Effect of storage period of red blood cell suspensions on helper T-cell subpopulations

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Background. The aim of this study was to investigate the immunological alterations that occur during the storage of erythrocyte suspensions which may lead to transfusion-related immunomodulation following allogeneic blood transfusion.

Materials and methods. One part of the erythrocyte suspensions obtained from donors was leucoreduced while the other part was not. The leucoreduced (LR) and non-leucoreduced (NL) erythrocyte suspensions were then further divided into three equal amounts which were stored for 0, 21 or 42 days prior to measurements, by enzyme-linked immunosorbent assays, of cytokine levels in their supernatants. T-helper (Th) lymphocyte subgroups and gene expression were analysed in the NL erythrocyte suspensions by flow cytometry and real-time polymerase chain reaction, respectively. Results were compared to those of storage day 0.

Results. By day 21, the number of Th2 cells had increased significantly and the numbers of Th1, Th22 and Treg cells had decreased significantly in the NL erythrocyte suspensions. On day 42 the numbers of Th2 and Treg cells in the NL suspensions were significantly increased while the number of Th1 cells was significantly decreased. The levels of transcription factors (TBX21, GATA3, and SPI.1) were significantly decreased on days 21 and 42, and AHR, FOXP3 and RORC2 levels were significantly increased on day 42 in NL erythrocyte suspensions. The decrease in interleukin-22 and increase in transforming growth factor-β levels found in NL erythrocyte suspensions on day 21 were statistically significant. Elevated levels of interleukin-17A were found in both LR and NL erythrocyte suspensions on day 42.

Discussion. Our results suggest that allogeneic leucocytes and cytokines may play significant roles in the development of transfusion-related immunomodulation.

Keywords: TRIM, immunomodulation, transfusion, Th cell subgroups, leucoreduction.

Introduction

When performed at the right time and in the correct conditions, allogeneic blood transfusion is a lifesaving intervention. Although the benefit of such transfusions is obvious, they are associated with a real risk of serious and life-threatening complications. These complications can be classified as infectious, immunological and non-immunological. Transfusion-related immunomodulation (TRIM) is listed among the immunological complications, and can be defined as a consequence of changes within the immune system of the transfusion recipient, induced by allogeneic blood transfusion. It was first described in patients waiting for renal transplantation, based on the observation that allogeneic blood transfusions prolonged graft survival[1]. TRIM also seems to be associated with increases in cancer recurrence, graft survival, post-operative bacterial infection, and short-term mortality rates, decreases in Crohn's disease recurrences and recurrent spontaneous abortion, and reactivation of certain latent infections such as those caused by cytomegalovirus and human immunodeficiency virus[2-15]. The role of allogeneic leucocytes[16,17], microchimerism[18,19], biological response modifiers (BRM)[20], cytokines[21-24], bioactive lipids[25,26], erythrocyte suspension (ES) supernatant[27,28], storage duration of blood components[29,30], soluble Fas ligand[21,31], soluble human leucocyte antigen (HLA) class I molecules[21,32,33], and similar potential factors in the development of TRIM have been investigated, but the essential mechanism underlying TRIM has not yet been elucidated[34]. Major changes that occur in the recipient's immune system can be summarised as a

decrease in the number of T-helper cells (Th), a reduction in T-cell response, a decrease in natural killer cell function, insufficient antigen presentation, suppression of lymphocyte blastogenesis, an increased production of anti-idiotypic antibodies and anti-clonotypic antibodies[35,36], a decrease in the CD4/CD8 ratio[35-37], decreased late type hypersensitivity reactions[35,36,38], decreases in cytokine production (interleukin-2 [IL-2], interferon-gamma [IFN-γ])[35,36,39], and an impairment of monocyte/macrophage phagocytic function[35,36,40]. Based on these immunological changes, it has been postulated that the effect of TRIM may originate from three sources: mononuclear cells (MNC) within the blood component[6,17,41], BRM-immunological mediators (BRM-IM) that accumulate within the blood component during storage[6,20,28,42], and soluble HLA class I peptides within the allogeneic plasma[6,33,42].

These factors are thought to cause TRIM via mechanisms such as clonal deletion[43,44], immunosuppression[16,28,42,45-47], anergy[48,49], microchimerism[50,51], transition of the immune response from Th1 to Th2[39,49,51-59], apoptosis[60-62]. In addition to above-mentioned factors, duration of ES storage, number of transfusion products, erythrocytes and erythrocyte-derived microparticles have also been associated with TRIM[63-83]. In general, allogeneic MNC and related structures are considered to be the main cause of TRIM. It is, therefore, thought that the effects of TRIM can be eliminated by leucoreduction. However, several components such as free haemoglobin, lipids, cytokines and microparticles that have been shown to pass through the filter used for leucoreduction and accumulate during storage in leucoreduced (LR) ES and LR platelet suspensions limit the effect of leucoreduction[27].

Cytokines constitute a group of BRM-IM which may also lead to the TRIM effect in the recipient. Cytokines are proteins produced by different cell types which mediate inflammatory and immune responses. They are the primary mediators that provide connections between the cells of the immune system. Alterations in cytokine levels during the storage of LR-ES and non-leucoreduced (NL) ES have been demonstrated in previous studies[84,85]. The cytokine content of NL-ES and LR-ES supernatants during storage may, therefore, provide valuable information regarding potential TRIM effects and the efficiency of leucoreduction.

In this study, we aimed to investigate the changes that occur in ES during the storage period and the relationship between leucoreduction and TRIM. To do this, MNC and BRM-IM that are considered the main cause of TRIM within the product were evaluated in order to detect the changes in Th subgroups and cytokine profiles which occur during storage of ES, their association with TRIM and the relationship between leucoreduction and TRIM.

Materials and methods

Donation and preparation of blood component samples

This study was approved by Uludag University School of Medicine Ethical Board (N. 2011-3/20). Ten units of whole blood were obtained from ten volunteers who met national blood donor selection criteria and were admitted to the "Dr. Raşit Durusoy" Blood Bank at the Uludag University School of Medicine. CPD/SAG-M quadruple paediatric component bags (Kansuk, Istanbul, Turkey) were used to store the blood donations. ES (with additive solution) and fresh plasma were obtained from the whole blood bags. The fresh plasma was not used in this study. NL-ES and LR-ES samples were prepared from ES for 0, 21 and 42 days of storage based on the algorithm shown in Figure 1. First, the ES was divided into two equal parts: one of these parts was then further divided, in three equal portions, into paediatric bags to be stored as the day 0, 21, and 42 NL-ES samples. The other part of the ES was connected under sterile conditions (Sterile Tube Connecting Device; Terumo, Lakewood, CO, USA) to a CPD/SAG-M quadruple paediatric component bag with whole blood filter (Kansuk). However, the main bag was disconnected from the new bag system before connection so that none of the solutions in this new bag system came into contact with the half unit of ES integrated into the system. After the connection procedure, the ES was filtered through the integrated 4 log leucocyte filter (Pall, Portsmouth, UK) and further divided into three equal portions as the LR-ES samples for 0, 21 and 42 days of storage. The NL-ES and LR-ES bags containing samples for storage for 21 and 42 days were stored in blood bank refrigerators (Nuve, Ankara, Turkey), while the day 0 samples were transferred to five test-tubes for laboratory analysis. All of the laboratory analyses were conducted on these samples. The same procedure was performed for laboratory analyses of the related samples stored for 21 and 42 days.

Th lymphocyte subgroups, specific transcription factors and plasma cytokine profiles were investigated in NL-ES samples. Th subgroups and specific transcription factors were not measured in LR-ES samples because the number of leucocytes within the product following 4log leucoreduction is theoretically expected to be insufficient. Only plasma cytokine profiles were evaluated in these samples.

Evaluation of T-helper subgroups in non-leucoreduced erythrocyte suspensions

Th1, Th2, Th9, Th17, Th22 and Treg cells were determined by flow cytometry based on surface markers and intracellular cytokine expression in each NL-ES sample. The monoclonal antibodies used in our study

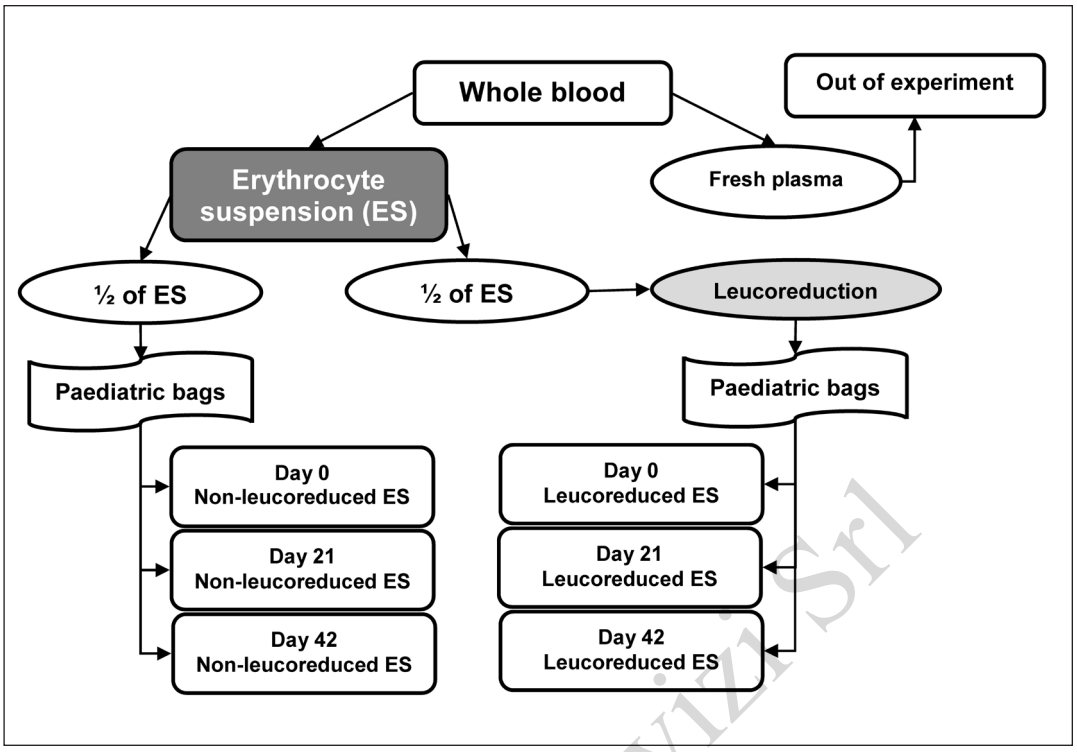

Figure 1 - Algorithm for preparing the samples from whole blood.

were IgG1 FITC, IgG1 APC, IgG1 PE, IgG2A PE, IgG1 AF647/CD3 FITC/CD4 PE (BioLegend, San Diego, CA, USA), IgG1-PE Cy5 (eBioscience, Waltham, MA, USA), CD3 APC, CD4 FITC, IFN-γ PE, IL-4 APC, IL-5 PE, IL-9 PE, IL-21 APC, IL-22 PE, IL-17 AF647 / CD3 FITC / CD4 PE (BioLegend), IL-13 FITC, IL-21 APC, Foxp3 PE Cy5, CD4 FITC / CD25 PE, CD127 APC (eBioscience), CD3 PerCP and CD4 PE (BD Biosciences, San Jose, CA, USA). Stained cells were evaluated using flow cytometry (Navios; Beckman Coulter, Indianapolis, IN, USA).

Evaluation of transcription factors in non-leucoreduced erythrocyte suspensions by polymerase chain reaction

Each NL-ES sample was analysed for the expression of specific transcription factors TBX21, GATA3, PU.1, RORC2, AHR and FOXP3 of Th cell subgroups. This process consisted of MNC isolation, total RNA isolation, complementary DNA (cDNA) synthesis and then real-time polymerase chain reaction (RT-PCR).

MNC were isolated by density gradient using Ficoll (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA). Total RNA was isolated from 5×10^6 cells using a commercially available kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and then used for cDNA synthesis with the commercial First Strand cDNA Synthesis Kit (New England BioLabs Inc, Ipswich, MA, USA). cDNA samples that were obtained were stored in a freezer at −20 °C until RT-PCR analysis.

Real-Time Ready (Roche, Mannheim, Germany) designed for this study were used to measure the expression of specific transcription factors by RT-PCR. Panels consisted of six genes related to the transcription factors (TBX21, GATA3, AHR, SPI.1, FOXP3, RORC2), five reference genes (HPRT1, RPL13A, ACTB, GAPDH, YWHAZ), positive and negative controls. The expression of specific transcription factors was measured using Light-Cycler 480 RT-PCR (Roche). Following the RT-PCR analysis, relative quantification of target gene expression was performed. All data were analysed using the ΔΔCt method.

Measurement of cytokine levels

For cytokine analysis, supernatants were obtained from NL-ES and LR-ES through centrifugation of test-tubes at 3,500 *g* for 10 minutes. The supernatants were then re-centrifuged at 15,000 *g* for 7 minutes to discard cell debris; plasma samples were transferred into clean test-tubes and stored at −80 °C in a freezer (Revco, St. Louis, MO, USA) until use. Interleukin (IL)-4, IL-6, IL-8, IL-9, IL-10, IL-13, IL-17, IL-22, tumour necrosis factor (TNF)-α, transforming-growth factor (TGF)-β and interferon (IFN)-γ levels were measured in these samples using commercial enzyme-linked immunosorbent assay (ELISA) kits (Bio-Legend)

according to the manufacturer's recommendations. Minimum detectable levels of the ELISA were as follows: 3.2 pg/mL for IL-4, 7.8 pg/mL for IL-6, 15.6 pg/mL for IL-8; 6 pg/mL for IL-9, 3.9 pg/mL for IL-10, 15.6 pg/mL for IL-13, 3.9 pg/mL for IL-17A, 62.5 pg/mL for IL-22, 15.6 pg/mL for TNF-α, 7.8 pg/mL for TGF-β, and 15.6 pg/mL for IFN-γ.

Statistical analysis

Continuous variables are presented as medians (range). Between-group comparisons were performed using the Mann-Whitney test, whereas within-group comparisons were performed using Wilcoxon's ranked-sum test. Percent changes (change between last measurement and first measurement divided by first measurement multiplied by 100) in variables that were measured in dependent time periods were also calculated, and the between-group comparisons were performed using the Mann-Whitney test. Statistical analyses were conducted using SPSS v.21 (IBM Corp., New York, NY, USA), and p-values of <0.05 are considered statistically significant.

Results

T-helper cell subgroups

Alterations in Th cell subgroups during the storage of NL-ES are presented in Table I. At day 21, there were reductions in Th cells expressing IFN-γ (p=0.005), IL-22 (p=0.028) and FOXP3 (p=0.034) when compared to day 0. There were increases in the number of Th cells expressing IL-4 (p=0.005), IL-5 (p=0.005) and Th cells that do not express CD127 (p=0.005). At day 42, there were reductions in Th cells expressing IFN-γ (p=0.007) when compared to day 0. There were increases in the number of Th cells expressing IL-4 (p=0.005), IL-5 (p=0.005), IL-17A (p=0.028) and Th cells that do not express CD127 (p=0.005).

Transcription factors

Changes in expression of specific transcription factors during the storage of NL-ES samples are presented in Table II. At day 21, there were reductions in the expression of TBX21 (p=0.005), GATA3 (p=0.005) and SPI.1 (p=0.007) when compared to day 0. At day 42, there were statistically significant reductions in TBX21 (p=0.005), GATA3 (p=0.005) and SPI.1 (p=0.005) gene expression and statistically significant increases in the expression of AHR (p=0.005), FOXP3 (p=0.005) and RORC2 (p=0.005) when compared to day 0.

Cytokine levels

IL-4, IL-6, IL-9, IL-10 and TNF-α could not be detected in any of the samples by ELISA. IL-22 and TGF-β levels were only detected in NL-ES plasma

Table I - Alterations of T-helper cell subgroups in stored erythrocyte suspensions (%).

		Day 0	Day 21	Day 42
$CD3^+CD4^+IFN\text{-}\gamma^+$	median	0.40	*0.00*	*0.00*
	(min:max)	(0.20:1.00)	*(0.00:0.10)*	*(0.00:0.30)*
$CD3^+CD4^+IL\text{-}4^+$	median	0.25	*2.00*	*3.70*
	(min:max)	(0.10:0.70)	*(0.50:5.90)*	*(1.70:11.80)*
$CD3^+CD4^+IL\text{-}5^+$	median	7.70	*20.80*	*44.30*
	(min:max)	(2.40:17.40)	*(2.90:34.20)*	*(20.00:67.30)*
$CD3^+CD4^+IL\text{-}9^+$	median	0.65	0.50	0.30
	(min:max)	(0.20:1.60)	(0.00:1.40)	(0.00:1.20)
$CD3^+CD4^+IL\text{-}13^+$	median	1.95	3.65	4.65
	(min:max)	(1.10:8.60)	(2.30:8.20)	(0.70:8.90)
$CD3^+CD4^+IL\text{-}17A^+$	median	0.90	2.15	*3.90*
	(min:max)	(0.20:2.30)	(0.50:7.50)	*(0.20:17.80)*
$CD3^+CD4^+IL\text{-}21^+$	median	6.65	6.40	6.15
	(min:max)	(1.20:9.00)	(2.50:21.70)	(0.40:20.30)
$CD3^+CD4^+IL\text{-}22^+$	median	1.05	*0.45*	0.55
	(min:max)	(0.50:3.00)	*(0.10:1.00)*	(0.10:6.50)
$CD4^+CD25^+highFoxp3^+$	median	2.70	*0.00*	29.40
	(min:max)	(0.00:24.30)	*(0.00:6.90)*	(0.00:64.80)
$CD4^+CD25^+highCD127^-$	median	0.30	*1.70*	*3.20*
	(min:max)	(0.20:1.60)	*(0.80:5.80)*	*(0.40:33.60)*

Results are presented as percentage rates, showing the median (minimum and maximum) values. p-values <0.05 are considered as statistically significant and significant changes are shown in italic.

samples (Table III). There were no significant changes in IL-8, IL-13 and IFN-γ levels in either NL-ES or LR-ES at day 21 or day 42 when compared to day 0. IL-22 levels were decreased (p=0.043) and TGF-β levels were increased (p=0.008) in NL-ES samples at day 21, when compared to day 0. At day 42, TGF-β levels were increased in NL-ES samples when compared to the day 0 samples. The increase in TGF-β levels was very close to being statistically significant (p=0.051). There was an increase in IL-17A levels in LR-ES samples which

Table II - Alterations in T-cell subgroup-specific transcription factors in stored erythrocyte suspensions.

ΔΔCt		**Day 0**	**Day 21**	**Day 42**
TBX21	median	0.00064	*0.00000*	*0.00023*
	(min:max)	(0.000:0.004)	*(0.000:0.001)*	*(0.000:0.002)*
GATA3	median	0.01750	*0.00147*	*0.00000*
	(min:max)	(0.006:0.027)	*(0.000:0.005)*	*(0.000:0.000)*
AHR	median	0.02410	0.00560	*0.38495*
	(min:max)	(0.011:0.098)	(0.000:0.411)	*(0.336:0.437)*
SPI.1	median	0.25925	*0.03665*	*0.00019*
	(min:max)	(0.167:0.564)	*(0.000:0.212)*	*(0.000:0.001)*
FOXP3	median	0.00273	0.00040	*0.05410*
	(min:max)	(0.002:0.006)	(0.000:0.062)	*(0.042:0.098)*
RORC2	median	0.00131	0.00000	*0.33805*
	(min:max)	(0.000:0.006)	(0.000:0.458)	*(0.232:0.492)*

Results are presented as ΔΔCt values, showing the median (minimum and maximum) values. p values <0.05 are considered as statistically significant and significant changes are shown in italic.

Table III - Alterations of cytokine levels in stored erythrocyte suspensions.

(pg/mL)		**IL-8**		**IL-13**	
		LR-ES	***NL-ES***	***LR-ES***	***NL-ES***
DAY 0	median	25.46	40.01	11.36	13.19
	(min:max)	(17.65:112.60)	(25.46:200.39)	(8.48:173.26)	(4.44:174.64)
DAY 21	median	34.56	63.50	16.05	16.38
	(min:max)	(12.45:102.84)	(27.41:1218.76)	(4.44:639.15)	(0.74:61.62)
DAY 42	median	28.71	36.51	23.28	21.43
	(min:max)	(20.26:495.62)	(27.41:2214.37)	(5.11:877.63)	(0.00:142.68)
		IL-17A		**IL-22**	
		LR-ES	***NL-ES***	***LR-ES***	***NL-ES***
DAY 0	median	5.58	4.24	0.00	3.67
	(min:max)	(2.55:9.78)	(2.93:14.41)	(0.00:0.00)	(0.00:650.46)
DAY 21	median	6.79	5.78	0.00	*0.00*
	(min:max)	(5.25:10.74)	(2.07:12.96)	(0.00:0.00)	*(0.00:2.33)*
DAY 42	median	9.20	*3.66*	0.00	0.00
	(min:max)	(6.30:13.93)	*(1.87:14.70)*	(0.00:0.00)	(0.00:454.40)
		IFN-γ		**TGF-β**	
		LR-ES	***NL-ES***	***LR-ES***	***NL-ES***
DAY 0	median	22.88	23.34	0.00	1.39
	(min:max)	(0.01:161.48)	(0.01:481.48)	(0.00:0.00)	(0.00:62.72)
DAY 21	median	26.60	13.81	0.00	*55.87*
	(min:max)	(0.00:365.67)	(0.55:380.09)	(0.00:0.00)	*(0.00:106.06)*
DAY 42	median	12.65	19.39	0.00	*36.84*
	(min:max)	(0.00:304.74)	(1.48:537.30)	(0.00:0.00)	*(0.00:162.61)*

Results (pg/mL) are presented as the median (minimum and maximum) values. p-values <0.05 are considered as statistically significant and significant changes are shown in italic. LR-ES: leucoreduced erythrocyte suspension; NL-ES: non-leucoreduced erythrocyte suspension; IL-8: interleukin-8; IL-13: interleukin-13; IL-17A: interleukin-17A; IL-22: interleukin-22; IFN-γ: interferon-gamma; TGF-β; transforming growth factor-beta.

was statistically significant (p=0.012). When percent changes in IL-8, IL-13, IL-17A and IFN-γ levels were compared between NL-ES and LR-ES groups, a greater increase in IL-17A levels was noted in LR-ES samples than in NL-ES samples at day 42 (p=0.050). IL-22 and TGF-β levels were excluded from this comparative analysis since these cytokines could not be detected in LR-ES samples.

Discussion

MNC within blood components and the supernatant are two major drivers of the TRIM phenomenon. Storage duration[61,62,72-74,86] and number of transfused products[7,71] are also thought to contribute to this effect. In a breakthrough study by Baumgartner *et al.*, NL-ES and LR-ES supernatants and MNC were mixed in a culture medium and all supernatants were found to induce Treg induction[28]. It was reported that this induction was not associated with leucoreduction, storage duration or cytokines. We investigated the changes in MNC and supernatant within ES over a period of storage and examined the effectiveness of leucoreduction. Differently from Baumgartner *et al.*, we aimed to investigate all Th subgroups and not only Treg cells. Analyses were performed directly on MNC in ES and supernatant rather than cell culture. Moreover, 4log leucocyte filters were used instead of 3log filters during the production of LR-ES.

Changes in T-helper cell profile

Previous studies reported contradictory results regarding the relationship between allogeneic MNC and the TRIM effect, some supporting[7,71,87-89], some against this phenomenon[90-97]. $CD4^+$ Th cells, being the primary cells mediating cellular or humoral immune responses, have been accused of being among those cells that are responsible. These cells develop from naïve Th cells in secondary lymphoid organs in response to specific antigen presentation and cytokine mediation[98].

Effector Th cells are classified in subgroups such as Th1, Th2, Th9, Th17, Th22, and Treg according to their expression of intracellular cytokines under the influence of specific transcription factors. We investigated all these Th subgroups in our study, studying their surface markers, intracellular cytokine profiles and specific transcription factors.

Th1 cells differentiate from naïve Th cells under the influence of the specific transcription factor TBX21 (T-bet), which is expressed following IL-12 and IFN-γ stimulation, and produce high amounts of IFN-γ[99-102]. Significant reductions in TBX21 and $CD3^+CD4^+$IFN-γ^+ levels in NL-ES noted on storage days 21 and 42 may suggest a reduction in the Th1 type response. This finding parallels those of previous studies demonstrating reductions in Th1 type cytokine levels in recipients following allogeneic blood transfusion[39,51,56-59].

Th2 cells differentiate from naïve Th cells under the influence of the specific transcription factor GATA3, which is expressed following IL-4 stimulation, and produce IL-4, IL-5, IL-9, IL-10, and IL-13[99,100,103,104]. Increased numbers of $CD3^+CD4^+$IL-4^+ and $CD3^+CD4^+$IL-5^+ cells suggest that Th2 type cells within the product increased during storage. This result supports the theory that allogeneic blood transfusion leads to TRIM by transforming the recipient's immune response from Th1 to Th2[39,49,51,54-59]. This transformation might lead to TRIM in the recipient as a result of a process which begins within the product. Furthermore, reduction in co-stimulant expression due to an enhanced recipient Th2 response might also be a contributing factor to the TRIM phenomenon[53,59]. However, the inverse relationship between increased intracellular cytokine levels and decreased GATA3 levels that we noted in our study appears to be a paradox. Nevertheless, it is known that IL-4, which is endogenously synthesised as a result of GATA3 activation, is sufficient for the persistence of Th2 differentiation[105]. It is possible that GATA3 is depleted after inducing the IL-4 production necessary for Th2 differentiation, which might explain the above-mentioned paradox.

Th9 cells differentiate from naïve Th cells under the influence of TGF-β and IL-4 or from Th2 cells under the influence of TGF-β[106]. Their specific transcription factor is SPI.1[107]. They mostly produce IL-9[108]. Although Th9 cells appear to be the main source of IL-9, this cytokine is expressed by Th2 and Th17 cells as well[109,110]. The significant reduction in SPI.1 gene expression observed in this study, as well as some reduction in $CD3^+CD4^+$IL-9^+ cell numbers at days 21 and 42 of storage, may indicate that Th9 activity in the product decreases. Considering the significant reduction noted in its transcription factor, the less than expected decrease in intracellular IL-9 might be due to additional expression of IL-9 from Th2 and Th17 cells[109,110].

Th17 cells differentiate from naïve Th cells under the influence of the specific transcription factor RORC2, which is expressed following TGF-β and IL-6 stimulation, and produce IL-17A, IL-17F, and IL-21[111,112]. Significant increases in RORC2 and $CD3^+CD4^+$IL-$17A^+$ levels at storage day 42 suggest an increase in Th17 activity within the product towards the end of the storage period. The increase in Th17 activity and decrease in Th1 activity might be due to the absolute antagonism between these two cell populations[113-115]. It has previously been reported that disturbance of the interaction between Th1 and Th17 cells and, especially, the absence of a Th1 response leads to a stronger autoimmune response in an experimental setting[115]. Reduced Th1 activity and increased Th17 activity may suggest a potential

relationship between NL-ES that have approached the end of the storage period and autoimmunity.

Th22 cells differentiate from naïve Th cells under the influence of the specific transcription factor AHR, which is expressed following IL-6 and TNF-α stimulation, and produce IL-22[104,116,117]. An insignificant decrease in AHR level on storage day 21 and the significant increase in AHR level at day 42, the significant reduction in $CD3^{+}CD4^{+}IL\text{-}22^{+}$ level at day 21 and insignificant reduction in $CD3^{+}CD4^{+}IL\text{-}22^{+}$ level at day 42 all suggest a decrease in Th22 activity within the product during its period of storage.

Treg cells either develop naturally in the thymus (natural Treg; nTreg) or differentiate from naïve T cells in the presence of TGF-β (inducible Treg; iTreg)[99,118]. Treg cells specifically express a transcription factor called FOXP3, and intracellular Foxp3 expression is considered to be the most specific Treg marker[99,118]. However, activated effector T cells may also temporarily express low levels of intracellular FOXP3 under certain conditions[118]. In contrast to these cells, which express high levels of CD127, Treg cells express CD127 at either very low levels or not at all[119-122]. Unlike other Th cells, Treg cells suppress the immune response. Their main functions consist of self-tolerance and establishing and maintaining immune homeostasis. We found that the level of the transcription factor FOXP3 was increased at day 42, the $CD4^{+}CD25^{+high}FOXP3^{+}$ level was decreased at day 21, and the $CD4^{+}CD25^{+high}$ $CD127^{-}$ levels were increased at both days 21 and 42 of storage. These findings suggest that Treg cell activity tends to increase towards the end of the period of storage. There might be a relationship between transfusion of NL-ES approaching the end of its storage period and the immunosuppressive effect of transfusion. It has been demonstrated that the Th2 immune response inhibits Treg differentiation through binding of GATA3 to the FOXP3 promoter region, thus blocking its expression[123]. Our results suggest that, by day 21 of storage, GATA3 may have caused a reduction in Treg activity via inhibition of FOXP3. The increase in FOXP3 and $CD4^{+}CD25^{+high}CD127^{-}$ levels together with the disappearance of GATA3 observed at day 42 support this suggestion.

Cytokines in the supernatant and effectiveness of leucoreduction

Evidence suggests that that several BRM-IM[21-24,27,81,124-128], soluble Fas ligand and soluble HLA class I molecules[6,55,129,130] which accumulate in ES during storage may have a role in TRIM. One of the potential drivers of TRIM effects are cytokines[21-24]. Changes in cytokine levels in ES supernatants during storage and their effects have been previously demonstrated in various studies[28,84,85,129,131,132]. The effectiveness of leucoreduction in the prevention of TRIM is unclear. Contradictory results have been reported in several studies in favour[7,71,89,133,134] and against[6,135-137] its effectiveness. We, therefore, measured cytokine profiles in LR-ES and NL-ES supernatants in order to examine the relationship of these profiles with the TRIM effect.

We measured IL-4, IL-6, IL-8, IL-9, IL-10, IL-13, IL-17A, IL-22, IFN-γ, TNF-α, and TGF-β levels by ELISA in this study. Among the listed cytokines, IL-4, IL-6, IL-9, IL-10 and TNF-α were not detected in any of the samples. This might be due to their rapid degradation, the low sensitivity of the ELISA for their detection, their short half-life or their consumption within the product during the storage period. IL-22 and TGF-β levels were only measurable in NL-ES supernatants.

IL-8, IL-13, IL-17A, IFN-γ levels could be determined in LR-ES; among these only IL-17A was found to be significantly increased at storage day 42. Comparative analysis revealed that this increase tended to be greater than the increase in NL-ES samples which was close to statistical significance. It could be suggested that increased IL-17A in LR-ES might contribute to the TRIM effect by activating recipient Th17 cells following transfusion by weakening the Th1 response[113-115] or by enhancing Th2 activity via transformation into Th17/Th2 cells[138]. If IL-17A does play a role in TRIM, this documented increase in IL-17A in LR-ES might be one of the reasons why leucoreduction is ineffective in preventing post-transfusion immunomodulation. The IL-8, IL-13 and IFN-γ levels together with IL-17A in samples not containing leucocytes were also interesting. It is possible that these cytokines originate either from sources within the product other than leucocytes, or leucocyte-related structures (exosomes, microparticles, etc.) that can permeate the leucocyte filter. However, it does not appear to be rational to expect these sources to produce higher levels of IL-17 in LR-ES samples than NL-ES supernatants containing leucocytes. Our results regarding IL-17A levels do, therefore, need questioning and further confirmation.

In NL-ES supernatants IL-8, IL-13, IL-17A, IL-22, IFN-γ, TGF-β levels could be measured; among these, only a decrease in IL-22 levels and an increase in TGF-β levels were found to be statistically significant at day 21 of storage. The increase in TGF-β levels in day 42 samples was close to statistical significance. This increase in TGF-β levels during the storage process supports previous studies suggesting a relationship between this cytokine and TRIM[28,129]. TGF-β, transferred to the recipient via allogeneic blood transfusion may reach high levels, especially in patients who receive multiple transfusions, and inhibit leucocyte activation or enhance the immunosuppressive features of TRIM

by inducing differentiation of naive Th cells into Treg cells. Therefore, in contrast to the above-mentioned hypothesis, the change in TGF-β levels, which were increased in NL-ES but undetectable in LR-ES supernatants, may be an indicator of the benefit of leucoreduction in preventing TRIM.

In summary, IL-17A and TGF-β accumulation in supernatants may contribute to TRIM via the mechanisms suggested above. This leads to the hypothesis that the TRIM effect could occur not only via allogeneic blood components but also through autologous ones, which have approached the end of their storage period. On the other hand, our comparative analysis suggests that leucoreduction may be effective in the prevention of TRIM. While increased TGF-β levels in NL-ES support this suggestion, the increased level of IL-17A in LR-ES is difficult to interpret. In any case, as direct immunosuppressive effects of TGF-β overcome the potential proposed effects of IL-17A, leucoreduction might ultimately be considered as effective.

Conclusions

In conclusion, significant amounts of molecules and cells are transferred to the recipient via allogeneic blood transfusion. The persistence and activity of these transferred elements within the circulation may predispose to the development of TRIM. Our results suggest that allogeneic leucocytes and cytokines both play roles in the development of TRIM. It appears that leucoreduction may at least be effective in preventing cytokine-mediated TRIM. However, factors other than leucocytes will always limit this preventive effect. Finally, the TRIM phenomenon is not encountered following every transfusion, and it is possible that different mechanisms underlie the effects in different individuals. Numerous product- and recipient-related variables lead to these effects. It should not be forgotten that, in addition to leucocytes in the product and BRM-IM accumulated within the supernatant, erythrocytes, solutions within the bag systems, concomitant diseases and demographic characteristics of the patients receiving blood transfusion may also play roles in the TRIM effect. Valuable additional data could be provided by future studies designed to take all of these factors into account.

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Authorship contributions

SHB and HBO designed the study. SHB, YH and LTK provided the blood samples. SHB and FB performed the experiments. SHB, FB, FG, GG and HBO analysed and interpreted the data, and wrote the manuscript. FG, YH and HBO critically reviewed, edited and approved the manuscript.

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References

1) Opelz G, Sengar DP, Mickey MR, Terasaki PI. Effect of blood transfusions on subsequent kidney transplants. Transplant Proc 1973; **5**: 253-9.
2) Acheson AG, Brookes MJ, Spahn DR. Effects of allogeneic red blood cell transfusions on clinical outcomes in patients undergoing colorectal cancer surgery. Ann Surg 2012; **256**: 235-44.
3) Velásquez JF, Cata JP. Transfusions of blood products and cancer outcomes. Rev Esp Anestesiol Reanim 2015; **62**: 461-7.
4) Bilgin YM, Brand A. Transfusion-related immunomodulation: a second hit in an inflammatory cascade? Vox Sang 2008; **95**: 261-71.
5) Blumberg N, Heal JM. Effects of transfusion on immune function. Cancer recurrence and infection. Arch Pathol Lab Med 1994; **118**: 371-9.
6) Vamvakas EC, Blajchman MA. Transfusion-related immunomodulation (TRIM): an update. Blood Rev 2007; **21**: 327-48.
7) van de Watering LM, Hermans J, Houbiers JG, et al. Beneficial effects of leukocyte depletion of transfused blood on postoperative complications in patients undergoing cardiac surgery: a randomized clinical trial. Circulation 1998; **97**: 562-8.
8) Sloand E, Kumar P, Klein HG, et al. Transfusion of blood components to persons infected with human immunodeficiency virus type 1: relationship to opportunistic infection. Transfusion 1994; **34**: 48-53.
9) Houbiers JG, van de Velde CJ, van de Watering LM, et al. Transfusion of red cells is associated with increased incidence of bacterial infection after colorectal surgery: a prospective study. Transfusion 1997; **37**: 126-34.
10) Chang H, Hall GA, Geerts WH, et al. Allogeneic red blood cell transfusion is an independent risk factor for the development of postoperative bacterial infection. Vox Sang 2000; **78**: 13-8.
11) Peters WR, Fry RD, Fleshman JW, Kodner IJ. Multiple blood transfusions reduce the recurrence rate of Crohn's disease. Dis Colon Rectum 1989; **32**: 749-53.
12) Mowbray JF, Gibbings C, Liddell H, et al. Controlled trial of treatment of recurrent spontaneous abortion by immunisation with paternal cells. Lancet 1985; **1**: 941-3.
13) Heiss MM, Mempel W, Delanoff C, et al. Blood transfusion-modulated tumor recurrence: first results of a randomized study of autologous versus allogeneic blood transfusion in colorectal cancer surgery. J Clin Oncol 1994; **12**: 1859-67.
14) Gantt CL. Red blood cells for cancer patients. Lancet 1981; **2**: 363.
15) Amato A, Pescatori M. Perioperative blood transfusions and recurrence of colorectal cancer. Cochrane Database Syst Rev 2006; **1**: CD005033.
16) Clark DA, Gorczynski RM, Blajchman MA. Transfusion-related immunomodulation due to peripheral blood dendritic cells expressing the CD200 tolerance signaling molecule and alloantigen. Transfusion 2008; **48**: 814-21.
17) Bordin JO, Heddle NM, Blajchman MA. Biologic effects of leukocytes present in transfused cellular blood products. Blood 1994; **84**: 1703-21.
18) Dzik WH. Mononuclear cell microchimerism and the immunomodulatory effect of transfusion. Transfusion 1994; **34**: 1007-12.
19) Beko KR, Tran HO, Hewitt CW, et al. Mechanisms of prior blood transfusion-cyclosporine-induced tolerance: a potential role for immune-cellular chimerism. Transplant Proc 1991; **23**: 147-8.

20) Nielsen HJ, Reimert CM, Pedersen AN, et al. Time-dependent, spontaneous release of white cell- and platelet-derived bioactive substances from stored human blood. Transfusion 1996; **36**: 960-5.
21) Ghio M, Contini P, Negrini S, et al. Down regulation of human natural killer cell-mediated cytolysis induced by blood transfusion: role of transforming growth factor-β(1), soluble Fas ligand, and soluble class I human leukocyte antigen. Transfusion 2011; **51**: 1567-73.
22) Kristiansson M, Soop M, Shanwell A, Sundqvist KG. Prestorage versus bedside white blood cell filtration of red blood cell concentrates: effects on the content of cytokines and soluble tumor necrosis factor receptors. J Trauma 1996; **40**: 379-83.
23) Hodge G, Markus C, Nairn J, Hodge S. Effect of blood storage conditions on leucocyte intracellular cytokine production. Cytokine 2005; **32**: 7-11.
24) Ghio M, Ottonello L, Contini P, et al. Transforming growth factor- 1 in supernatants from stored red blood cells inhibits neutrophil locomotion. Blood 2003; **102**: 1100-7.
25) Silliman CC, Moore EE, Kelher MR, et al. Identification of lipids that accumulate during the routine storage of prestorage leukoreduced red blood cells and cause acute lung injury. Transfusion 2011; **51**: 2549-54.
26) Silliman CC, Voelkel NF, Allard JD, et al. Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model. J Clin Invest 1998; **101**: 1458-67.
27) Grimshaw K, Sahler J, Spinelli SL, et al. New frontiers in transfusion biology: identification and significance of mediators of morbidity and mortality in stored red blood cells. Transfusion 2011; **51**: 874-80.
28) Baumgartner JM, Silliman CC, Moore EE, et al. Stored red blood cell transfusion induces regulatory T cells. J Am Coll Surg 2009; **208**: 110-9.
29) Adamson JW. New blood, old blood, or no blood? N Engl J Med 2008; **358**: 1295-6.
30) Mynster T, Dybkjoer E, Kronborg G, Nielsen HJ. Immunomodulating effect of blood transfusion: is storage time important? Vox Sang 1998; **74**: 176-81.
31) Puppo F, Contini P, Ghio M, et al. Soluble human MHC class I molecules induce soluble Fas ligand secretion and trigger apoptosis in activated CD8(+) Fas (CD95)(+) T lymphocytes. Int Immunol 2000; **12**: 195-203.
32) Magee CC, Sayegh MH. Peptide-mediated immuno-suppression. Curr Opin Immunol 1997; **9**: 669-75.
33) Ghio M, Contini P, Ubezio G, et al. Blood transfusions with high levels of contaminating soluble HLA-I correlate with levels of soluble CD8 in recipients' plasma; a new control factor in soluble HLA-I-mediated transfusion-modulated immunomodulation? Blood Transfus 2014; **12** (Suppl 1): 105-8.
34) Vamvakas EC, Blajchman MA. Deleterious clinical effects of transfusion-associated immunomodulation: fact or fiction? Blood 2001; **97**: 1180-95.
35) Brunson ME, Alexander JW. Mechanisms of transfusion-induced immunosuppression. Transfusion 1990; **30**: 651-8.
36) Blajchman MA, Bordin JO. Mechanisms of transfusion-associated immunosuppression. Curr Opin Hematol 1994; **1**: 457-61.
37) Kaplan J, Sarnaik S, Gitlin J, Lusher J. Diminished helper/suppressor lymphocyte ratios and natural killer activity in recipients of repeated blood transfusions. Blood 1984; **64**: 308-10.
38) Nielsen HJ, Hammer JH, Moesgaard F, Kehlet H. Comparison of the effects of SAG-M and whole-blood transfusions on postoperative suppression of delayed hypersensitivity. Can J Surg 1991; **34**: 146-50.
39) Wood ML, Gottschalk R, Monaco AP. Effect of blood transfusion on IL-2 production. Transplantation 1988; **45**: 930-5.
40) Waymack JP, Gallon L, Barcelli U, Alexander JW. Effect of blood transfusions on macrophage function in a burned animal model. Curr Surg 1986; **43**: 305-7.
41) Blajchman MA, Bardossy L, Carmen R, et al. Allogeneic blood transfusion-induced enhancement of tumor growth: two animal models showing amelioration by leukodepletion and passive transfer using spleen cells. Blood 1993; **81**: 1880-2.
42) Ghio M, Contini P, Mazzei C, et al. Soluble HLA class I, HLA class II, and Fas ligand in blood components: a possible key to explain the immunomodulatory effects of allogeneic blood transfusions. Blood 1999; **93**: 1770-7.
43) Terasaki PI. The beneficial transfusion effect on kidney graft survival attributed to clonal deletion. Transplantation 1984; **37**: 119-25.
44) Naji A. Induction of tolerance by intrathymic inoculation of alloantigen. Curr Opin Immunol 1996; **8**: 704-9.
45) Puppo F, Ghio M, Contini P, et al. Fas, Fas ligand and transfusion immunomodulation. Transfusion 2001; **41**: 416-8.
46) Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998; **281**: 1305-8.
47) Singal DP, Leber B, Harnish DG, et al. Molecular genetic basis for the antiidiotypic antibody response associated with successful renal allograft survival in humans. Transplant Proc 1991; **23**: 1059-61.
48) Mincheff MS, Meryman HT, Kapoor V, et al. Blood transfusion and immunomodulation: a possible mechanism. Vox Sang 1993; **65**: 18-24.
49) Kirkley SA. Proposed mechanisms of transfusion-induced immunomodulation. Clin Diagn Lab Immunol 1999; **6**: 652-7.
50) Hu A, Li Q, Shi H, et al. Donor-derived bone marrow transfusion produces mixed chimerism and promotes a Th2 shift in Th1/Th2 balance in rat heterotopic small bowel transplantation. Dig Liver Dis 2012; **44**: 988-94.
51) Gafter U, Kalechman Y, Sredni B. Blood transfusion enhances production of T-helper-2 cytokines and transforming growth factor beta in humans. Clin Sci 1996; **91**: 519-23.
52) Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol 1991; **146**: 108-13.
53) Perez RV, Swanson C, Morgan M, et al. Portal venous transfusion up-regulates Kupffer cell cyclooxygenase activity: a mechanism of immunosuppression in organ transplantation. Transplantation 1997; **64**: 135-9.
54) Blumberg N, Heal JM. The transfusion immunomodulation theory: the Th1/Th2 paradigm and an analogy with pregnancy as a unifying mechanism. Semin Hematol 1996; **33**: 329-40.
55) Lannan KL, Sahler J, Spinelli SL, et al. Transfusion immunomodulation--the case for leukoreduced and (perhaps) washed transfusions. Blood Cells Mol Dis 2013; **50**: 61-8.
56) Pandey P, Chaudhary R, Aggarwal A, et al. Transfusion-associated immunomodulation: Quantitative changes in cytokines as a measure of immune responsiveness after one time blood transfusion in neurosurgery patients. Asian J Transfus Sci 2010; **4**: 78-85.
57) Zhu X, Yu B, You P, et al. Ubiquitin released in the plasma of whole blood during storage promotes mRNA expression of Th2 cytokines and Th2-inducing transcription factors. Transfus Apher Sci 2012; **47**: 305-11.
58) Guo J-R, Xu F, Jin X-J, et al. Impact of allogenic and autologous transfusion on immune function in patients with tumors. Asian Pac J Cancer Prev 2014; **15**: 467-74.
59) Babcock GF, Alexander JW. The effects of blood transfusion on cytokine production by TH1 and TH2 lymphocytes in the mouse. Transplantation 1996; **61**: 465-8.
60) Feng JF, Chen F, Liu H, Liu J. Induction of immune tolerance by pre-infusion of apoptotic lymphocytes derived from peripheral blood of donor rats before liver transplantation. Minerva Chir 2013; **68**: 183-9.

61) Cheng J, Zhou L, Qin YS, et al. Donor apoptotic lymphocyte transfusion-induced liver allograft tolerance by up-regulation of CD4(+)CD25(+) regulatory T cells in peripheral blood. Transplant Proc 2009; **41**: 3893-7.
62) Dzik WH. Apoptosis, TGF beta and transfusion-related immunosuppression: Biologic versus clinical effects. Transfus Apher Sci 2003; **29**: 127-9.
63) Long K, Meier C, Bernard A, et al. T-cell suppression by red blood cells is dependent on intact cells and is a consequence of blood bank processing. Transfusion 2014; **54**: 1340-7.
64) Cholette JM, Pietropaoli AP, Henrichs KF, et al. Longer RBC storage duration is associated with increased postoperative infections in pediatric cardiac surgery. Pediatr Crit Care Med 2015; **16**: 227-35.
65) Salpeter SR, Buckley JS, Chatterjee S. Impact of more restrictive blood transfusion strategies on clinical outcomes: a meta-analysis and systematic review. Am J Med 2014; **127**: 124-31.
66) Holst LB, Petersen MW, Haase N, et al. Restrictive versus liberal transfusion strategy for red blood cell transfusion: systematic review of randomised trials with meta-analysis and trial sequential analysis. BMJ 2015; **350**: h1354.
67) Woods BI, Rosario BL, Chen A, et al. The association between perioperative allogeneic transfusion volume and postoperative infection in patients following lumbar spine surgery. J Bone Joint Surg Am 2013; **95**: 2105-10.
68) Kriebardis A, Antonelou M, Stamoulis K, Papassideri I. Cell-derived microparticles in stored blood products: innocent-bystanders or effective mediators of post-transfusion reactions? Blood Transfus 2012; **10** (Suppl 2): 25-38.
69) Sadallah S, Eken C, Schifferli JA. Erythrocyte-derived ectosomes have immunosuppressive properties. J Leukoc Biol 2008; **84**: 1316-25.
70) Lion N, Crettaz D, Rubin O, Tissot J-D. Stored red blood cells: a changing universe waiting for its map(s). J Proteomics 2010; **73**: 374-85.
71) Bilgin YM, van de Watering LMG, Eijsman L, et al. Double-blind, randomized controlled trial on the effect of leukocyte-depleted erythrocyte transfusions in cardiac valve surgery. Circulation 2004; **109**: 2755-60.
72) Purdy FR, Tweeddale MG, Merrick PM. Association of mortality with age of blood transfused in septic ICU patients. Can J Anaesth 1997; **44**: 1256-61.
73) Zallen G, Offner PJ, Moore EE, et al. Age of transfused blood is an independent risk factor for postinjury multiple organ failure. Am J Surg 1999; **178**: 570-2.
74) Koch CG, Li L, Sessler DI, et al. Duration of red-cell storage and complications after cardiac surgery. N Engl J Med 2008; **358**: 1229-39.
75) Hendrickson JE, Hod EA, Spitalnik SL, et al. Storage of murine red blood cells enhances alloantibody responses to an erythroid-specific model antigen. Transfusion 2010; **50**: 642-8.
76) Hendrickson JE, Hod EA, Hudson KE, et al. Transfusion of fresh murine red blood cells reverses adverse effects of older stored red blood cells. Transfusion 2011; **51**: 2695-702.
77) Yazdanbakhsh K, Bao W, Zhong H. Immunoregulatory effects of stored red blood cells. Hematol Am Soc Hematol Educ Progr 2011; **2011**: 466-9.
78) Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 2010; **10**: 490-500.
79) Brusko TM, Wasserfall CH, Agarwal A, et al. An integral role for heme oxygenase-1 and carbon monoxide in maintaining peripheral tolerance by CD4+CD25+ regulatory T cells. J Immunol 2005; **174**: 5181-6.
80) Chauveau C, Rémy S, Royer PJ, et al. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. Blood 2005; **106**: 1694-702.
81) Cairo G, Pietrangelo A. Iron regulatory proteins in pathobiology. Biochem J 2000; **352**: 241-50.
82) Hod EA, Zhang N, Sokol SA, et al. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood 2010; **115**: 4284-92.
83) Atzil S, Arad M, Glasner A, et al. Blood transfusion promotes cancer progression: a critical role for aged erythrocytes. Anesthesiology 2008; **109**: 989-97.
84) Weisbach V, Wanke C, Zingsem J, et al. Cytokine generation in whole blood, leukocyte-depleted and temporarily warmed red blood cell concentrates. Vox Sang 1999; **76**: 100-6.
85) Stack G, Baril L, Napychank P, Snyder EL. Cytokine generation in stored, white cell-reduced, and bacterially contaminated units of red cells. Transfusion 1995; **35**: 199-203.
86) Offner PJ, Moore EE, Biffl WL, et al. Increased rate of infection associated with transfusion of old blood after severe injury. Arch Surg 2002; **137**: 711.
87) Opelz G, Vanrenterghem Y, Kirste G, et al. Prospective evaluation of pretransplant blood transfusions in cadaver kidney recipients. Transplantation 1997; **63**: 964-7.
88) Wallis JP, Chapman CE, Orr KE, et al. Effect of WBC reduction of transfused RBCs on postoperative infection rates in cardiac surgery. Transfusion 2002; **42**: 1127-34.
89) Blumberg N, Zhao H, Wang H, et al. The intention-to-treat principle in clinical trials and meta-analyses of leukoreduced blood transfusions in surgical patients. Transfusion 2007; **47**: 573-81.
90) Collier AC, Kalish LA, Busch MP, et al. Leukocyte-reduced red blood cell transfusions in patients with anemia and human immunodeficiency virus infection: the Viral Activation Transfusion Study: a randomized controlled trial. JAMA 2001; **285**: 1592-601.
91) Hiesse C, Busson M, Buisson C, et al. Multicenter trial of one HLA-DR-matched or mismatched blood transfusion prior to cadaveric renal transplantation. Kidney Int 2001; **60**: 341-9.
92) van Hilten JA, van de Watering LM, van Bockel JH, et al. Effects of transfusion with red cells filtered to remove leucocytes: randomised controlled trial in patients undergoing major surgery. BMJ 2004; **328**: 1281.
93) Nathens AB, Nester TA, Rubenfeld GD, et al. The effects of leukoreduced blood transfusion on infection risk following injury: a randomized controlled trial. Shock 2006; **26**: 342-7.
94) Vamvakas EC. White-blood-cell-containing allogeneic blood transfusion and postoperative infection or mortality: an updated meta-analysis. Vox Sang 2007; **92**: 224-32.
95) Lange MM, van Hilten JA, van de Watering LM, et al. Leucocyte depletion of perioperative blood transfusion does not affect long-term survival and recurrence in patients with gastrointestinal cancer. Br J Surg 2009; **96**: 734-40.
96) van de Watering LM, Brand A, Houbiers JG, et al. Perioperative blood transfusions, with or without allogeneic leucocytes, relate to survival, not to cancer recurrence. Br J Surg 2001; **88**: 267-72.
97) Skånberg J, Lundholm K, Haglind E. Effects of blood transfusion with leucocyte depletion on length of hospital stay, respiratory assistance and survival after curative surgery for colorectal cancer. Acta Oncol 2007; **46**: 1123-30.
98) Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. Annu Rev Immunol 2010; **28**: 445-89.
99) Jäger A, Kuchroo VK. Effector and regulatory T-cell subsets in autoimmunity and tissue inflammation. Scand J Immunol 2010; **72**: 173-84.
100) Wan YY. Multi-tasking of helper T cells. Immunology 2010; **130**: 166-71.
101) Yamane H, Paul WE. Memory CD4+ T cells: fate determination, positive feedback and plasticity. Cell Mol Life Sci 2012; **69**: 1577-83.
102) Romagnani S. Lymphokine production by human T cells in disease states. Annu Rev Immunol 1994; **12**: 227-57.

103) Parronchi P, De Carli M, Manetti R, et al. IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. J Immunol 1992; **149**: 2977-83.
104) Noelle RJ, Nowak EC. Cellular sources and immune functions of interleukin-9. Nat Rev Immunol 2010; **10**: 683-7.
105) Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. J Exp Med 2005; **202**: 793-804.
106) Schmitt E, Germann T, Goedert S, et al. IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. J Immunol 1994; **153**: 3989-96.
107) Chang H-C, Sehra S, Goswami R, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. Nat Immunol 2010; **11**: 527-34.
108) Cosmi L, Maggi L, Santarlasci V, et al. T helper cells plasticity in inflammation. Cytometry A 2014; **85**: 36-42.
109) Elyaman W, Bradshaw EM, Uyttenhove C, et al. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. Proc Natl Acad Sci U S A 2009; **106**: 12885-90.
110) Nowak EC, Weaver CT, Turner H, et al. IL-9 as a mediator of Th17-driven inflammatory disease. J Exp Med 2009; **206**: 1653-60.
111) Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor-β induces development of the TH17 lineage. Nature 2006; **441**: 231-4.
112) Veldhoen M, Hocking RJ, Atkins CJ, et al. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006; **24**: 179-89.
113) Nakae S, Iwakura Y, Suto H, Galli SJ. Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. J Leukoc Biol 2007; **81**: 1258-68.
114) Tanaka K, Ichiyama K, Hashimoto M, et al. Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-gamma on STAT3 and Smads. J Immunol 2008; **180**: 3746-56.
115) Peck A, Mellins ED. Plasticity of T-cell phenotype and function: the T helper type 17 example. Immunology 2010; **129**: 147-53.
116) Duhen T, Geiger R, Jarrossay D, et al. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat Immunol 2009; **10**: 857-63.
117) Eyerich S, Eyerich K, Pennino D, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J Clin Invest 2009; **119**: 3573-85.
118) Fritzsching E, Kunz P, Maurer B, et al. Regulatory T cells and tolerance induction. Clin Transplant 2009; **23**: 10-4.
119) Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006; **203**: 1701-11.
120) Banham AH. Cell-surface IL-7 receptor expression facilitates the purification of FOXP3(+) regulatory T cells. Trends Immunol 2006; **27**: 541-4.
121) Kang SM, Tang Q, Bluestone JA. $CD4^+$ $CD25^+$ regulatory T cells in transplantation: progress, challenges and prospects. Am J Transplant 2007; **7**: 1457-63.
122) Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006; **203**: 1693-700.
123) Mantel P-Y, Kuipers H, Boyman O, et al. GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. PLoS Biol 2007; **5**: e329.
124) Theurl I, Fritsche G, Ludwiczek S, et al. The macrophage: a cellular factory at the interphase between iron and immunity for the control of infections. Biometals 2005; **18**: 359-67.
125) Schaible UE, Kaufmann SHE. Iron and microbial infection. Nat Rev Microbiol 2004; **2**: 946-53.
126) Walker EM, Walker SM. Effects of iron overload on the immune system. Ann Clin Lab Sci 2000; **30**: 354-65.
127) Jacobi KE, Wanke C, Jacobi A, et al. Determination of eicosanoid and cytokine production in salvaged blood, stored red blood cell concentrates, and whole blood. J Clin Anesth 2000; **12**: 94-9.
128) Silliman CC, Clay KL, Thurman GW, et al. Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase. J Lab Clin Med 1994; **124**: 684-94.
129) Hodge GL, Hodge SJ, Nairn J, et al. Poststorage leuko-depleted plasma inhibits T-cell proliferation and Th1 response in vitro: characterization of TGFbeta-1 as an important immunomodulatory component in stored blood. Transplantation 2005; **80**: 95-101.
130) Cata JP, Wang H, Gottumukkala V, et al. Inflammatory response, immunosuppression, and cancer recurrence after perioperative blood transfusions. Br J Anaesth 2013; **110**: 690-701.
131) Shanwell A, Kristiansson M, Remberger M, Ringdén O. Generation of cytokines in red cell concentrates during storage is prevented by prestorage white cell reduction. Transfusion 1997; **37**: 678-84.
132) Karam O, Tucci M, Toledano BJ, et al. Length of storage and in vitro immunomodulation induced by prestorage leukoreduced red blood cells. Transfusion 2009; **49**: 2326-34.
133) Friese RS, Sperry JL, Phelan HA, Gentilello LM. The use of leukoreduced red blood cell products is associated with fewer infectious complications in trauma patients. Am J Surg 2008; **196**: 56-61.
134) Hébert PC, Fergusson D, Blajchman MA, et al. Clinical outcomes following institution of the Canadian universal leukoreduction program for red blood cell transfusions. JAMA 2003; **289**: 1941-9.
135) Vamvakas EC. Meta-analysis of randomized controlled trials investigating the risk of postoperative infection in association with white blood cell-containing allogeneic blood transfusion: the effects of the type of transfused red blood cell product and surgical setting. Transfus Med Rev 2002; **4**: 304-14.
136) Vamvakas EC, Blajchman MA. Prestorage versus poststorage white cell reduction for the prevention of the deleterious immunomodulatory effects of allogeneic blood transfusion. Transfus Med Rev 2000; **14**: 23-33.
137) Phelan HA, Sperry JL, Friese RS. Leukoreduction before red blood cell transfusion has no impact on mortality in trauma patients. J Surg Res 2007; **138**: 32-6.
138) Cosmi L, Maggi L, Santarlasci V, et al. Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. J Allergy Clin Immunol 2010; **125**: 222-30e1-4.

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