SUPPLEMENTARY APPENDIX

# Red blood cell alloimmunization is influenced by the delay between Toll-like receptor agonist injection and transfusion

Rahma Elayeb,[1,2,4] Marie Tamagne,[1,2,4] Philippe Bierling,[1,2,3,4] France Noizat-Pirenne,[1,2,3,4] and Benoît Vingert[1,2,4]

[1]Établissement Français du Sang, Créteil; [2]Institut Mondor de Recherche Biomédicale, Inserm U955, Equipe 2, Créteil; [3]Université Paris Est, Faculté de Médecine, Créteil; and [4]Laboratory of Excellence GR-Ex, Paris, France

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Correspondence: benoit.vingert@efs.sante.fr

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# Supplemental figures:

| | Whole blood % | Number of transfused residual leukocytes |
|---|---|---|
| $CD3^+$ T cells | [a] 77.5 ± 7.3 | 48 ± 90 |
| CD8 T cells $CD3^+CD8^+$ | 12.8 ± 2.5 | 5 ± 10 |
| CD4 T cells $CD3^+CD4^+$ | 85.3 ± 2.9 | 41 ± 80 |
| Treg $CD3^+$ $CD25^+$ $Foxp3^+$ | 2.1 ± 0.6 | 1 ± 1 |
| NKT cells $CD3^+$ $NK1.1^+$ | 0.3 ± 0.2 | 2 ± 2 |
| NK cells $NK1.1^+$ | 0.2 ± 0.1 | 1 ± 1 |
| DC $CD11c^+$ cells | 7.4 ± 7.8 | 11 ± 11 |
| DC $CD11c^+$ $CD8^+$ $DEC205^+$ | 4.6 ± 5.1 | 1 ± 1 |
| DC $CD11c^+$ $CD8^+$ $DEC205^-$ | 2.8 ± 2.0 | 2 ± 2 |
| DC $CD11c^+$ $CD8^-$ | 43 ± 19.5 | 5 ± 5 |
| pDC $CD11c^+$ | 49.7 ± 18.5 | 4 ± 5 |
| Macrophages $F4/80^+$ | 0.3 ± 0.1 | 1 ± 1 |
| Granulocytes $CD11b^+$ $GR1^+$ | 0.6 ± 0.4 | 1 ± 1 |
| Monocytes $CD11b^+$ $CD115^+$ | 0.9 ± 0.4 | 1 ± 1 |

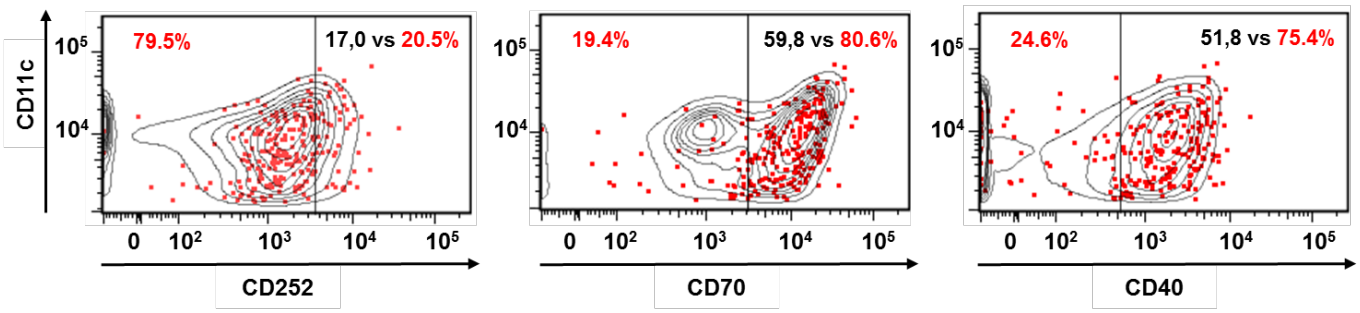
[a] Mean ± standard deviation

**Supplementary table 1. Residual leukocytes after blood leukoreduction**

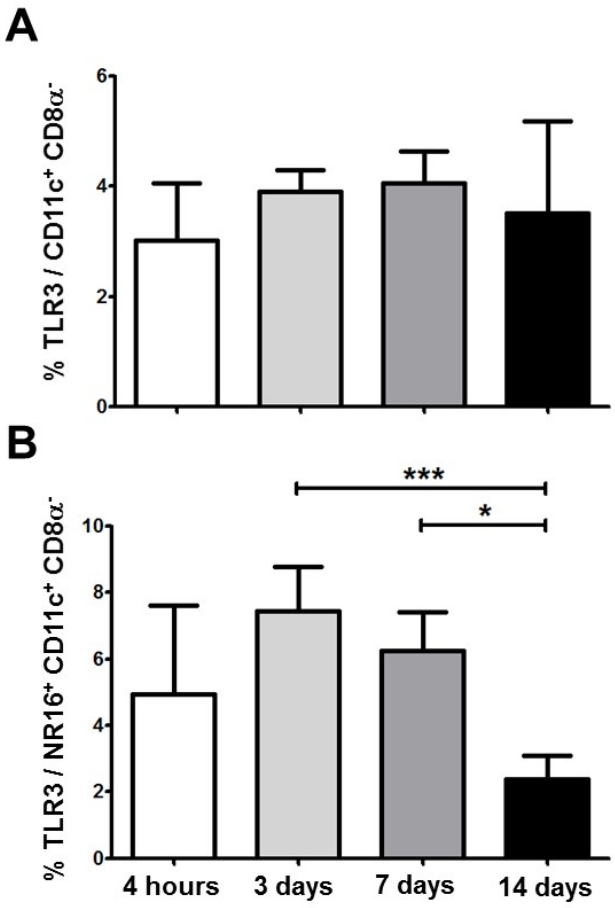
| | Before leukoreduction pg/ml | After leukoreduction pg/ml |
|---|---|---|
| IL13 | [a] 283.8 ± 506.2 | 372.3 ± 658.3 |
| IL1α | 381.6 ± 320.1 | 334.5 ± 363.4 |
| IL22 | 24.1 ± 46.6 | 13.4 ± 28.9 |
| IL2 | 272.6 ± 278.5 | 244.4 ± 316.1 |
| IL5 | 242.8 ± 394.2 | 202.6 ± 380.6 |
| IL21 | 282.5 ± 671.9 | 437.9 ± 1064.5 |
| IL6 | 214.3 ± 483.0 | 209.6 ± 457.5 |
| IL10 | 46.4 ± 122.0 | 215.1 ± 783.6 |
| IL27 | 1139.9 ± 1427.6 | 637.4 ± 599.2 |
| IL17 | 104.5 ± 237.4 | 137.0 ± 299.1 |
| IL4 | 189.0 ± 365.7 | 229.6 ± 466.4 |
| IFNγ | 851.2 ± 1180.6 | 593.3 ± 909.4 |
| TNFα | 281.6 ± 491.3 | 124.8 ± 221.9 |
| MCP3 | 67.2 ± 115.3 | 79.1 ± 301.3 |
| MCP1 | 197.1 ± 614.0 | 273.0 ± 658.5 |
| MIP1α | 12.7 ± 50.4 | 44.5 ± 155.9 |
| MIP1β | 352.9 ± 421.1 | 365.5 ± 558.0 |
| RANTES | 280.7 ± 532.0 | 321.4 ± 844.4 |
| GM-CSF | 176.6 ± 259.4 | 188.0 ± 165.8 |

[a] Mean ± standard deviation

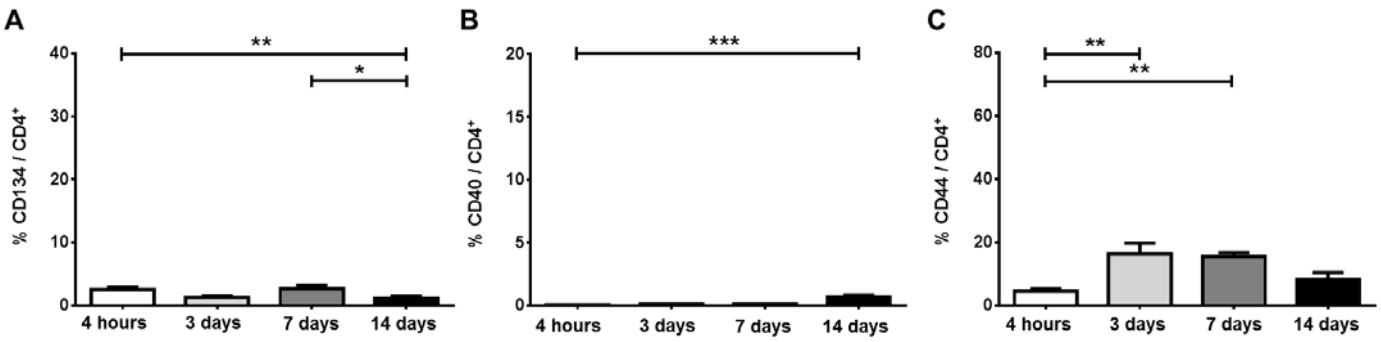
**Supplementary table 2. Cytokine concentration after blood leukoreduction**




**Supplementary Figure S1.** A representative example of CD252, CD70 and CD40 expression on splenic CD11c$^+$ DCs. For the 7 days group, the expression of CD252, CD70 and CD40 was assessed 48 hours after transfusion on NR16-presenting CD11c$^+$ DCs (red dot plot) and total CD11c$^+$ DCs (black contour plot).




**Supplementary Figure S2. The effect of the timing of Poly(I:C) delivery on TLR3 expression by splenic CD11c$^+$ CD8$\alpha^-$ DCs after transfusion.** At 4 hours, 3 days, 7 days or 14 days after Poly(I:C) intraperitoneal injection, mice were transfused with HEL RBCs and spleens were harvested 48 hours later. The expression of TLR3 was measured in total (A) and NR16-presenting (B) CD11c$^+$ CD8$\alpha^-$ DCs. Comparisons were performed using the Kruskal Wallis test and Dunn's post test. * $P$<0.05; *** $P$<0.005.




**Supplementary Figure S3. The effect of the timing of Poly(I:C) injection on total CD4$^+$ T cells.** Mice received transfusions of HEL RBCs 4 hours, 3 days, 7 days or 14 days after Poly(I:C) intraperitoneal injection. Their spleens were harvested 48 hours later. The expression of CD134 (A), CD40 (B) and CD44 (C) was measured *ex vivo* on total splenic CD4$^+$ T cells, for each of the injection time points before transfusion. Comparisons were performed with the Kruskal Wallis test and Dunn's post test. * $P$<0.05; ** $P$<0.01; *** $P$<0.005. Representative data from two independent experiments on five mice each are shown (mean ± SD).

# Supplemental Methods

### Blood collection and leukoreduction

Blood from HOD mice was collected in acid-citrate dextrose solution B (BD Biosciences San Diego, CA) and washed with PBS. A RBC concentrate was obtained using a leukoreduction filter designed for pediatric patients (Pall Medical, Port Washington, NY). Blood from B10BR mice was sampled using hematocrit tubes with heparin sodium to collect RBCs (Drummond Scientific Company, Broomall, PA).

After leukoreduction, residual leukocyte subtypes and cytokines were analyzed and compared with those present in whole blood (Supplementary Tables 1 & 2). The phenotype of the leukocyte subtypes was characterized by flow cytometry with antibodies specific for T lymphocytes (CD3, CD8, CD4, CD25, Foxp3), NK cells (NK1.1), dendritic cells (CD11c, CD8, DEC205), macrophages (F4/80), granulocytes (CD11b, GR1) and monocytes (CD11b, CD15). Cytokine concentrations were determined in a multiplex assay (eBioscience).

### Purification of splenocytes

Spleens were harvested two days after transfusion. Purified splenocytes were obtained after the treatment of the spleen with D-type Collagenase (400 MandL/ml, Roche Diagnostics, Foster City, CA) and DNAse (50µg/ml, Roche Diagnostics) for 30 minutes at 37°C. Fetal bovine serum (FBS) was then added, before dilaceration of the spleen. Cell strainers (40 µm) were used for cell filtration in complete medium (MC): RPMI 1640-glutamate, penicillin-streptomycin (50 U/mL), Hepes 10 mM, sodium pyruvate (Life Technologies) and 5% FBS. Cells were counted on Malassez slides (C-Chip,

Digital Biology, Japan) and cell viability was assessed by trypan blue staining (0.02%, Sigma-Aldritch).

**DC enrichment**

Splenic DCs were enriched by negative selection using a commercial kit (BD Biosciences) containing a cocktail of antigen-specific antibodies (CD2, CD3ε, CD45R/B220, CD49b, CD147, Ly-6G, Ly-6C/Gr-1 and TER-119).

**NR16 peptide**

HEL immunodominant peptide, NR16 ($HEL_{46-61}$: NTDGSTDYGILQINSR), was synthesized by PolyPeptide Laboratories (Strasbourg, France), at a purity of 97.5%.

**Flow cytometry and data analysis**

LSR II and Canto II (BD Biosciences) flow cytometers were used for data acquisition of all the different staining protocols. The data were then analyzed with FlowJo software (V10, Tree Star, Ashland, OR).

**Statistical analysis**

Statistical analysis was conducted with Prism 5.03 (GraphPad Software, La Jolla, CA) and comparisons between groups were performed with the Mann Whitney or Kruskal Wallis tests with a Dunn's post test. $P<0.05$ was considered significant.