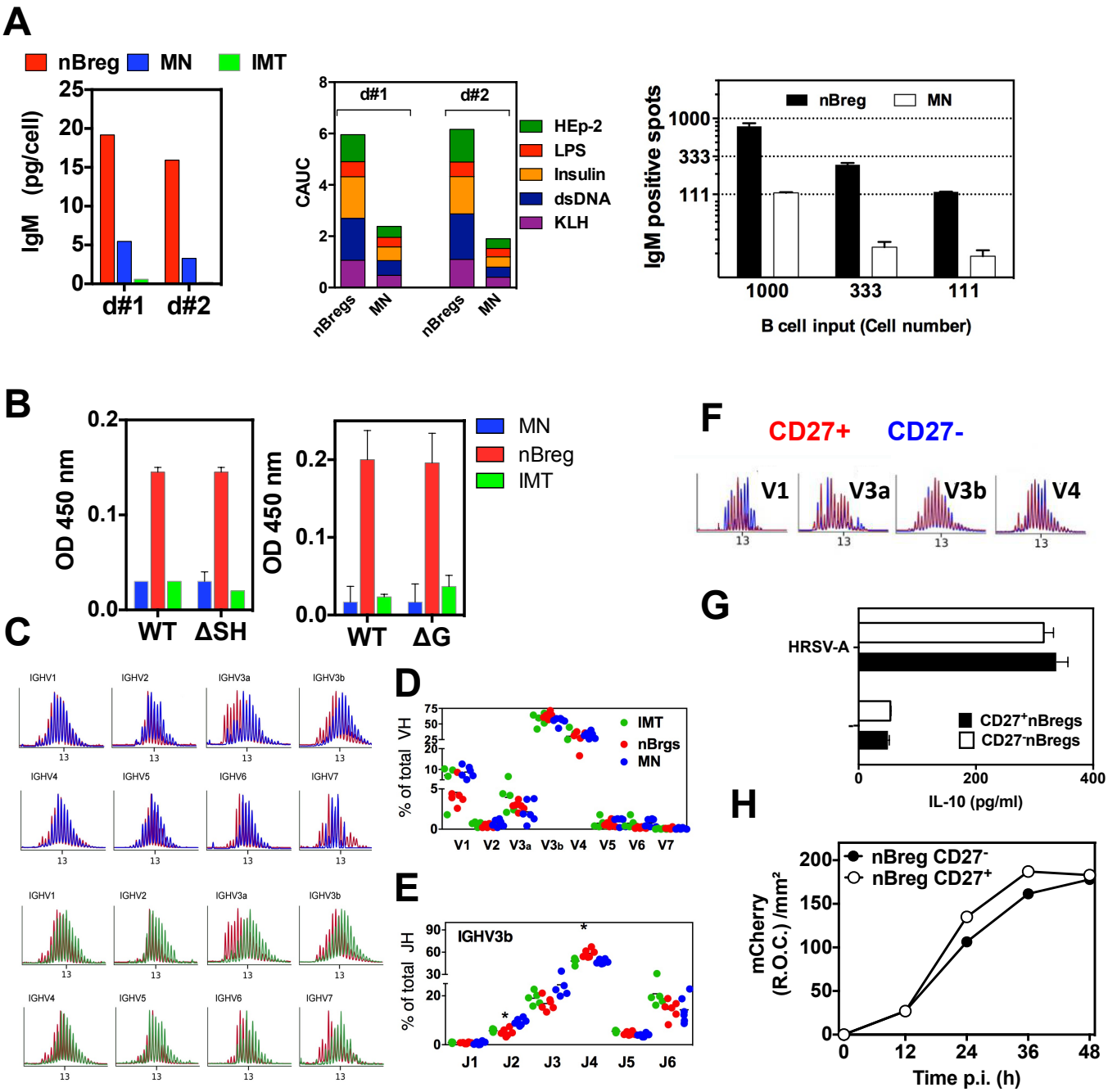


Fig. S6 (related to Fig. 5): B cell reactivity and repertoire analysis

(A) Indicated B cell subset (3X10^6/ml) was stimulated for 6 days with CpG, and concentration of IgM was determined. IgM produced by nBregs and MN were tested at 4-0.4 and 0.04 µg/ml for polyreactivity against the indicated Ag by ELISA and results are plotted as CAUC. Alternatively, B subsets, was analyzed using an enzymatic ELISPOT assay to evaluate the frequency IgM secreting cells after 48 h. (B) IgM (100 ng/ml) produced by nBregs, IMT and MN neonatal B cell were tested by ELISA for recognition of WT HRSV-A vs. (B) ΔSH and (C) ΔG mutants. (C) CDR3 length profiles (in AA) of one neonatal sample nBreg subset (red) for the various IGVH are compared by overlay with MN (blue) and IMT (green) B cell subsets. For the three neonatal B cell subsets, (D) the IgM V usage and (E) the J usage repertoire was analyzed for IgM V3b (IGHV3b) (*P<0.05). Each dot represents one donor (n=5). (F-H) nBregs were sorted as CD27-positive and negative cell fractions and subjected to repertoire analysis as in Fig. 5 and to RSV infection and IL-10 response. CDR3 length spectra are shown for major IGHV gene family (V1, V3a, V3b and V4). (G) nBregs subsets were exposed to HRSV-A and IL-10 was detected by ELISA at 48 h. (H) nBregs subsets were exposed to rHRSV-Ch and infection was monitored by following mCherry expression by fluorescent live microscopy for 48h.

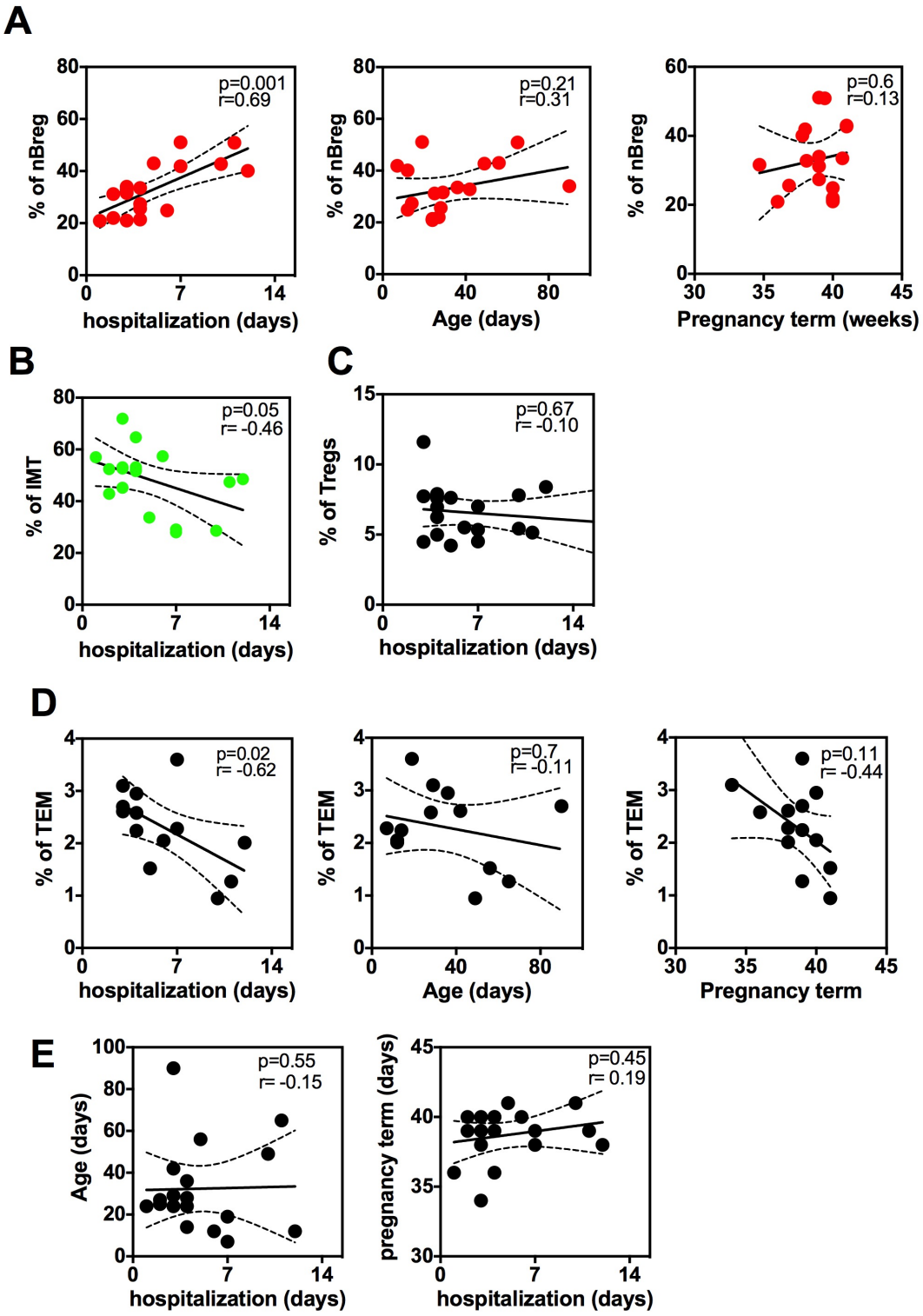


Figure S7 (related to Fig. 7) : RSV-positive patient cohort analysis. Correlation analysis of blood cell parameters of patients suffering of acute bronchiolitis with duration of ICU hospitalization, age of patients and pregnancy term. Immunological parameters correspond to those presented in Figure 7.

Supplementary Experimental procedures

HRSV mutants

To generate the rHRSV-ΔG-Cherry virus, the first ATG of the G gene was substituted by ACA by site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene). Mutagenesis was performed using the pJET2.1 vector in which the HRSV G gene was cloned at XhoI-StuI sites, with the following primers: forward primer: CGTTGGGGCAAATGCAAACACATCCAAAAA CAAGGACCAACGC; reverse primer: GCGTTGGTCCTTGTTTTTGGATGTGTTTGCATTTGCC CCAACG (sequence changes were boxed). The modified sequence was then sub-cloned in the pACNR-rHRSV-Cherry vector (Genbank accession N° KF713492.1) to engineer the pACNR-rHRSV-ΔG–Cherry vector. Sequence analysis was carried out to control the integrity of this vector. The recombinant rHRSV-ΔG-Cherry virus was recovered by co-transfecting the pACNR-rHRSV-ΔG–Cherry vector together with plasmids expressing the RSV N, P, M2-1 and L proteins in BSRT7/5 cells (Buchholz et al., 1999) as previously described (Rameix-Welti, 2014). Rescued viruses were passaged and amplified on Vero cells grown at 37°C with 5% CO_2 in EMEM (Gibco) supplemented with 2% foetal calf serum (FCS). To control that rHRSV-ΔG–Cherry no longer express G, immunofluorescence was carried out after virus titration on Vero cells (described below). Briefly, 6 days postinfection cells were wash with PBS 1X, fixed with PBS- PFA 4% and labelled with either a polyclonal anti-N serum (Castagne et al., 2004) or anti-G monoclonal antibodies (AbD Serotec). Fluorescent plaques were observed using an inverted fluorescence microscope. For rHRSV-ΔSH, SH gene together with corresponding Gene Start and Gene End signals was deleted from the full-length cDNA clone of HRSV subgroup A previously described (Rameix-Welti et al., 2014) using standard cloning procedures. Resulting sequence is available in the Genbank nucleotide database with accession code KU707921. rHRSV-ΔSH was rescued and amplified as previously described. Viral genome sequence was verified at passage 3. Viruses were titrated on Vero cells at 37°C using a plaque assay procedure derived from the one previously described (Rameix-Welti et al., 2014).

RSV detection in nasal washes.

For RSV expression, B cell subsets were directly sorted from the nasal washes in a Lysis Solution (Lysis Enhancer and Resuspension Buffer at a ratio 1:10) (CellsDirect™ One-Step qRT-PCR Kit, Invitrogen). Sequence-specific pre-amplification was performed using TaqMan PreAmp Master Mix (Invitrogen). Unincorporated primers were inactivated by Exonuclease I treatment (New England Biolabs). RSV nucleoprotein gene N analysed by qPCR with 2x Sso Fast EvaGreen Supermix With Low ROX (Bio-Rad Laboratories) using primers in 48:48 Dynamic Arrays on a Biomark System (Fluidigm). Quantitative data for the viral N was normalized to house keeping genes mRNA content (β-actin and GAPDH). *RSV N* forward primer AGATCAACTTCTGTCATCCAGCAA and reverse primer TTCTGCACATCATAATTAGGAG TATCAAT were used.

B cell repertoire analysis.
We characterized the IgM repertoire at the molecular level in various B-cell subsets from cord blood. IGHV gene usage and CDR3 analysis were performed using the Immunoscope method coupled with real-time PCR to
provide quantitative information on the IGHV and IGHJ gene usage. Briefly, PCR reactions were performed by combining a primer and a specific fluorophore-labeled probe for the constant region CHμ with one of eight primers co
vering the different IGHV1-7 genes. V3 was divided in two subgroups: V3a (V3-15,49,72,73) and V3b (V3-d,07,09,11,13,20,21,23,30,30.3,33,43, 48,53,64,66,74). Reactions were performed using Taqman 7300. PCR products were subjected
to run-off reactions with a nested fluorescent primer specific for the constant region gene. The fluorescent products were separated and analyzed on an ABI-PRISM 3730 DNA analyzer to determine CDR3 lengths. The IGHV3a/C amplification prod
ucts were cloned, sequenced, and analyzed according to the procedure described previously (Lim et al., 2008). A more detailed analysis of V-Cμ H-chain transcripts was performed to examine the usage of IGHD families and the IGHJ gene as well as the

of the IGHV-D and IGHJ-IGHD junction regions. IMGT/junction analysis was used to accurately identify the different regions of the junctions: 3'V-region, D-region(s), and 5'J-region. IGVH CDR3 length was analyzed in nBregs,
and NM B cells. Each profile represents the CDR3 length distribution for a given IGVH family. One-way ANOVA was used for group comparisons; P values <0.05 were considered statistically significant. List of primers is detailed below.

Polyreactivity ELISA

IgM (3-4ug/ml) from nBregs or MN were tested for polyreactivity using high-binding 96-well ELISA plates (Costar) coated with 10 μg/ml of LPS from E. coli (Sigma, L2637), Keyhole Limpet Hemocyanin (KLH) (Sigma, H8283), ssDNA from dsDNA (heated at 95°C for 30 min), 5 μg/ml Human insulin (Sigma, I9278), HEp-2 whole cell lysates (Prigent et al., 2016) and purified HIV-1 (YU-2) gp140 trimers gp140 (Mouquet et al, 2011). (2.5μg/ml). ELISA
done as previously described. HRSV-F protein (4μg/ml) was described (McLellan et al., 2011 ; McLellan et al., 2013)

Buchholz, et al. J Virol *73*, 251-259.
Castagne, N.,et al. The Journal of general virology *85*, 1643-1653.
Lim, A. et al. Int Immunol *20*, 105-116.
McLellan, et al. Science 340 (6136): 1113–17. doi:10.1126/science.1234914.
McLellan et al. Journal of Virology 85 (15): 7788–96. doi:10.1128/JVI.00555-11.
Rameix-Welti, M.A. et al. Nature communications *5*, 5104.
Prigent, J. et al., European journal of immunology *46*, 2340-2351.
Mouquet, H. et al. PloS one *6*, e24078.

Supplentary Experimental procedures are related to reagents and info of the experimental procedure

List of V, J and Cmu specific primers for repertoire analysis.

	Primer Sequence	Specificity	Location
IGHV subgroup			
V1	AGTGAAGGTCTCCTGCAAGGC	V1-02,08,18,58,69,e	FR1
	AGTGAAGGTTTCCTGCAAGGC	V1-03,45,46	FR1
	AGTGAARRTCTCCTGCAAGGT	V1-f,24	FR1
V2	AACCCACASAGACCCTCAC	V2-05,70,26	FR1
V3a	GCAGATTCACCATCTCAAGAGATG	V3-15,49,72	FR3
	GCAGGTTCACCATCTCCAGAGATG	V3-73	FR3
V3b	GCCGATTCACCATCTCCAGAGA	V3-07,09,13,20,21,30,30.3 33,43,48,53,74	FR3
	GCAGATTCACCATCTCCAGAGA	V3-d,64,66	FR3
	GCCGATTCACCATCTCCAGGGA	V3-11	FR3
	GCAGGTTCACCATCTCCAGAGA	V3-23	FR3
V4	CTACAACCCGTCCCTCAAGAGT	V4-04,28,30-2,30-4,31,34,b	FR3
	CTACAACCCCTCCCTCAAGAGT	V4-59,61	FR3
V5	GTGAAAAAGCCCGGGGAG	V5-51,a	FR1
V6	TCCGGGGACAGTGTCTCT	V6-01	FR1
V7	GGTGCAATCTGGGTCTGAGT*T*	V7-04.1	FR1
IGHJ subgroup			
J1	CCCTGGCCCCAGTGCT*G	J1	
J2	CCACGGCCCCAGAGATC*G	J2	
J3	CCCTTGGCCCCAGAYATCAAAA*G	J3a,b	
J4	GGTTCCTTGGCCCCAGTA*G	J4a	
	GGTTCCCTGGCCCCAGTA*G	J4b	
	GGTCCCTTGGCCCCAGTA*G	J4d	
J5	TGGCCCCAGGRGTCGAA*C	J5a,b	
J6	CCTTGCCCCCAGACGTCCA*T	J6a	
	CCTTGGCCCCAGACGTCCA*T	J6b	
	CCTTTGCCCCAGACGTCCA*T	J6c	
IGH mu chain			
	CAGCCAACGGCCACGC	IGHM.01,02,03	CH1
	6Fam-GGAGACGAGGGGGAAAAGG		CH1
	6Fam-CCGTCGGATACGAGC-MGB		CH1

Supplentary Experimental procedures are related to reagents and info of the experimental procedure

Demographic and diagnostics data of children

	RSV group	positive	negative
Diaganostics	RSV+	36	10
	Other viruses	0	0
	Bacteria	6	0
Sampling	Blood	29	10
	NPA	13	0
Age (months)	median	1.18	1.15
	IQR	0.63-1.73	0.16-2.03
Gestational age at birth (weeks)	median	39	38
	IQR	38-40	37-39.29
Weight (kg)	median	4.0	3.9
	IQR	3.34-5.03	3.46-4.65

Supplentary Experimental procedures are related to reagents and info of the experimental procedure

List of antibodies used for mass cytometry

141 Pr	CD196	REA190	Miltenyi
142 Nd	CD19	LT19	Miltenyi
143 Nd	CD11c	mj4-27g12	Miltenyi
143 Nd	CD123	AC145	Miltenyi
143 Nd	CD56	REA196	Miltenyi
143 Nd	IgA	IS11-8E10	Miltenyi
144 Nd	CD38	IB6	Miltenyi
145 Nd	CD4	RPT-T4	Biolegend
146 Nd	CD161	191B8	Miltenyi
146 Nd	CD20	LT20	Miltenyi
146 Nd	CD8	BW135/80	Miltenyi
147 Sm	CD11b	M1/70.15.11.5	Miltenyi
147 Sm	CD20	LT20	Miltenyi
148 Nd	CD86	FM95	Miltenyi
149 Sm	CD15	VIMC6	Miltenyi
150 Nd	CD10	97C5	Miltenyi
150 Nd	CD304	AD5-17F6	Miltenyi
151 Eu	CD70	REA292	Miltenyi
151 Eu	CD5	AC145	Miltenyi
152 Eu	CD23	M-L23,4	Miltenyi
152 Eu	CD4	VIT4	Miltenyi
153 Eu	CD62L	145/15	Miltenyi
154 Sm	CD27	M-T271	Miltenyi
158 Gd	CD40	HB14	Miltenyi
159 Tb	CD197	REA108	Miltenyi
159 Tb	CD9	SN4	Miltenyi
160 Gd	CD14	TUK4	Miltenyi
161 Dy	CD33	AC104.3E3	Miltenyi
162 Dy	CD11c	mj4-27g12	Miltenyi
164 Dy	CD15	VIMC6	Miltenyi
164 Dy	CD161	191B8	Miltenyi
164 Dy	CD56	REA196	Miltenyi
165 Ho	CD43	DF-T1	Miltenyi
166 Er	CD24	32D12	Miltenyi
167 Er	CD5	UCHT2	Miltenyi
168 Er	CD138	44F9	Miltenyi
169 Tm	CD45RA	T6D11	Miltenyi
169 Tm	IgG	IS11-3B2.2.3	Miltenyi
170 Er	CD3	UCHT1	Biolegend
172 Yb	anti-IgM	PJ2-22H3	Miltenyi
173 Yb	CD21	HB5	Miltenyi
173 Yb	CD40	HB14	Miltenyi
174 Yb	anti-IgD	IgD26	Miltenyi
174 Yb	HLA-DR	AC122	Miltenyi
175 Lu	CD10	97C5	Miltenyi
175 Lu	HLA-DR	AC122	Miltenyi
176 Yb	CD1c	AD5-8E7	Miltenyi

Supplentary Experimental procedures are related to reagents and info of the experimental procedure

Erk1/2 (T202/Y204)	MILAN8R	eBioscience
CXCR3	G025H7	Biolegend
CX3CR1	2A9-1	Miltenyi Biotec
CCR6	11A9	BD
CD10	ebioCB-CALLA	eBioscience
CD123	6H6	eBioscience
CD127	ebioRDR5	eBioscience
CD14	HCD14	Biolegend
CD19	J3-129	eBioscience
CD20	2H7	eBioscience
CD25	BC96	Biolegend
CD27	L128	BD
CD3	OKT3	eBioscience
CD304	AD5-17F6	Miltenyi Biotec
CD4	RPA-T4	eBioscience
CD45RA	HI100	Biolegend
CD45RO	UCHL1	eBioscience
CD5	L17F12	eBioscience
CD79a (Tyr182)	D1B9	Cell signaling technology
IFN-g	45.B3	Biolegend
IL-4	MP4-25D2	BD
IL-13	JES10-5A2	Biolegend
IL-17A	BL168	Biolegend
IL-22	22URTI	eBioscience
TNF-a	MAb11	eBioscience
IL-10	JES3-9D7	eBioscience
IFN-α	LT27:295	Miltenyi Biotec
CD80	2D10	Biolegend
HLA-DR	L243	Biolegend