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

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ABSTRACT

Murine models of red blood cell transfusion show that inflammation associated with viruses or methylated DNA promotes red blood cell alloimmunization. In vaccination studies, the intensity of antigen-specific responses depends on the delay between antigen and adjuvant administration, with a short delay limiting immune responses. In mouse models of alloimmunization, the delay between the injection of Toll-like receptor agonists and transfusion is usually short. In this study, we hypothesized that the timing of Toll-like receptor 3 agonist administration affects red blood cell alloimmunization. Poly(I:C), a Toll-like receptor 3 agonist, was administered to B10BR mice at various time points before the transfusion of HEL-expressing red blood cells. For each time point, we measured the activation of splenic HEL-presenting dendritic cells, HEL-specific CD4$^+$ T cells and anti-HEL antibodies in serum. The phenotype of activated immune cells depended on the delay between transfusion and Toll-like receptor-dependent inflammation. The production of anti-HEL antibodies was highest when transfusion occurred 7 days after agonist injection. The proportion of HEL-presenting CD8α^+ dendritic cells producing interleukin-12 was highest in mice injected with poly(I:C) 3 days before transfusion. Although the number of early-induced HEL-specific CD4$^+$ T cells was similar between groups, a high proportion of these cells expressed CD134, CD40 and CD44 in mice injected with poly(I:C) 7 days before transfusion. This study clearly shows that the delay between transfusion and Toll-like receptor-induced inflammation influences the immune response to transfused red blood cells.

Introduction

Sickle cell disease (SCD) is a devastating condition which still relies on red blood cell (RBC) transfusion. The main immunological complication of transfusion in SCD patients is alloimmunization against RBC antigens, leading to life-threatening post-transfusion hemolysis. Alloimmunization is more frequent in SCD patients than in other patients and represents a major concern in transfusion medicine.[1] The high incidence of alloimmunization in this population is partly explained by the large disparity of blood groups between European donors and recipients of African descent. However, some SCD patients never become immunized, and can be qualified as "low responders".

The immune mechanisms underlying red blood cell alloimmunization are poorly understood.[2] In humans, several genotypes of class II major histocompatibility complex (MHC II) could be implicated in alloimmunization against specific antigens but controversy remains regarding this.[3,4] Little is known about the role of CD4$^+$ T cells in alloimmunization,[5] except for Treg cells.[6-8] Recently, we showed that the phenotype of CD4$^+$ T cells from SCD patients differs according to whether

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the patients have been alloimmunized or not.[9]

Most knowledge about the mechanisms of alloimmunization has been provided by mouse models. However, it has been shown that SCD does not increase the rate of alloimmunization in mice.[10] Despite important differences in the immune system between mice and humans, mouse models enable the investigation of different parameters separately, and provide hypotheses that can be tested in humans. Murine models of post-transfusion alloimmunization have been developed, such as those expressing transgenic human antigens, e.g. glycophorin A, or non-human antigens, e.g. hen egg lysozyme (HEL), at the erythrocyte membrane.[11]

In mouse models, Toll-like receptor (TLR) stimulation promotes alloimmunization. Prior to transfusion, the injection of CpG, a TLR9 agonist, facilitates the production of alloantibodies.[12] Moreover, the injection of poly(I:C), a TLR3 agonist, also promotes alloimmunization in mice that are transfused.[13,14] TLR3 and TLR9 are implicated in immunity to dsRNA viruses and bacterial infections, respectively.[15] Poly(I:C) stimulates splenic CD11c$^+$ dendritic cells (DC) to consume transfused RBC, and modifies the expression of co-stimulatory molecules on these DC.[16] However, no study has yet tried to identify the RBC antigen-presenting DC and to characterize their phenotype. In the absence of TLR agonists, splenic macrophages consume RBC, preventing the production of alloantibodies.[14] Indeed, transfusion in the absence of inflammation can lead to tolerance to RBC antigens.[17] However, in murine models of vaccination, the administration of TLR agonist enables the maturation of DC, leading to the establishment of immune responses rather than tolerance.[18] Two main subsets of CD11c$^+$ DC, CD8α^+ and CD8α^- DC, have been described in the spleen[19] and are distinct in terms of function: the CD8α^+ population produces interleukin (IL)12.[20] IL12 directly affects CD4$^+$ T-cell responses because it induces Th1 polarization, leading to the production of IL2 and interferon (IFN)γ.[19,20]

Poly(I:C) injection directly modulates the function of CD4$^+$ T cells and stimulates cytokine production and lymphoproliferation.[21,22] In a mouse model of transfusion, poly(I:C) was confirmed to promote the lymphoproliferation of HEL-specific CD4$^+$ T cells following transfusion.[14] Using this TLR3 agonist, Longhi *et al.*[22] found that the antigen-specific immune response was weak if inflammation occurred during antigen capture and presentation. Indeed, Th1-type immune responses were weak when poly(I:C) injection preceded antigen administration by a period of 6 h.[22] It is, therefore, likely that the delay between antigen and adjuvant administration affects antigen-specific responses. In currently used mouse models of alloimmunization, poly(I:C) is typically injected 4 h before transfusion.[13,14,16,17,23]

In human transfusions, the role of viral and bacterial infections in the induction of alloimmunization has not been documented. However, in SCD patients, a recent study showed that an inflammatory state at the time of transfusion, independently of direct TLR stimulation, can influence RBC alloimmunization.[24] The underlying inflammatory state of the patient and TLR signaling may, therefore, be central to the process of RBC alloimmunization.

Here, we hypothesized that RBC alloimmunization is influenced by the delay between transfusion and the administration of a TLR3 agonist. To test this, B10BR mice were transfused at various time points after the administration of poly(I:C) with HEL-expressing RBC obtained from HOD mice. For each delay, the production of anti-HEL antibodies was measured in the recipient mice and the function of splenic HEL-presenting DC and HEL-specific CD4$^+$ T cells was analyzed. We report here that the time between transfusion and TLR3 stimulation influences the immune response to transfused RBC.

Methods

Mice

B10BR mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and have class II MHC IAk. HOD mice (transgenic RBC-specific expression of HEL, ovalbumin, and Duffy b anchoring HEL to the RBC membrane) are issued from the FVB background and have class II MHC IAb. Mice were housed and bred at the *Institut Mondor de Recherche Biomédicale* (IMRB) conventional animal facility, in pathogen-free conditions. Transfusion-recipient mice were used at 7 to 9 weeks of age, in homogeneous mixed-sex groups. All procedures were approved by the local ethics committee.

Transfusion and treatment of mice

Mice received a 100 μL intraperitoneal injection of phosphate-buffered saline or a TLR3 agonist, poly(I:C) (100 μg, Amersham Piscataway, NJ, USA), at various time points (4 h, 3 days, 7 days or 14 days before transfusion). The mice then received a 100 μL transfusion of fresh HOD RBC concentrate into the lateral tail vein. All mice were sacrificed 2 days or 28 days after transfusion, and the spleen was harvested. Blood was collected from the retro-orbital vein before death. Serum was isolated by centrifugation and frozen at -20°C.

Flow cross-matching and enzme-linked immunosorbent assay for the detection of anti-HEL responses

The presence of anti-HEL antibodies was evaluated by flow cross-matching. Serum from B10BR mice, harvested 28 days after transfusion, was diluted 1:10 and incubated with RBC expressing HEL (HOD) antigen or control RBC (B10BR). Anti-HEL antibodies were detected by flow cytometry, with allophycocyanin (APC)-labeled antibodies against total Ig (BD Biosciences, Franklin Lakes, NJ, USA). Serum from HEL immunized mice with aluminum salts incubated with HEL-expressing RBC (HEL$^+$ RBC) was used as a positive control. Adjusted mean fluorescence intensity (MFI) was calculated as follows: adjusted MFI = (MFI of the serum incubated with HEL$^+$ RBC) – (MFI after incubation with HEL$^-$ RBC). Enzyme-linked immunosorbent assay (ELISA) was performed in a HEL-coated plate, to determine the total amount of IgG antibody against HEL in the serum. The alkaline-phosphatase-conjugated secondary antibody used for detection was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Antibody binding was detected by reaction with the pNPP substrate (Sigma-Aldrich, St-Louis, MO, USA). Anti-HEL antibody (Raybiotech, GA, USA), with known amounts of the IgG isotype, was used as a standard.

Dendritic cell immunostaining and intracellular cytokine staining

After DC enrichment, at least 2x10^4 cells were cultured overnight in complete medium supplemented with brefeldin A (0.5 μg/well) to determine the production of cytokines. Before staining, the Fc receptors were blocked with CD16/CD32 antibodies (0.5 μg, eBioscience, San Diego, CA, USA). Cultured DC were incubated (15 min, 4°C) with antibodies against the following

membrane proteins, to determine their phenotype: CD8α-PE-CF594 (BD Biosciences), CD40-PE-Cy7, CD252-AF647 (Biolegend, San Diego, CA, USA), CD11c-APC-eF780, and CD70-PerCP-eF710 (eBioscience). DC were fixed and permeabilized with a commercial kit (eBioscience) for intramembranous staining with CD283-PE (Biolegend, San Diego, CA, USA), IL12-FITC and IFNγ-AF700 (BD Biosciences). The HEL protein contains an immunodominant peptide, NR16 (HEL_{46-61}: NTDGSTDYGILQIN-SR). NR16-presenting DC were studied using an AW3.18 antibody. This antibody recognizes the MHC II I-A^k-NR16 complex.[25] The AW3.18 antibody was detected with a biotinylated anti-IgG1 antibody (Jackson ImmunoResearch) and streptavidin-BV421 (Biolegend). The proportions of $CD8\alpha^+$ and $CD8\alpha^-$ DC among total $CD11c^+$ cells were calculated as follows: (number of CD8α cells / total number of $CD11c^+$ cells) x 100.

Ex vivo *staining of NR16-specific CD4⁺ T cells by MHC II tetramers*

Splenocytes were incubated with APC-labeled class II MHC I-A^k tetramers (4 μg/mL, 90 min, 4°C) in phosphate-buffered saline – bovine serum albumin (1%, Sigma-Aldrich) supplemented with azide (0.02% Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies against membrane proteins, CD134-BV421 (Biolegend), CD40-PE-Cy5, CD3-APC-eF780 (eBioscience), CD4-PE-CF594 and CD44-PerCP-Cy5.5 (BD Biosciences), were added in the last 30 min of tetramer staining. A negative control was obtained by staining with a tetramer loaded with class II-associated invariant chain peptide (CLIP).

Results

Anti-HEL antibody production and the timing of poly(I:C) delivery

To determine how the interval between poly(I:C) delivery and transfusion influences the induction of alloimmunization, we injected B10BR mice with poly(I:C) at 4 h, 3 days, 7 days or 14 days before transfusion.

As in previous studies relating to the induction of anti-RBC antibodies as efficiently as possible in mouse

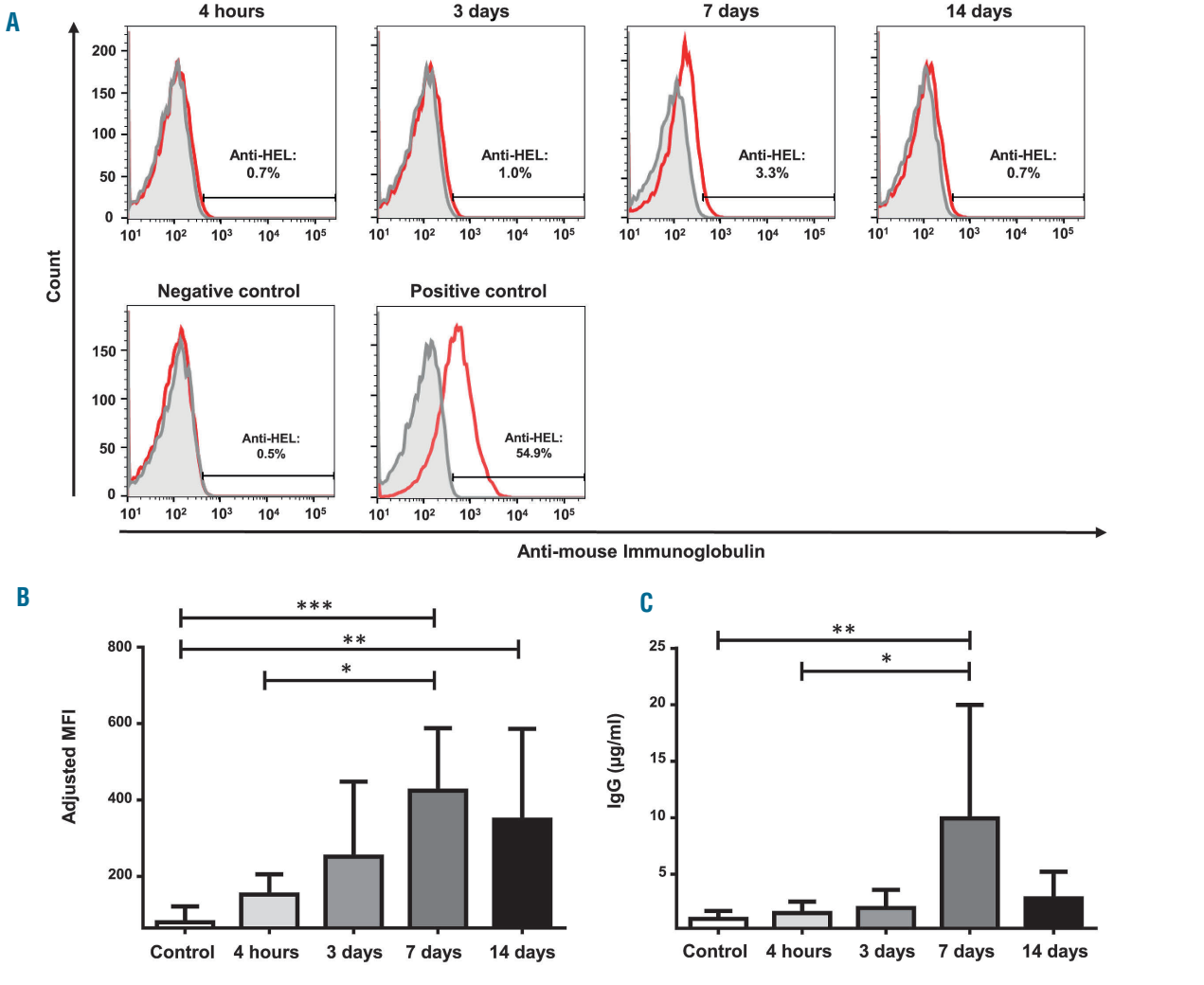

Figure 1. Anti-HEL response 28 days after transfusion in mice injected with poly(I:C) at various times before RBC transfusion. At 4 h, 3 days, 7 days or 14 days after poly(I:C) or phosphate-buffered saline (control represented at 4 h) intraperitoneal injection, mice were transfused with HEL RBC and sera were obtained 28 days later. (A) Histograms of representative experiments showing the detection of anti-HEL antibodies by flow cross-matching with sera incubated with HEL^+ RBC (red line) or HEL^- RBC (black line). Serum from HEL-immunized mice was used as a positive control. Serum from HEL-naïve B10BR mice was used as a negative control. Sera were tested for the presence of anti-HEL antibodies by (B) flow cross-matching or by (C) ELISA. Comparisons were performed using the Kruskal-Wallis test and Dunn post test. *P<0.05; **P<0.01; ***P<0.005. Representative data from two independent experiments in six mice each are shown (mean ± SD).

models,[12,13] we evaluated the production of anti-HEL antibodies by flow cross-matching and ELISA, 28 days after the transfusion of blood from donor HOD mice (Figure 1). The adjusted MFI was calculated as described above, to eliminate the background noise of each sample. The HEL antibody response was significantly higher in mice injected with poly(I:C) 7 days before transfusion than in those injected 4 h before transfusion (42.5±16.4 *versus* 15.2±5.3, respectively; $P<0.05$) (Figure 1B). The anti-HEL IgG response was assessed by ELISA (Figure 1C). The titer of these antibodies was also significantly higher in mice receiving the injection 7 days before transfusion than in those receiving the injection 4 h before transfusion (0.99±1.01 μg/mL *versus* 0.15±0.10 μg/mL, respectively; $P<0.05$).

Effect of poly(I:C) injection before transfusion on the proportion of NR16-presenting dendritic cells

We evaluated the effect of the timing of poly(I:C) injection (4 h, 3 days, 7 days or 14 days before transfusion) on CD8α$^+$ and CD8α$^-$ splenic CD11c$^+$ DC 48 h after transfusion. These two subsets are the main subpopulations of DC in the spleen. We determined the proportion of NR16-presenting DC in the CD11c$^+$ and CD8α$^{-/+}$ subsets, using the AW3.18 antibody (Figure 2A). NR16-presenting DC represented 33.9±13.5% of cells in the CD11c$^+$ DC population in mice injected with poly(I:C) 7 days before transfusion, but only 14.6±9.1% in mice injected with poly(I:C) 4 h before transfusion ($P<0.05$, Figure 2B). Regarding the different subpopulations, the proportion of NR16-presenting cells in the CD8α$^-$ subpopulation was highest in mice injected with poly(I:C) 7 days before transfusion (31.1±6.0%, $P<0.05$). By contrast, the proportion of NR16-presenting cells in the CD8α$^+$ subpopulation was highest in mice injected only 4 h before (34.6±16.7%). The proportion of CD8α$^+$ NR16-presenting cells decreased as the delay between poly(I:C) injection and transfusion increased, and reached its lowest value of 4.0±1.8% in mice injected with poly(I:C) 14 days before transfusion (Figure 2C).

The functions of NR16-presenting dendritic cells are influenced by the timing of poly(I:C) delivery

IL12 expression by the total CD8α$^{+/-}$ DC subset was not affected by the timing of poly(I:C) delivery (*data not shown*). However, to examine the function of NR16-pre-

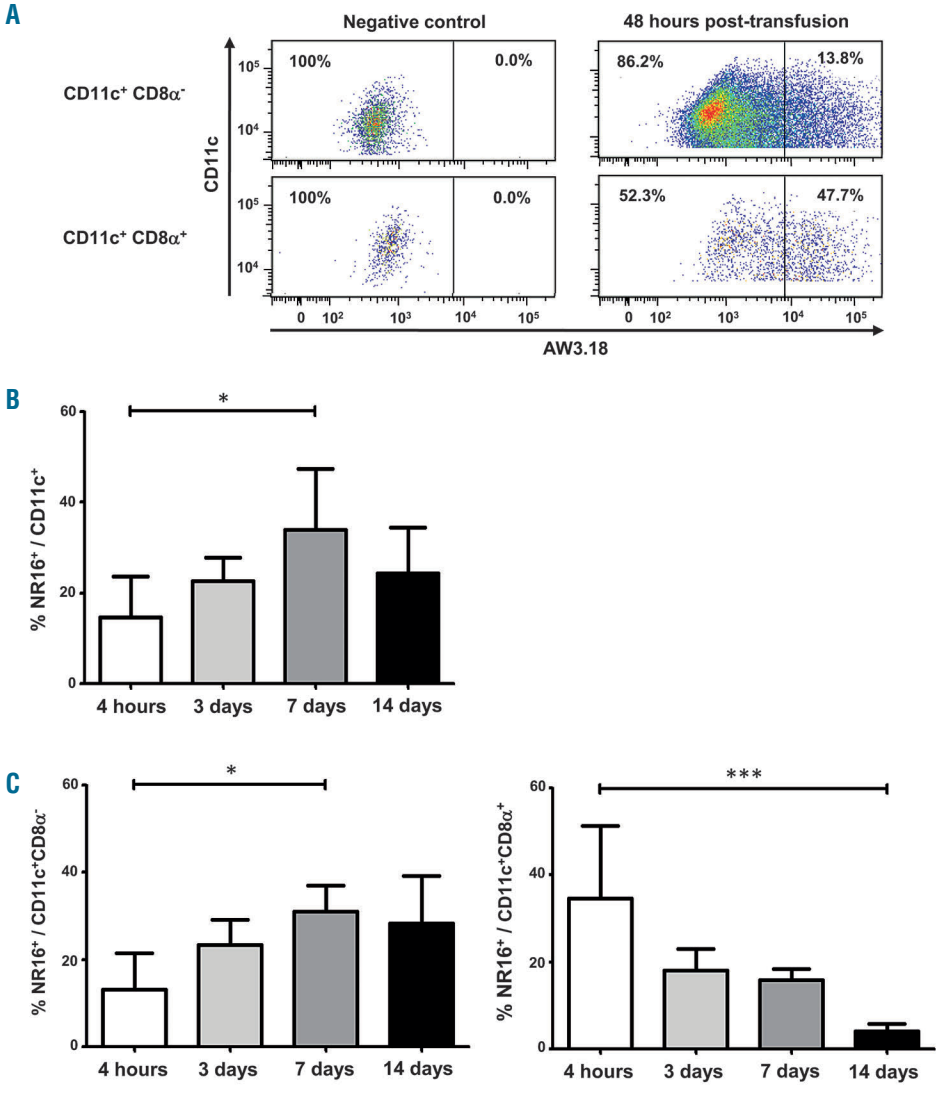

Figure 2. Proportion of splenic NR16-presenting cells in the CD11c$^+$ DC and CD8α$^{+/-}$ subpopulations, after transfusion following poly(I:C) injection. At 4 h, 3 days, 7 days or 14 days after poly(I:C) intraperitoneal injection, mice were transfused with HEL RBC and spleens were harvested 48 h later. (A) The AW3.18 antibody was used to detect NR16-presenting CD11c$^+$ DC in the CD8α$^-$ (top) and CD8α$^+$ (bottom) subpopulations. NR16 is the immunodominant peptide of HEL. Gating is shown for mice injected with poly(I:C) 4 h before transfusion. AW3.18$^-$ cells isolated from mice that received intravenous PBS instead of transfusion were used as a negative control. (B) The proportions of NR16-presenting cells in the CD11c$^+$ DC population are given. (C) The proportions of NR16-presenting cells in the CD8α$^-$ CD11c$^+$ (left) and CD8α$^+$ CD11c$^+$ (right) DC populations are given. Comparisons were performed using the Kruskal Wallis test and Dunn post test. $P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$. Representative data from two independent experiments in five mice each are shown (mean ± SD).

senting DC, we also measured IL12 production in both the CD8α⁺ and CD8α⁻ subsets, by intracellular staining (Figure 3A). The proportion of NR16-presenting CD8α⁺ CD11c⁺ DC producing IL12 was significantly higher in mice that received poly(I:C) 3 days before transfusion than in those injected only 4 h before transfusion (8.3±5.5% *versus* 21.8±5.2%, respectively; $P<0.05$). As expected, the CD8α⁻ subpopulation produced low amounts of IL12; however, its production was not influenced by the timing of poly(I:C) delivery (Figure 3B).

We also investigated IFNγ production by the two subpopulations: IFNγ expression in the NR16-presenting CD8α⁻ subpopulation was significantly weaker in mice injected with poly(I:C) 3 days or 7 days before transfusion than in those that received poly(I:C) 4 h before transfusion (*data not shown*, $P<0.05$ and $P<0.01$, respectively). IFNγ production by the CD8α⁺ subpopulation was not affected by the timing of poly(I:C) injection (*data not shown*).

The timing of poly(I:C) delivery affects the phenotype of NR16-presenting dendritic cells

We also evaluated the effect of the timing of poly(I:C) injection on the two NR16-presenting DC subpopulations 48 h after transfusion, by measuring the expression of the co-stimulatory molecules CD252, CD70 and CD40.

In both the CD8α⁻ and CD8α⁺ subpopulations, CD252 expression decreased as the delay between poly(I:C) injection and transfusion increased (Figure 4A). CD252 expression on NR16-presenting CD8α⁻ DC was highest in mice injected with poly(I:C) 4 h before transfusion and lowest in those injected 14 days before transfusion (45.0±5.6% to 2.6±1.0%, respectively; $P<0.005$). We observed a similar pattern for CD8α⁺ DC (77.1±8.2% in mice injected 4 h and 9.7±2.7% in those injected 14 days before transfusion; $P<0.005$).

In both the CD8α⁻ and CD8α⁺ subpopulations, the timing of poly(I:C) delivery had little effect on CD70 expression (Figure 4B). The proportion of CD8α⁻ NR16-presenting DC expressing CD70 was slightly higher in mice injected with poly(I:C) 7 days than in those injected 4 h before transfusion (75.3±24.3% *versus* 63.1±9.6%, respectively; $P<0.05$). The proportion of CD8α⁺ NR16-presenting cells expressing CD70 was significantly lower in mice that received poly(I:C) 3 or 7 days than in those that received it 4 h before transfusion ($P<0.01$ and $P<0.005$, respectively) (Figure 4B).

CD40 expression followed the same pattern as CD70 expression. The proportion of cells in the CD8α⁻ DC subpopulation expressing CD40 was slightly higher in mice injected with poly(I:C) 14 days before transfusion than in those injected 4 h prior to the transfusion (72.7±5.3% *versus* 60.8±11.4%; $P<0.05$). In the CD8α⁺ subset, the proportion of cells expressing CD40 was significantly lower in mice injected 7 days than in those injected 4 hours before transfusion (56.6±2.2% *versus* 81.0±10.3%; $P<0.005$) (Figure 4C). Furthermore, as expected, poly(I:C) played the same activating role in the total DC population and in NR16-presenting DC (a representative example for the 7-day group is presented in *Online Supplementary Figure S1*). Indeed, the levels of CD252, CD70 and CD40 expression were equivalent on total DC at all four time points studied, and changes in these levels followed the same pattern as those observed in NR16-presenting DC (*data not shown*).

We also measured the effect of poly(I:C) delivery time on the intracellular expression of TLR3 in the CD11c⁺ DC subpopulations (Figure 5A). In the total CD8α⁺ subpopula-

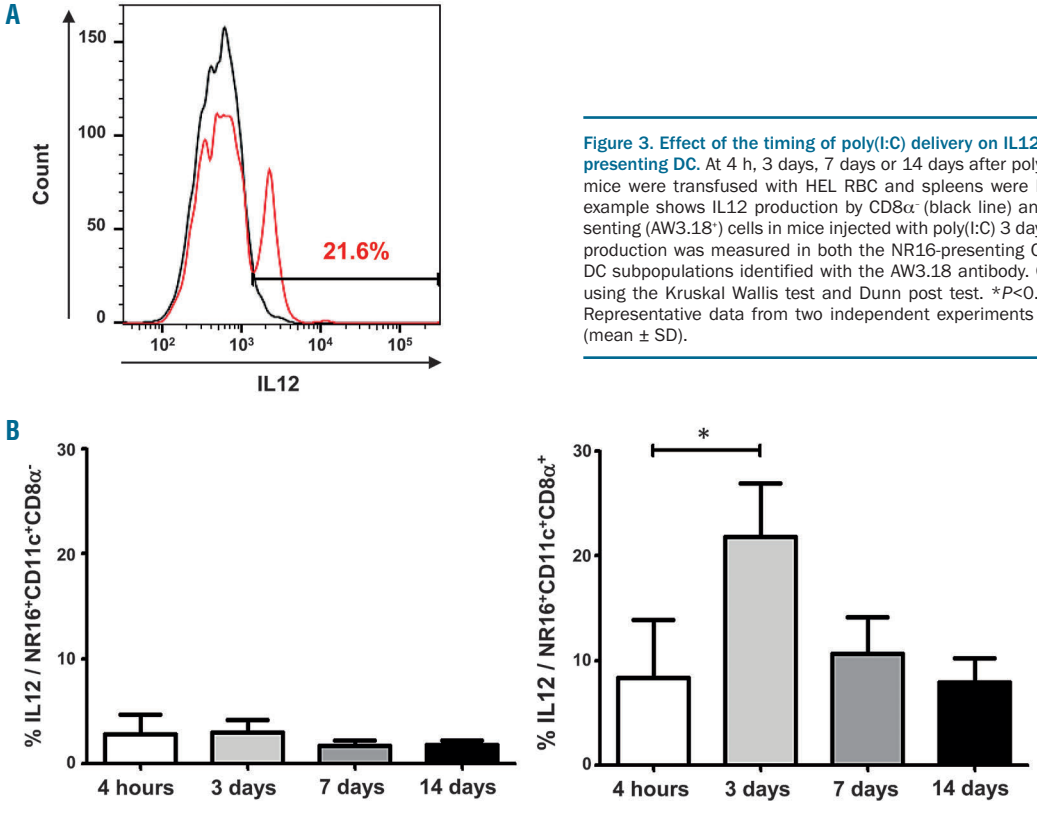

Figure 3. Effect of the timing of poly(I:C) delivery on IL12 production by splenic NR16-presenting DC. At 4 h, 3 days, 7 days or 14 days after poly(I:C) intraperitoneal injection, mice were transfused with HEL RBC and spleens were harvested 48 h later. (A) The example shows IL12 production by CD8α⁻ (black line) and CD8α⁺ (red line) NR16-presenting (AW3.18⁺) cells in mice injected with poly(I:C) 3 days before transfusion. (B) IL12 production was measured in both the NR16-presenting CD8α⁻ (left) and CD8α⁺ (right) DC subpopulations identified with the AW3.18 antibody. Comparisons were performed using the Kruskal Wallis test and Dunn post test. *$P<0.05$; **$P<0.01$; ***$P<0.005$. Representative data from two independent experiments in five mice each are shown (mean ± SD).

tion, TLR3 expression was higher in mice injected with poly(I:C) 3 or 7 days before transfusion than in those injected 4 h before (P<0.01) (Figure 5B). TLR3 expression in the NR16-presenting CD8α⁺ subpopulation was also higher in mice that received poly(I:C) 3 or 7 days before transfusion than in those that received it only 4 h before (P<0.01 and P<0.05, respectively) (Figure 5B).

As expected, the CD8α⁻ subpopulation expressed low amounts of TLR3 and its expression was not influenced by the timing of poly(I:C) delivery (*Online Supplementary Figure S2*). In the NR16-presenting CD8α⁻ subpopulation, TLR3 expression was weakest in the mice that received poly(I:C) 14 days before transfusion (*Online Supplementary Figure S2*).

The timing of poly(I:C) injection influences the abundance and phenotype of early NR16-specific CD4⁺ T cells

We harvested the spleen 48 h after transfusion to investigate CD4⁺ T-cell induction at an early stage of TCR engagement. We used MHC class II tetramers (Tet) to study how the timing of poly(I:C) injection influences NR16-specific CD4⁺ T effector cells *ex vivo* (Figure 6A). NR16-Tet⁺ cells were present in all four sets of conditions, but there were no important differences between groups. Indeed, the percentage of NR16-Tet⁺ cells was similar in mice injected with poly(I:C) 7 or 14 days before transfusion and those injected 4 h before, and it was only slightly lower in those injected 3 days before (P<0.05 *versus* injection at 4 h) (Figure 6B).

We, therefore, analyzed the phenotype of NR16-Tet⁺ cells by evaluating expression of the activation molecules CD134, CD40 and CD44. The expression of CD134, CD40 and CD44 was lower on total T CD4⁺ cells than on NR16-Tet⁺ cells (Figure 6C). The proportion of total cells expressing CD134, CD40 or CD44 was smaller than that of NR16-Tet⁺ cells for each of the injection time points studied (*Online Supplementary Figure S3*).

The proportion of NR16-Tet⁺ cells expressing CD134 was highest in mice injected 7 days before transfusion and

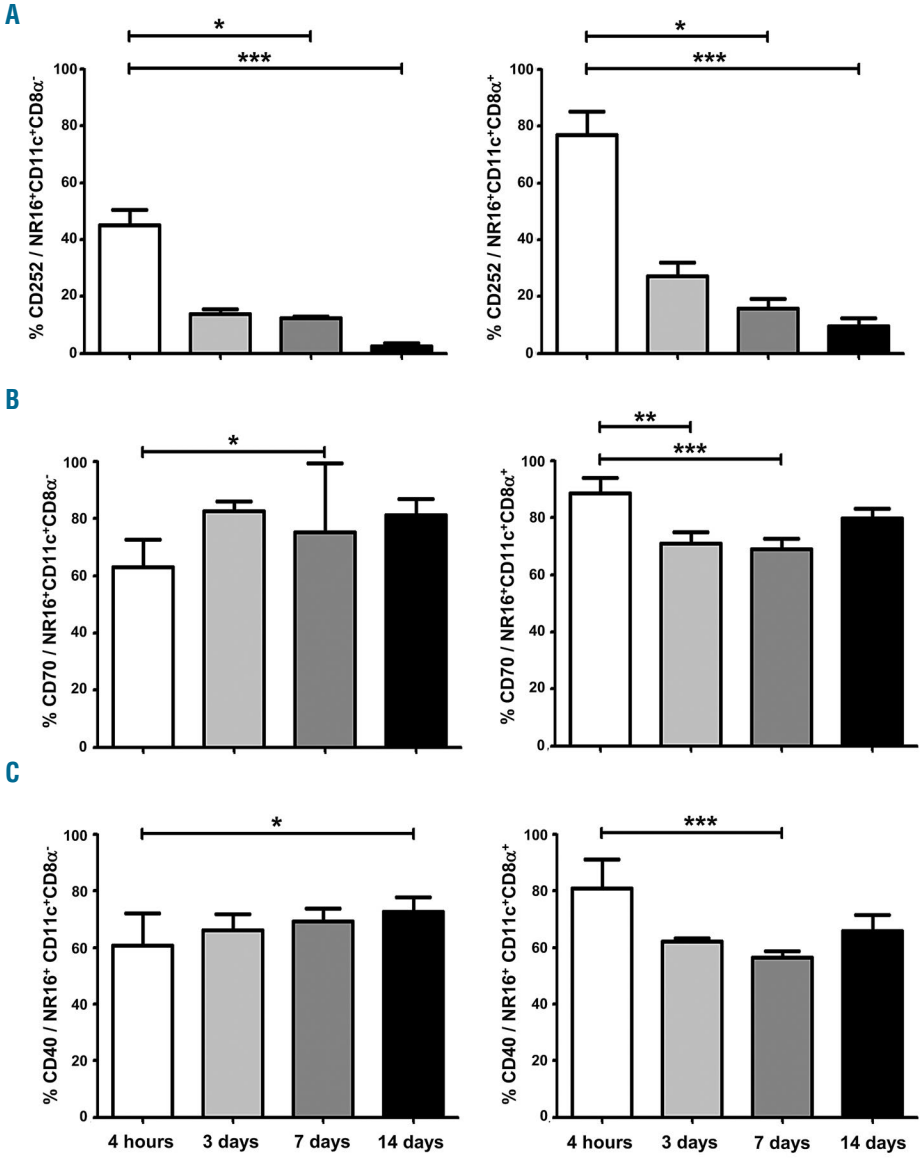

Figure 4. The timing of poly(I:C) delivery influences the phenotype of NR16-presenting DC. At 4 h, 3 days, 7 days or 14 days after poly(I:C) intraperitoneal injection, mice were transfused with HEL RBC and spleens were harvested 48 h later. The expression of (A) CD252, (B) CD70 and (C) CD40 was measured on NR16-presenting CD11c⁺ CD8α⁻ (left column) and CD8α⁺ (right column) DC. Comparisons were performed using the Kruskal Wallis test and Dunn post test. *P<0.05; **P<0.01; ***P<0.005. Representative data from two independent experiments in five mice each are shown (mean ± SD).

lowest in those injected 4 h before (26.8±6.2% *versus* 4.4±1.6%, respectively; $P<0.01$) (Figure 6D). The proportion of NR16-Tet$^+$ cells expressing CD40 also tended to be correlated with the timing of poly(I:C) injection, and was significantly higher in mice injected 14 days before transfusion than in those injected 4 h before (8.5±6.7% *versus* 0.7±1.1%, respectively; $P<0.01$) (Figure 6D). CD44 was expressed on NR16-Tet$^+$ cells, but the proportion of cells expressing this molecule was not significantly influenced by the timing of poly(I:C) injection (Figure 6D).

Discussion

The HEL antibody response was significantly higher in mice injected with poly(I:C) 7 days before transfusion than in those injected 4 h before transfusion. To understand why antibody production depends on the delay between transfusion and poly(I:C) injection, we first explored DC subpopulations and their activation phenotypes. Poly(I:C) is widely used to promote the maturation of both mouse and human DC.[22,26,27] In mouse studies, poly(I:C) induces complete activation of DC after 12 to 24 h.[22,28] In humans, poly(I:C) induces a mature surface phenotype, which can still be detected at 72 h.[29] TLR3 signaling stimulates the expression of co-stimulatory molecules on antigen-presenting cells, potentially enhancing CD4$^+$ T-cell responses.[26] We, therefore, also investigated antigen-specific CD4$^+$ T-cell responses.

In murine models of transfusion, poly(I:C) promotes the consumption of RBC by CD11c$^+$ DC in the spleen.[16] However, no studies have examined whether these DC present RBC antigens. We, therefore, explored whether the phenotypic modifications of CD11c$^+$ DC observed by Hendrickson *et al.*[16] were also detectable in HEL-presenting CD11c$^+$ DC. Specifically, we examined whether the delay between poly(I:C) injection and transfusion affected the phenotype and function of HEL-presenting DC. In vaccination studies, immune responses are weak if the antigen is administered within 6 h of agonist injection.[22] We studied CD11c$^+$ DC subpopulations in the spleen, which can be differentiated by their CD8α expression.[20] We found that the timing of agonist injection influenced the ratio of CD8α$^+$ to CD8α$^-$ NR16-presenting CD11c$^+$ DC. Poly(I:C) and the CD8α$^+$ DC subset play an important role in the induction of Th1 polarization.[19,22] Our observation was, therefore, surprising because we expected to find a high proportion of NR16-presenting CD8α$^+$ CD11c$^+$ DC in mice that received poly(I:C) 7 days before transfusion, rather than CD8α$^-$ CD11c$^+$ DC. Given that the CD8α$^-$ subset outnumbers the CD8α$^+$ subset in these conditions, it is possible that the CD8α$^-$ subset is more efficient at antigen uptake and presentation. Alternatively, either only the CD8α$^+$ subset is primordial, independently of its proportion, or both subsets are implicated in RBC alloimmunization. These two subsets of DC can be differentiated by their expression of TLR3, with the CD8α$^+$ subset expressing high levels of TLR3.[19] TLR3 expression in the NR16-presenting CD8α$^+$ subset was positively correlated with the delay between transfusion and poly(I:C) injection, suggesting a higher sensitivity of the CD8α$^-$ subset to TLR3 agonists.

However, these two DC subsets are also distinct in terms of function, which may be important for alloimmunization. Indeed, upon activation, the CD8α$^+$ subpopulation produces high levels of IL12, whereas the CD8α$^-$ subpopulation does not.[20] We found that the proportion of NR16-presenting CD8α$^+$ DC expressing IL12 was significantly higher in mice injected with poly(I:C) 3 days before transfusion than in those injected 4 h before. This result is consistent with studies showing that poly(I:C) stimulates CD8α$^+$ DC to produce IL12,[19,27] which may contribute substantially to RBC alloimmunization. Indeed, activated DC produce IL12, which induces the differentiation of naïve CD4$^+$ T cells into T follicular helper (Tfh) cells.[30] Tfh cells are specialized T helper cells that regulate antibody production and the development of memory B cells.[31] The production of anti-HEL antibodies in mice receiving blood

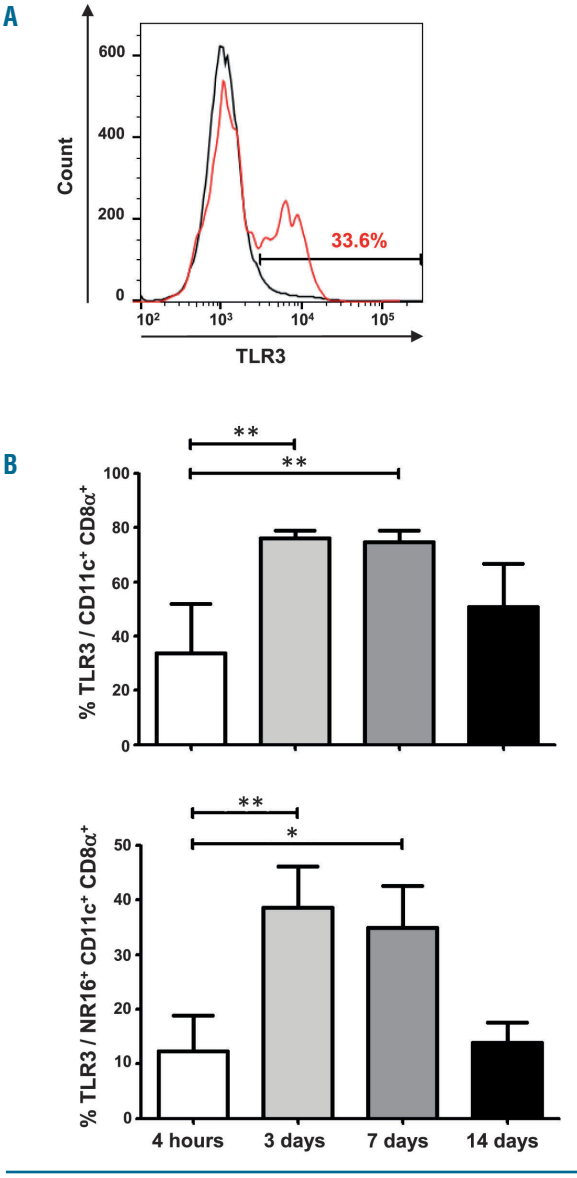

Figure 5. The effect of the timing of poly(I:C) delivery on TLR3 expression by splenic CD11c$^+$ CD8α$^+$ DC after transfusion. At 4 h, 3 days, 7 days or 14 days after poly(I:C) intraperitoneal injection, mice were transfused with HEL RBC and spleens were harvested 48 h later. (A) TLR3 expression on the CD8α$^-$ (black line) and CD8α$^+$ (red line) CD11c$^+$ subpopulations in mice injected with poly(I:C) 4 h before transfusion is shown. The expression of TLR3 was measured in (B) total and (C) NR16-presenting CD11c$^+$CD8α$^+$ DC. Comparisons were performed using the Kruskal Wallis test and Dunn post test. *$P<0.05$; **$P<0.01$. Representative data from two independent experiments in five mice each are shown (mean ± SD).

transfusions may reflect changes in the phenotype of NR-16-presenting CD8α⁺ DC. However, we cannot exclude a role for CD8α⁻ DC in alloimmunization, because this subset produces IL10, which is important for B-cell differentiation and immunoglobulin switching.[32] In this study, due to differences in phenotype observed between the different time periods studied, we can conclude that DC are involved in the induction of alloimmunization, but that these cells may not affect the magnitude of this phenomenon.

Poly(I:C) tightly regulates the expression of co-stimulatory molecules at the surface of DC.[22] In transfused mice, poly(I:C) induces the expression of CD70, CD252 and CD40 molecules on DC.[16] The expression of CD252 on DC promotes CD4⁺ T-cell expansion about 48 h after antigen stimulation.[33] However, in this study, the level of CD252 expression was low in both subpopulations 48 h after transfusion in mice that received poly(I:C) 3 or more days before transfusion. Given that this model differs from classical models used to study antigen stimulation, it is likely that the exosomes issued from RBC or platelets also modulate immune responses to RBC antigens.[34-36]

However, other tumor necrosis factor receptors, notably CD70 and CD40, are required for complete CD4⁺ T-cell

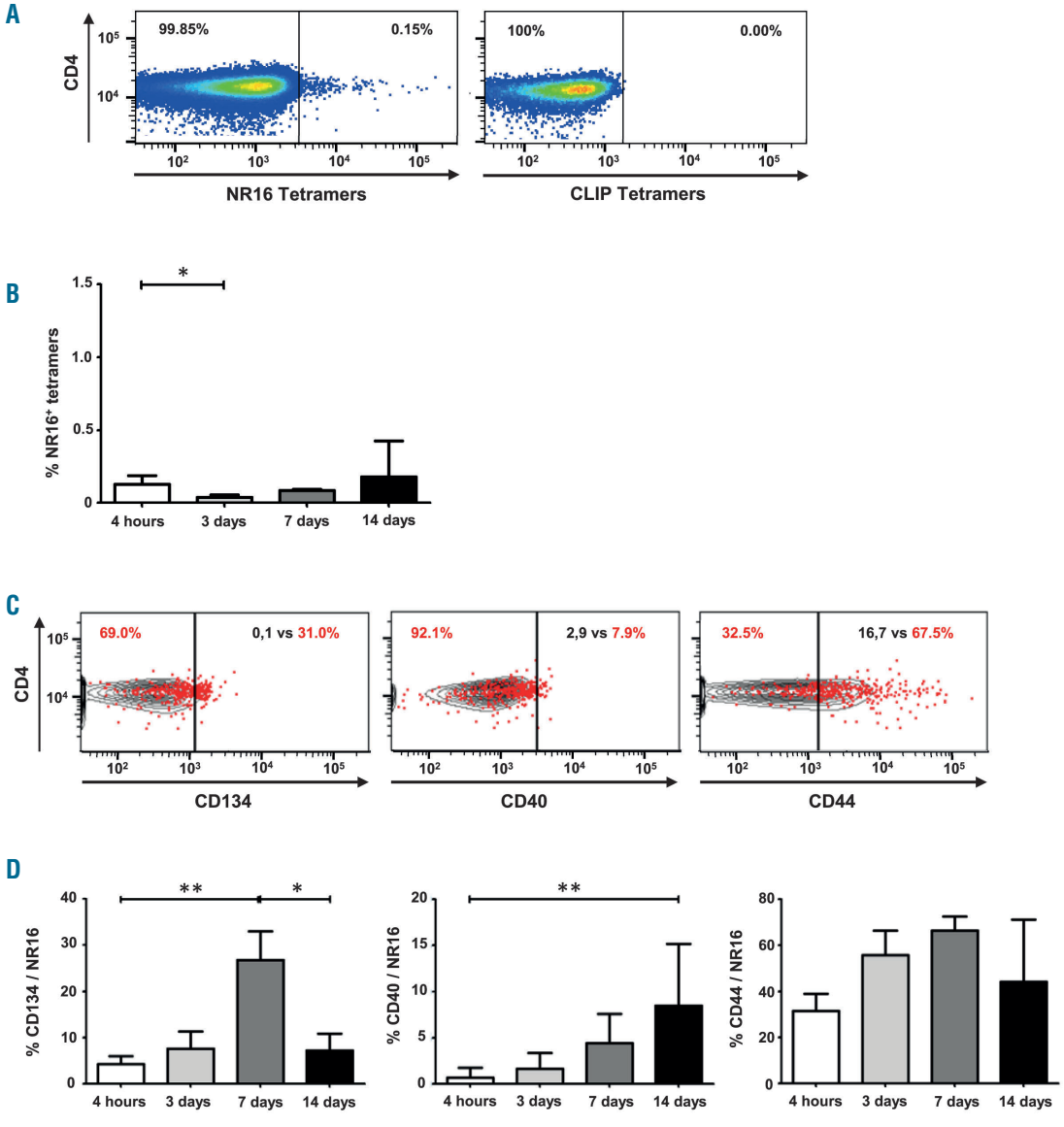

Figure 6. The effect of the timing of poly(I:C) injection on NR16-specific CD4⁺ T cells. Mice were transfused with HEL RBC 4 h, 3 days, 7 days or 14 days after poly(I:C) intraperitoneal injection and spleens were harvested 48 h later. (A) The abundance of NR16-specific CD4⁺ T cells was measured *ex vivo* by class II MHC tetramers of the immunodominant NR16-peptide from HEL. Plots corresponding to mice injected with poly(I:C) 4 h before transfusion are shown. Tetramers of class II-associated invariant chain peptide (CLIP) were used as a specificity control for NR16 tetramers. (B) NR16-specific CD4⁺ T cells (NR16⁺ tetramers) were detected using tetramers of class II MHC and are reported as a percentage of total CD4⁺ T cells. (C) The expression of CD134, CD40 and CD44 was measured *ex vivo* on NR16-specific CD4⁺ T (red dot plot) and total CD4⁺ T cells (black contour plot). Plots for mice injected with poly(I:C) 7 days before transfusion are shown. (D) The phenotype of NR16-specific CD4⁺ T cells (NR16) in mice injected with poly(I:C) at various time points before transfusion. Comparisons were performed using the Kruskal Wallis test and Dunn post test. **P*<0.05; ***P*<0.01. Representative data from two independent experiments of five mice each are shown (mean ± SD).

activation.[37] Indeed, the injection of immunogenic protein and TLR3/9 agonists combined with an anti-CD40 antibody upregulates CD70 expression at the DC membrane.[37] This combination enables the binding of the CD70 ligand, CD27, which induces the differentiation of naïve-CD4+ T cells and memory T lymphocytes.[38-40] Although we observed minor variations between groups regarding the expression of CD70 and CD40 on both subsets of NR16-presenting DC, the proportion of cells expressing these molecules remained high in all conditions.

The expression of these co-stimulatory molecules on DC is associated with antigen-specific CD4+ T-cell responses.[26] Furthermore, poly(I:C) directly affects the lymphoproliferation of RBC-specific CD4+ T cells in a mouse model of transfusion.[14] For these reasons, we used class II MHC tetramers to investigate antigen-specific CD4+ T-cell responses 48 h after transfusion. The timing of poly(I:C) delivery did not appear to affect the proportion of NR16-specific CD4+ T cells. However, the CD4+ T-cell population was analyzed 48 h after transfusion, which may explain the small variation observed for CD4+ T-cell numbers.

Nevertheless, both the activation and differentiation of NR16-specific CD4+ T cells were affected by the timing of agonist injection. Co-stimulatory molecules expressed by DC and CD4+ T cells are important for the activation and differentiation of immune system cells.[33,41] Among these co-stimulatory molecules, CD134 (OX40) is expressed soon after the stimulation of the TCR on CD4+ T cells and binds to its ligand, CD252 (OX40L), on the DC membrane. We found that the proportion of NR16-specific CD4+ T cells expressing CD134 was highest in mice injected with poly(I:C) 7 days before transfusion. CD40 expression was also positively associated with the duration of the delay between TLR-agonist injection and transfusion. Both CD134 and CD40 play a role in CD4+ T-cell responses and autoimmunity.[33,42-44] This finding is consistent with the high frequency of autoantibodies against RBC antigens in alloimmunized SCD patients.[45,46] Moreover, autoimmunity has also been described in SCD patients.[47-49]

The strong expression of these two molecules may affect alloimmunization, especially when poly(I:C) is injected 7 days before transfusion. Specifically, the injection of TLR3 agonists combined with CD40 stimulation facilitates the secretion of pro-inflammatory cytokines by CD4+ T cells by promoting the expression of CD134.[40,50] Cross-linking between CD134 on CD4+ T cells and CD252 on activated B cells results in B-cell proliferation and the secretion of all Ig isotypes.[51] This high level of expression of CD134 at 7 days and its involvement in B-cell antibody secretion suggest a direct link between the CD4+ T-cell response and the level of antibody production, which were concordant for the same time interval between agonist injection and transfusion. Finally, the relationship between CD4+ T cells and B cells might be stronger than that between CD4+ T cells and DC in RBC alloimmunization.

In conclusion, our study confirms that the degree of alloimmunization depends on the delay between TLR3-induced inflammation and exposure to RBC antigens, with the largest effect observed at 7 days, for CD4+ T cells. TLR3 and TLR9 have been shown to affect alloimmunization in mouse models.[12,14] However, it remains unknown whether exposure to RNA viruses or bacterial infections in the week before a transfusion increases the risk of alloimmunization in humans. We need to evaluate this risk in SCD patients, who experience inflammation and infection more frequently than the general population of individuals undergoing transfusion.

The role of vaccination should also be taken into account. Indeed, in addition to the conventional adjuvants used in vaccination (aluminum salts, oil-emulsion or liposomes), which encourage antigen presentation and the activation of antigen-presenting cells,[52] TLR agonists have also been proposed as new adjuvants for vaccines in clinical trials.[52,53] It is likely that these new vaccines need to be used with caution in polytransfused SCD patients to limit RBC alloimmunization.

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References

1. Silvy M, Tournamille C, Babinet J, et al. Red blood cell immunization in sickle cell disease: evidence of a large responder group and a low rate of anti-Rh linked to partial Rh phenotype. Haematologica. 2014;99(7): e115-117.
2. Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. Blood. 2012; 120(3):528-537.
3. Ansart-Pirenne H, Zeliszewski D, Lee K, Martin-Blanc S, Rouger P, Noizat-Pirenne F. Identification of immunodominant alloreactive T-cell epitopes on the Jka red blood cell protein inducing either Th1 or Th2 cytokine expression. Blood. 2004; 104(10):3409-3410.
4. Hoppe C, Klitz W, Vichinsky E, Styles L. HLA type and risk of alloimmunization in sickle cell disease. Am J Hematol. 2009; 84(7):462-464.
5. Stephen J, Cairns LS, Pickford WJ, Vickers MA, Urbaniak SJ, Barker RN. Identification, immunomodulatory activity, and immunogenicity of the major helper T-cell epitope on the K blood group antigen. Blood. 2012; 119(23):5563-5574.
6. Vingert B, Tamagne M, Desmarets M, et al. Partial dysfunction of Treg activation in sickle cell disease. Am J Hematol. 2014; 89(3):261-266.
7. Zhong H, Bao W, Friedman D, Yazdanbakhsh K. Hemin controls T cell polarization in sickle cell alloimmunization. J Immunol. 2014;193(1):102-110.
8. Bao W, Zhong H, Li X, et al. Immune regulation in chronically transfused allo-antibody responder and nonresponder patients with sickle cell disease and β-thalassemia major. Am J Hematol. 2011;86(12):1001-1006.
9. Vingert B, Tamagne M, Habibi A, et al. Phenotypic differences of CD4+ T cells in response to red blood cell immunization in transfused sickle cell disease patients. Eur J Immunol. 2015;45(6):1868-1879.
10. Hendrickson JE, Hod EA, Perry JR, et al. Alloimmunization to transfused HOD red blood cells is not increased in mice with

sickle cell disease. Transfusion. 2012;52(2):231-240.
11. Desmarets M, Noizat-Pirenne F. Murine models in blood transfusion: allo-immunization, hemolysis. Transfus Clin Biol. 2011;18(2):115-123.
12. Yu J, Heck S, Yazdanbakhsh K. Prevention of red cell alloimmunization by CD25 regulatory T cells in mouse models. Am J Hematol. 2007;82(8):691-696.
13. Hendrickson JE, Desmarets M, Deshpande SS, et al. Recipient inflammation affects the frequency and magnitude of immunization to transfused red blood cells. Transfusion. 2006;46(9):1526-1536.
14. Hendrickson JE, Roback JD, Hillyer CD, Easley KA, Zimring JC. Discrete Toll-like receptor agonists have differential effects on alloimmunization to transfused red blood cells. Transfusion. 2008;48(9):1869-1877.
15. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637-650.
16. Hendrickson JE, Chadwick TE, Roback JD, Hillyer CD, Zimring JC. Inflammation enhances consumption and presentation of transfused RBC antigens by dendritic cells. Blood. 2007;110(7):2736-2743.
17. Smith NH, Hod EA, Spitalnik SL, Zimring JC, Hendrickson JE. Transfusion in the absence of inflammation induces antigen-specific tolerance to murine RBCs. Blood. 2012;119(6):1566-1569.
18. Steinman RM. Decisions about dendritic cells: past, present, and future. Annu Rev Immunol. 2012;30:1-22.
19. Shortman K, Heath WR. The CD8+ dendritic cell subset. Immunol Rev. 2010;234(1):18-31.
20. Coquerelle C, Moser M. DC subsets in positive and negative regulation of immunity. Immunol Rev. 2010;234(1):317-334.
21. Gelman AE, Zhang J, Choi Y, Turka LA. Toll-like receptor ligands directly promote activated CD4+ T cell survival. J Immunol. 2004;172(10):6065-6073.
22. Longhi MP, Trumpfheller C, Idoyaga J, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med. 2009;206(7):1589-1602.
23. Hendrickson JE, Saakadze N, Cadwell CM, et al. The spleen plays a central role in primary humoral alloimmunization to transfused mHEL red blood cells. Transfusion. 2009;49(8):1678-1684.
24. Fasano RM, Booth GS, Miles M, et al. Red blood cell alloimmunization is influenced by recipient inflammatory state at time of transfusion in patients with sickle cell disease. Br J Haematol. 2015;168(2):291-300.
25. Dadaglio G, Nelson CA, Deck MB, Petzold SJ, Unanue ER. Characterization and quantitation of peptide-MHC complexes produced from hen egg lysozyme using a monoclonal antibody. Immunity. 1997;6(6):727-738.
26. de Jong EC, Vieira PL, Kalinski P, et al. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals. J Immunol. 2002;168(4):1704-1709.
27. Lichtenegger FS, Mueller K, Otte B, et al. CD86 and IL-12p70 are key players for T helper 1 polarization and natural killer cell activation by Toll-like receptor-induced dendritic cells. PLoS One. 2012;7(9):e44266.
28. Tsujimoto H, Efron PA, Matsumoto T, et al. Maturation of murine bone marrow-derived dendritic cells with poly(I:C) produces altered TLR-9 expression and response to CpG DNA. Immunol Lett. 2006;107(2):155-162.
29. Verdijk RM, Mutis T, Esendam B, et al. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. J Immunol. 1999;163(1):57-61.
30. Schmitt N, Morita R, Bourdery L, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. Immunity. 2009;31(1):158-169.
31. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41(4):529-542.
32. Defrance T, Vanbervliet B, Brière F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. J Exp Med. 1992;175(3):671-682.
33. Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol. 2005;23:23-68.
34. Seghatchian J, Krailadsiri P. Red cell storage lesion assessed by the levels of potassium, haemoglobin and annexin V in supernatants. Transfus Apher Sci. 2002;26(2):139-143.
35. Danesh A, Inglis HC, Jackman RP, et al. Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting T-cell responses in vitro. Blood. 2014;123(5):687-696.
36. Sadallah S, Amicarella F, Eken C, Iezzi G, Schifferli JA. Ectosomes released by platelets induce differentiation of CD4+T cells into T regulatory cells. Thromb Haemost. 2014;112(6):1219-1229.
37. Sanchez PJ, McWilliams JA, Haluszczak C, Yagita H, Kedl RM. Combined TLR/CD40 stimulation mediates potent cellular immunity by regulating dendritic cell expression of CD70 in vivo. J Immunol. 2007;178(3):1564-1572.
38. Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. Nat Immunol. 2000;1(5):433-440.
39. Arimoto-Miyamoto K, Kadowaki N, Kitawaki T, Iwata S, Morimoto C, Uchiyama T. Optimal stimulation for CD70 induction on human monocyte-derived dendritic cells and the importance of CD70 in naive CD4(+) T-cell differentiation. Immunology. 2010;130(1):137-149.
40. Kurche JS, Burchill MA, Sanchez PJ, Haluszczak C, Kedl RM. Comparison of OX40 ligand and CD70 in the promotion of CD4+ T cell responses. J Immunol. 2010;185(4):2106-2115.
41. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4 T cells in immunity to viruses. Nat Rev Immunol. 2012;12(2):136-148.
42. Bourgeois C, Rocha B, Tanchot C. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. Science. 2002;297(5589):2060-2063.
43. Munroe ME. Functional roles for T cell CD40 in infection and autoimmune disease: the role of CD40 in lymphocyte homeostasis. Semin Immunol. 2009;21(5): 283-288.
44. Johnson S, Zhan Y, Sutherland RM, et al. Selected Toll-like receptor ligands and viruses promote helper-independent cytotoxic T cell priming by upregulating CD40L on dendritic cells. Immunity. 2009;30(2):218-227.
45. Garratty G. Autoantibodies induced by blood transfusion. Transfusion. 2004;44(1):5-9.
46. Noizat-Pirenne F, Bachir D, Chadebech P, et al. Rituximab for prevention of delayed hemolytic transfusion reaction in sickle cell disease. Haematologica. 2007;92(12):e132-135.
47. Michel M, Habibi A, Godeau B, et al. Characteristics and outcome of connective tissue diseases in patients with sickle-cell disease: report of 30 cases. Semin Arthritis Rheum. 2008;38(3):228-240.
48. Toly-Ndour C, Rouquette AM, Obadia S, et al. High titers of autoantibodies in patients with sickle-cell disease. J Rheumatol. 2011;38(2):302-309.
49. Maamar M, Tazi-Mezalek Z, Harmouche H, Mounfaloti W, Adnaoui M, Aouni M. Systemic lupus erythematosus associated with sickle-cell disease: a case report and literature review. J Med Case Rep. 2012;6(1):366.
50. Kurche JS, Haluszczak C, McWilliams JA, Sanchez PJ, Kedl RM. Type I IFN-dependent T cell activation is mediated by IFN-dependent dendritic cell OX40 ligand expression and is independent of T cell IFNR expression. J Immunol. 2012;188(2):585-593.
51. Stüber E, Strober W. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. J Exp Med. 1996;183(3):979-989.
52. Leroux-Roels G. Unmet needs in modern vaccinology: adjuvants to improve the immune response. Vaccine. 2010;28 (Suppl 3):C25-36.
53. Dubensky TW Jr, Reed SG. Adjuvants for cancer vaccines. Semin Immunol. 2010;22(3):155-161.