Primary T-cell responses against SARS-CoV-2 in patients with hematological disorders

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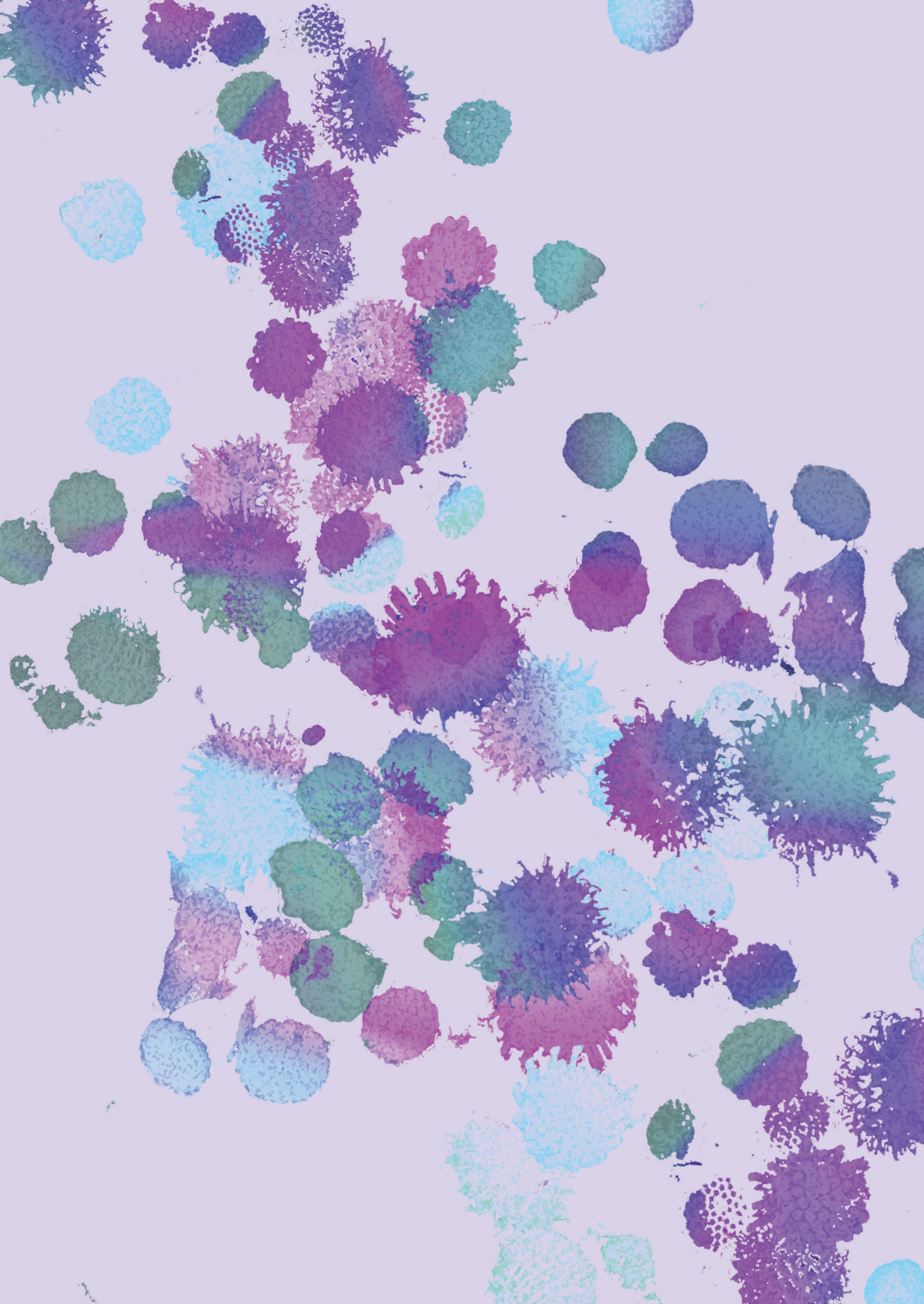

SARS-COV-2 MRNA VACCINATION OF APLASTIC ANEMIA PATIENTS IS SAFE AND EFFECTIVE

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(2022). *Am J Hematol*. DOI: 10.1002/ajh.26780

Chapter 3

To the editor,
Vaccines are an essential part of the fight against the COVID-19 pandemic. Especially immunocompromised patients at risk for a severe or fatal course of SARS-CoV-2 infection are expected to benefit from vaccination. While studies on SARS-CoV-2 mRNA vaccines have shown that healthy subjects are able to mount both effective humoral and cellular immune responses to these vaccines,(1) the effectiveness and safety of SARS-CoV-2 vaccines for immunocompromised patients remains unclear. Acquired aplastic anemia (AA) is an example of a disease that results in an immunocompromised state. AA patients are immunocompromised either due to the disease itself which is characterized by profound pancytopenia caused by immune mediated bone marrow failure, or due to the immunosuppressive treatment (IST) consisting of horse-derived anti-thymocyte globulin (hATG) in combination with cyclosporine A (CsA) that they received.(2) This immunocompromised state of AA patients argues that it is important to vaccinate these patients with a SARS-CoV-2 mRNA vaccine in order to prevent severe COVID-19. However, anecdotal case studies have reported AA relapse after vaccination and therefore the international guidelines recommend caution when vaccinating AA patients after IST irrespective of the time between last IST and vaccination.(3) Furthermore, it is not known whether previous IST affects the ability to mount an adequate immune response to a vaccine in these patients. These considerations create a dilemma whether to vaccinate AA patients after IST with SARS-CoV-2 mRNA vaccines.

In this study we investigated the occurrence of relapse as well as the ability to mount both a humoral and T-cell response to SARS-CoV-2 mRNA vaccination in 18 AA patients treated with IST at a median time of 11.1 years (range 0.3-39) before SARS-CoV-2 vaccination (**Table S1**). At the time of vaccination, 14 AA patients were transfusion-independent and successfully tapered from IST. Three patients were transfusion-independent but IST-dependent, and one patient was both transfusion- and IST-dependent. All IST-dependent patients (N=4) received CsA at time of vaccination. The AA patients and healthy controls (HCs; N=9) received two SARS-CoV-2 mRNA vaccines (mRNA-1273 (Moderna) or BNT162b2 (Pfizer-BioNtech) vaccines). Whole blood was sampled prior and post vaccination to measure blood counts, and to isolate serum and peripheral blood mononuclear cells (PBMCs) to measure SARS-CoV-2-specific IgG antibodies and T-cells (see **supplementary material and methods**).

To investigate whether AA patients relapsed after SARS-CoV-2 mRNA vaccination, hemoglobin (Hb), thrombocyte, and neutrophil values were determined in peripheral blood. Samples were taken pre-vaccination, post-vaccination (median 27 days after the second vaccination) and at follow-up (median 9.1 months after the first vaccination).

The blood values were stable post-vaccination and remained stable without the need for transfusion during the follow-up period in all 17 patients that were transfusion-independent at start of the study (**Figure 1A**). The transfusion-frequency remained stable in the patient that was transfusion-dependent at the start of the study. These results indicate that no signs of AA relapse are present up to 9 months after first vaccination, which is in accordance with a previous study investigating mRNA vaccination in 16 AA patients.(4) This suggests that the case reports describing AA relapse observed after vaccination may be rare incidents or incidents unrelated to vaccination.

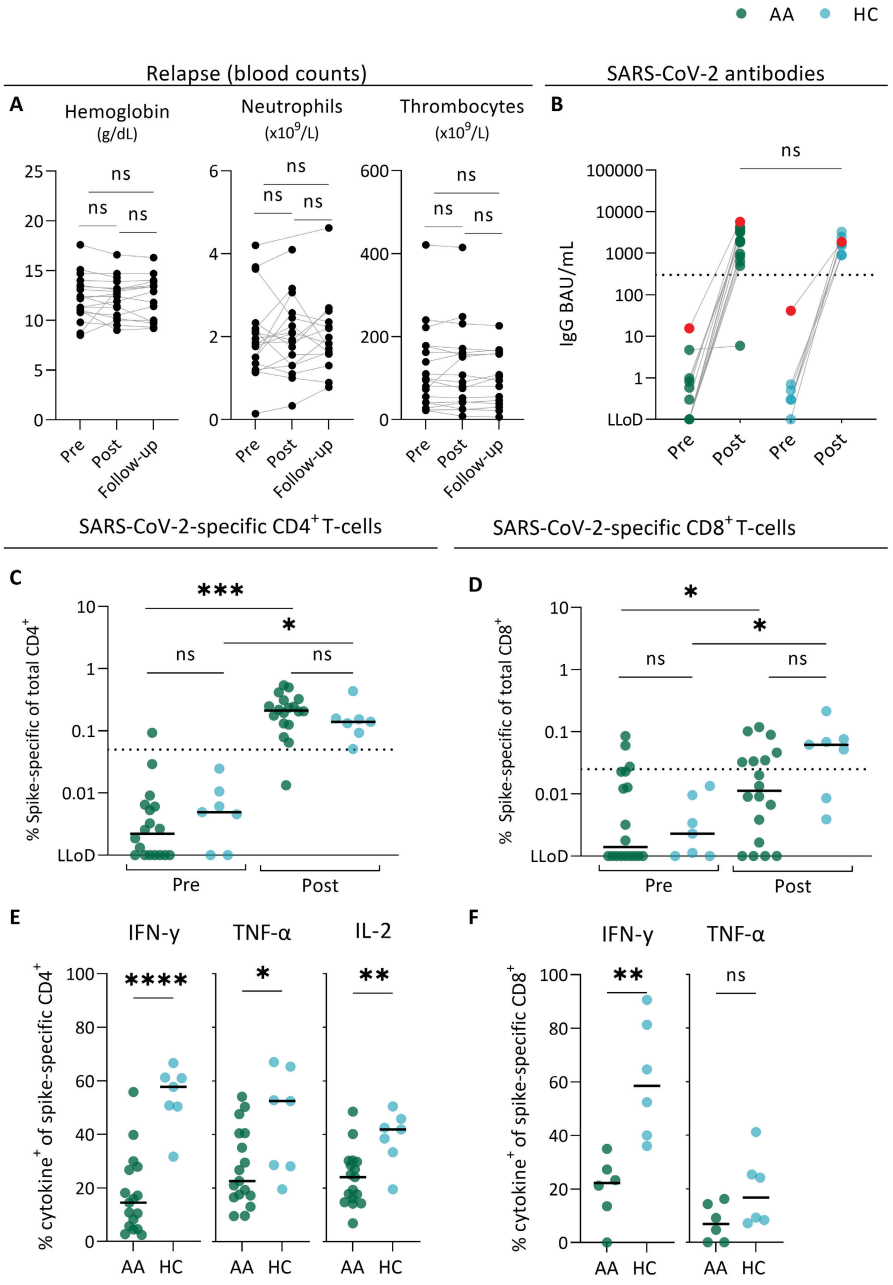

Figure 1 Blood counts, humoral responses and T-cell responses following SARS-CoV-2 mRNA vaccination in aplastic anemia patients and healthy controls

(A) Hemoglobin, neutrophils and thrombocytes shown pre-vaccination, post vaccination (median 27.1 days after second vaccination) and at follow-up (median 9.1 months after start vaccination). Blood value data at follow-up was not available for 3 patients, therefore the statistical comparisons of pre/post with follow-up blood values was only performed for the 15 AA patients for whom data was available. (B) SARS-CoV-2 spike IgG response according to WHO standardization of AA patients (green; n=18) and HCs (light blue; n=9). The red dots correspond to individuals that were positive for SARS-CoV-2 IgG before vaccination. Post-vaccination SARS-CoV-2 spike IgG levels were determined in serum of AA patients (median 27.1 days (range 11-49)) and HCs (median 21.4 days (range 18-24)) after second vaccination. Dotted line shows threshold of an adequate IgG response of 300 BAU/mL. (C) Percentage of SARS-CoV-2 spike-specific $CD4^+$ T-cells of total $CD4^+$ T-cells pre- and post-vaccination in AA patients (green) and HC (light blue). Dotted line shows a threshold for a $CD4^+$ T-cell response of 0.05%. (D) Percentage of SARS-CoV-2 spike-specific $CD8^+$ T-cells of total $CD8^+$ T-cells pre- and post-vaccination in AA patients (green) and HC (light blue). The percentage of SARS-CoV-2 spike-specific CD4+ and CD8+ T-cells was corrected for the background signal in the negative control (DMSO). Dotted line shows a threshold for a $CD8^+$ T-cell response of 0.025%. (E) The percentages of IFN-γ, TNF-α and IL-2 producing spike-specific CD4+ T-cells in AA patients (green) and HC (light blue). (F) The percentages of IFN-γ and TNF-α producing spike-specific $CD8^+$ T-cells in AA patients (green) and HC (light blue). Horizontal bars in figures C-F represent the median. ns: $p>0.05$, *: $p\leq0.05$, **: $p\leq0.01$, ***: $p\leq0.001$
Abbreviations: AA, aplastic anemia; HC, healthy controls; ns, not significant; LLoD, Lowest limit of detection; BAU, binding antibody units; DMSO, dimethyl sulfoxide; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL-2, interleukin 2.

The humoral immune response of AA patients to SARS-CoV-2 mRNA vaccination was measured by determining SARS-CoV-2 anti-Spike IgG levels pre- and post-vaccination. 17 of 18 AA patients had an adequate SARS-CoV-2 IgG antibody response (defined as >300 BAU/ml) after vaccination which was similar to HCs (**Figure 1B**). The patient with antibody levels below threshold had recently received hATG, still received CsA, and was the oldest person (79 years) in the AA patient cohort. An inversed correlation between age and Spike-IgG was found (**Table S2**), indicating that the amount of Spike-IgG decreased with increasing age. For other factors, such as time between IST (hATG treatment) and vaccination, absolute number of B-cells, absolute number of $CD4^+$ and $CD8^+$ T-cells, no significant correlations were observed (**Table S2**). In short, the majority of AA patients is able to generate an adequate antibody response and which is accordance with previous literature.(4)

Spike-specific $CD4^+$ and $CD8^+$ T cell responses were measured by incubating PBMCs with a SARS-CoV-2 spike peptide pool, followed by intracellular cytokine staining for flow cytometry. The frequency of SARS-CoV-2 spike-specific $CD4^+$ and $CD8^+$ T -cells was determined before and after vaccination which showed a significant increase in both AA patients and healthy controls (**Figure 1C-D**). The SARS-CoV-2 spike-specific $CD4^+$ and $CD8^+$ T-cell frequencies between AA patients and HCs were not significantly different after vaccination, although a trend towards a lower frequency of SARS-CoV-2 specific $CD8^+$ T-cells in AA patients could be observed (**Figure 1C-D**). As expected, the $CD4^+$ and $CD8^+$ T-cell responses directed against the broad cytomegalovirus, Epstein-barr virus, influenza and extended peptide pool (CEFX) did not differ pre- and post-vaccination in AA patients and HCs, and frequencies of CEFX-specific $CD4^+$ and $CD8^+$

T-cell were comparable for both cohorts (**Figure S1A-B**). Percentages of SARS-CoV-2 spike-specific $CD4^+$ and $CD8^+$ T-cells that produce interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) or interleukin-2 (IL-2) were significantly lower in the AA patients than in healthy controls (**Figure 1E-F**). Interestingly, this trend of reduced cytokine production was also observed for the CEFX-specific $CD4^+$ T-cells in AA patients that produced significantly reduced levels of TNF-α and IL-2 compared to healthy controls (**Figure S1C-D**). In conclusion, spike-specific $CD4^+$ and $CD8^+$ T cell frequencies were comparable between AA patients and healthy controls. However, the percentage of spike-specific $CD4^+$ and $CD8^+$ T cells that produced IFN-γ, TNF-α or IL-2 was lower in AA patients compared to healthy controls.

Reduced T-cell cytokine production can be caused by multiple factors. Age, time between IST (hATG treatment) and vaccination and absolute numbers of the $CD4^+$ and $CD8^+$ T-cell compartment at the time of vaccination were not significantly correlated to the reduced cytokine production seen after IST (**Table S2**). Since CsA is a known inhibitor of T-cell proliferation and cytokine production, we investigated whether CsA could be responsible for the decreased cytokine production of the SARS-CoV-2 specific T-cells. Although the frequency of $CD4^+$ and $CD8^+$ SARS-CoV-2 spike-specific T-cells was comparable between AA patients who received CsA at time of vaccination and AA patients who did not receive CsA at time of vaccination, we observed that 3 AA patients who received CsA at time of vaccination tended to have lower percentages of IFN-γ, TNF-α and IL-2 producing SARS-CoV-2 spike-specific $CD4^+$ T-cells (**Figure S2A**). Interestingly, these AA patients tended to have higher spike-IgG antibody levels (median: 3431 BAU/mL) compared to patients who no longer received CsA (median: 1912 BAU/mL) at the time of vaccination (**Figure S2B**). Due to the low number of patients that received CsA at time of vaccination (n=3) both trends could not be statistically confirmed.

For the AA patients that did not receive CsA during vaccination we cannot fully explain the lower percentage of cytokine producing SARS-CoV-2 spike-specific T-cells in comparison to HCs. We cannot exclude the possibility that the reduced cytokine production is the result of a lingering effect of the disease or the IST these patients have received. Although no correlation was found between the spike-specific T-cell response and time that patients last received hATG or CsA, hATG or CsA may have had a permanent effect on the repertoire of the T lymphocytes. Based on the analyses of the major T lymphocyte subsets, no obvious difference could be detected (**Figure S3**). However, it is also possible that the difference is more subtle and could therefore not be detected based on the T-cell markers used in this study and the sample size of the study population. Importantly, it is not known whether the reduced cytokine

production influences the effectiveness of vaccines in AA patients and whether this might increase by additional vaccination doses.

In summary, no indications of AA relapse was observed up to 9 months after the first mRNA vaccination. Additionally, 17 of 18 AA patients were able to mount an adequate humoral response and demonstrated comparable magnitudes of spike-specific $CD4^+$ T-cells and spike-specific $CD8^+$ T-cells. Our study sheds another light on the current view on the risk/benefit discussion for vaccination of AA patients as the results indicate that SARS-CoV-2 mRNA vaccines are more beneficial to AA patients than potentially harmful. The reduced cytokine production by the T-cells further underlines the importance of vaccinating AA patients to protect against a possible severe course of SARS-CoV-2 infection. Larger cohort studies are needed to further study the chance of AA relapse after SARS-CoV-2 mRNA vaccination and vaccine efficacy in AA patients not only after successfully tapered IST but also in AA patients recently treated with hATG who are still using CsA. Furthermore, it has to be determined whether additional vaccination doses result in improved cytokine production by spike-specific T-cells which could affect the vaccination scheme for AA patients.

ACKNOWLEDGEMENTS

Flow cytometry was performed at the Flow cytometry Core Facility (FCF) of Leiden University Medical Center (LUMC) in Leiden, the Netherlands.

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SUPPLEMENTARY MATERIAL AND METHODS

Patients and healthy controls

18 patients with AA and 9 healthy controls (HC) were recruited. AA patients were diagnosed and treated with first-line IST consisting of hATG (either ATGAM (Pfizer) or lymphoglobulin (Sanofi)) in combination with CsA according to the Dutch guidelines. Peripheral blood samples were collected after informed consent was given in accordance to local ethical guidelines and the Declaration of Helsinki. This study was approved by the Medical Ethical Committee of the Leiden University Medical Center (Protocol number: B22.029). Patients and HCs below 18 years of age were excluded. The patients received two SARS-CoV-2 mRNA vaccines (either the mRNA-1273 (Moderna) or the BNT162b2 (Pfizer-Biontech)) vaccines. The AA patients and the HCs were age- and sex-matched as much as possible. See Table S1 for patient characteristics.

SARS-CoV-2-specific IgG

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Isopaque and cryo-preserved until further use. Serum was collected from fresh blood and subsequently stored at -80°C. The humoral response to the SARS-CoV-2 mRNA vaccination was measured in serum using a commercial chemiluminescent microparticle immunoassay (CMIA); AdviseDx SARS-CoV-2 Spike immunoglobulin G (IgG) II (Abbott Alinity). An adequate antibody response was defined as a result above >300 BAU/ml anti-spike IgG which was based on neutralization capacity in a previous study.(5)

SARS-CoV-2-specific T-cells

SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T-cells were measured as previously described. (6) In short, PBMCs were overnight incubated with a 15-mer with 11 amino acid overlapping SARS-CoV-2 spike peptide pool (SB-peptide) or a CEFX peptide pool (JPT/LUMC) consisting of peptides from cytomegalovirus (CMV), Epstein-barr virus (EBV), influenza and other common pathogens(**Table S3-4**). After overnight incubation in the presence of Brefeldin A (Sigma), the cells were intracellularly stained for activation markers and cytokines for flow cytometry measurement (**Table S5**). SARS-CoV-2-specific $CD4^+$ T-cells were detected by increased expression of CD137 and/or CD154 compared to negative control (DMSO), and SARS-CoV-2-specific $CD8^+$ T-cells as increased CD137 and CD69 expression compared to negative control (**Figure S4**). In parallel, PBMCs were incubated without the presence of stimulators to measure differentiation state of the T-cells (CD45RA and/or CCR7 expression). A $CD4^+$ T-cell response was considered positive above 0.05% and a $CD8^+$ T-cell response above 0.025%. These percentages were based on the healthy cohort in this study.

SUPPLEMENTARY FIGURES

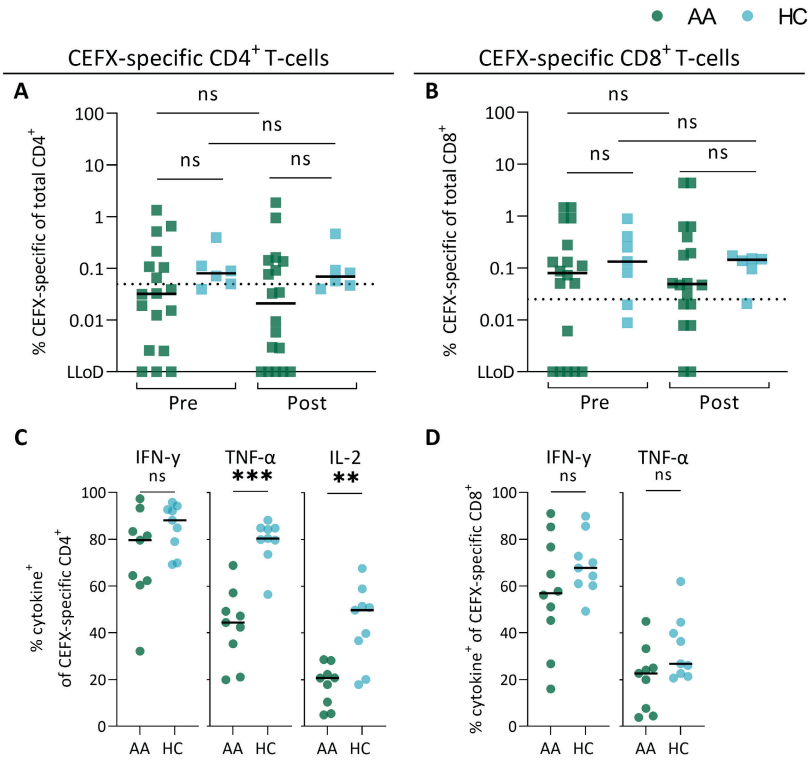

Figure S1 CEFX-specific T-cell responses in aplastic anemia patients and healthy controls

(A) Percentage of CEFX-specific $CD4^+$ T-cells of total $CD4^+$ T-cells pre- and post-vaccination in AA patients (green) and HC (light blue). Dotted line shows a threshold for a $CD4^+$ T-cell response of 0.05%. (B) Percentage of CEFX-specific $CD8^+$ T-cells of total $CD8^+$ T-cells pre- and post-vaccination in AA patients (green) and HC (light blue). The percentages of CEFX-specific CD4+ and CD8+ T-cells were corrected for the background signal in the negative control (DMSO). Dotted line shows a threshold for a $CD8^+$ T-cell response of 0.025%. (C) The percentages of IFN-γ, TNF-α and IL-2 producing CEFX-specific CD4+ T-cells in AA patients (green) and HC (light blue). (D) The percentages of IFN-γ and TNF-α producing CEFX-specific $CD8^+$ T-cells in AA patients (green) and HC (light blue). Horizontal bars in figures C-F represent the median. ns: $p>0.05$, *: $p\leq0.05$, **: $p\leq0.01$, ***: $p\leq0.001$

Abbreviations: AA, aplastic anemia; HC, healthy controls; ns, not significant; LLoD, Lowest limit of detection; DMSO, dimethyl sulfoxide; CEFX: Cytomegalovirus, Epstein-barr virus, influenza and extended peptide pool; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL-2, interleukin 2.

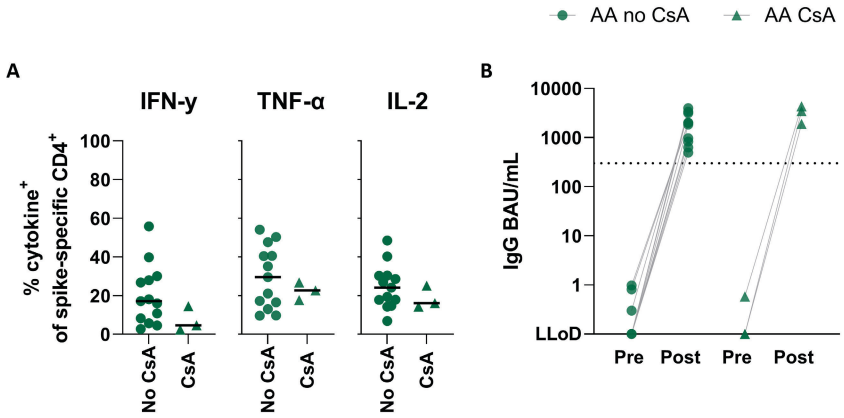

Figure S2 Humoral and T-cell response during SARS-CoV-2 vaccination in AA patients treated with Csa compared to patients not treated with CsA

(A) The percentages of type 1 cytokine (TNF-α, IFN-γ, IL-2) producing SARS-CoV-2 spike-specific $CD4^+$ T-cells of AA patients who did not receive CsA (No CsA: green circle) and AA patients who received CsA (CsA; green triangle) at time of vaccination. (B) SARS-CoV-2 spike IgG response of AA patients who did not receive CsA and who received CsA at time of vaccination. AA patients who were Spike-IgG positive pre-vaccination were excluded from these comparisons. Percentages of cytokine-producing $CD4^+$ T-cells and antibody titers were determined at median 27.1 days (11-49 days) after the second vaccination. Dotted line shows threshold of an adequate IgG response of 300 BAU/ml.

Abbreviations: AA, aplastic anemia; CsA, cyclosporin A; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL-2, interleukin 2; LLoD, lower limit of detection.

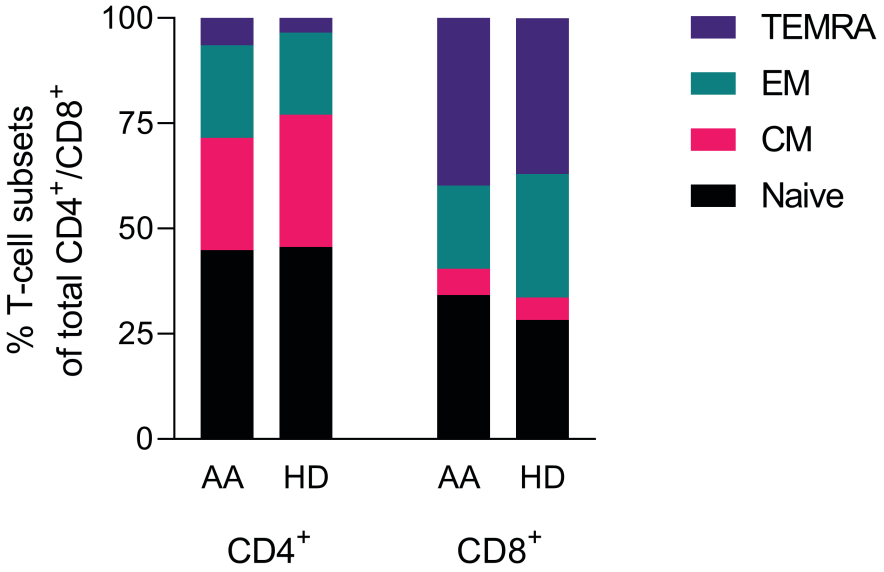

Figure S3 CD4⁺ and CD8⁺ T-cell subset frequencies in AA patients (n=18) and HC's (n=8) before vaccination

Median T-cell subset frequencies are shown. No significant differences between AA patients and HCs in the T-cell subsets could be found. Naïve T-cell subset is defined as $CCR7^{+}CD45RA^{+}$. CM T-cell subset is defined as $CCR7^{+}CD45RA^{-}$. EM T-cell subset is defined as $CCR7^{-}CD45RA^{-}$. TEMRA T-cell subset is defined as $CCR7^{-}CD45RA^{+}$.

Abbreviations: AA, aplastic anemia; HC, healthy controls; CM, central memory; EM, effector memory; TEMRA, terminally differentiated effector memory.

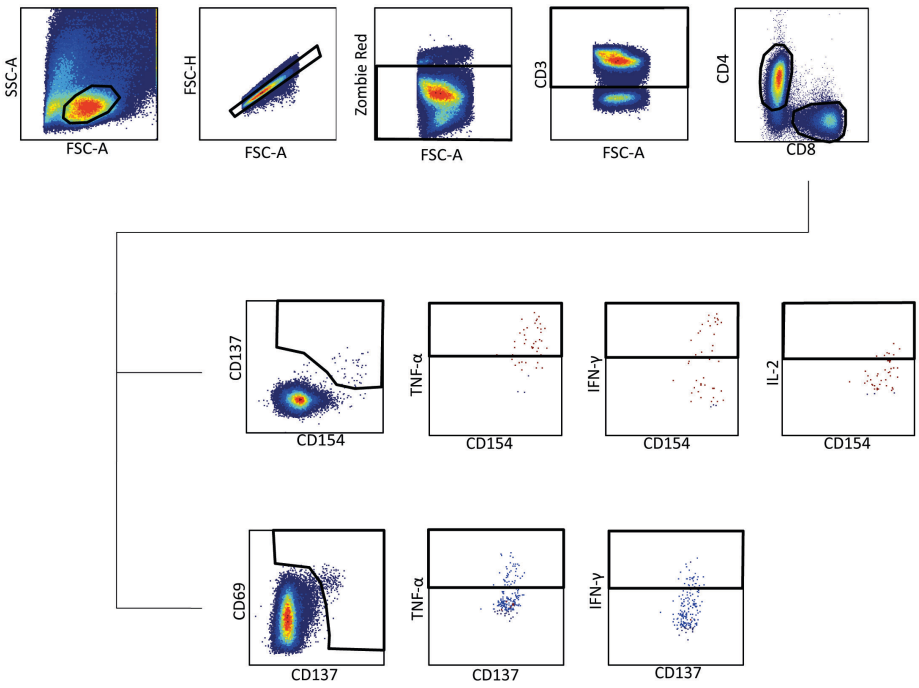

Figure S4 Representative example of gating strategy used to identify peptide-specific CD4⁺ and CD8⁺ T-cells

Cells were first gated using the side scatter area (SSC-A) and forward scatter area (FSC-A) parameters. Subsequently, doublets were excluded using the forward scatter height (FSC-H) and FSC-A parameters. Dead cells were then removed using the Zombie Red-A live dead marker. Next, CD3⁺ cells were selected and CD4⁺ and CD8⁺ T-cells were identified within the CD3⁺ gate. Peptide specific CD4⁺ T-cells and CD8⁺ T-cells were subsequentially gated using CD137⁺ and CD154⁺ and CD69⁺ and CD137⁺, respectively. Finally, cytokine-producing peptide-specific CD4⁺ and CD8⁺ T-cells were gated based on TNF-α, and IFN-γ positivity. The gating strategy on a representative AA patient sample is shown.

Abbreviations: SSC-A, side scatter area; FSC-A, forward scatter area; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL-2, interleukin 2.

SUPPLEMENTARY TABLES

Table S1 List of patient characteristics

	Aplastic anemia patients (n=18)	**Healthy controls (n=9)**
Age (median, range in years)	52 (21-80)	44 (33-59)
Gender (N, male:female)	8:10	5:4
Disease status at vaccination N (%)		
Transfusion-independent and IST-independent (%)	14 (77.8)	N/A
Transfusion-independent and IST dependent* (%)	3 (16.7)	N/A
Transfusion-dependent and IST dependent* (%)	1 (5.6)	N/A
Time between hATG and vaccination in years (median, range in years)	11.1 (0.3-39.0)	N/A
Sample time post second vaccination (median, range in days)	27.1 (11-49)	21.2 (18-24)
Follow-up time after first vaccination (median, range in months) n=15	9.1 (4.7-12.7)	N/A

*IST-dependent patients received cyclosporin A at the time of vaccination

Table S2 Spearman's rank sum testing to correlate spike-specific T-cell responses to patient characteristics

Correlation	**R value**	**P value**
% spike-specific $CD4^+$ T-cells *versus*		
Absolute # of $CD4^+$ T-cells pre vaccination	-0.44	0.08
% Naïve $CD4^+$ T-cells	-0.46	0.06
Age	-0.08	0.77
Date of last hATG administration	0.36	0.15
% spike-specific $CD8^+$ T-cells *versus*		
Absolute # of $CD8^+$ T-cells pre vaccination	0.11	0.69
% Naïve $CD8^+$ T-cells	0.15	0.56
Age	-0.09	0.73
Date of last hATG administration	-0.01	0.98
% TNF-α^+ spike-specific $CD4^+$ T-cells *versus*		
Absolute # of $CD4^+$ T-cells pre vaccination	-0.03	0.91
Age	-0.27	0.33

Correlation	R value	P value
Date of last hATG administration	0.12	0.67
% IFN-γ^+ spike-specific CD4$^+$ T-cells *versus*		
Absolute # of CD4$^+$ T-cells pre vaccination	-0.34	0.21
Age	-0.23	0.40
Date of last hATG administration	0.22	0.43
% IL-2$^+$ spike-specific CD4$^+$ T-cells *versus*		
Absolute # of CD4$^+$ T-cells pre vaccination	-0.06	0.83
Age	-0.30	0.27
Date of last hATG administration	0.40	0.14
SARS-CoV-2 IgG antibodies (BAU/mL) *versus*		
Age	-0.67	0.020
Date of last hATG administration	0.26	0.41
Activated CD4$^+$ T-cells	0.33	0.29
Activated CD8$^+$ T-cells	-0.10	0.76
Absolute # CD4$^+$ T-cells	0.39	0.24
Absolute # CD8$^+$ T-cells	0.60	0.06
Absolute # B-cells	0.25	0.45

Table S3 List of peptides used in the CEFX peptide pool mix

Pathogen	Antigen	Supplier	Cat #	Peptide characteristics
CMV	pp65	JPT	Custom-made	15-mers, 11 aa overlapping
Influenza A	NP1	JPT	N/A	15-mers, 11 aa overlapping, NCBI: ABB79814
EBV	BZLF1	JPT	PM-EBV-BZLF1	15-mers, 11 aa overlapping
EBV Class I	Pool	LUMC	Custom made	9-mers, known epitopes (Table S2)
Pool	Pool	JPT	PM-CEFX-2	15-mers, 11 aa overlapping

Table S4 List of EBV class I peptides used for the CEFX peptide pool

Antigen	Amino Acid Sequence	HLA Restriction	Supplier	Peptide Characteristics
LMP2	ESEERPPTPY	A*01:01	LUMC	9-mer
BMLF1	GLCTLVAML	A*02:01	LUMC	9-mer
LMP2	CLGGLLTMV	A*02:01	LUMC	9-mer
LMP2	FLYALALLL	A*02:01	LUMC	9-mer
BRLF1	RVRAYTYSK	A*03:01	LUMC	9-mer
EBNA3A	RLRAEAQVK	A*03:01	LUMC	9-mer
EBNA3B	IVTDFSVIK	A*11:01	LUMC	9-mer
EBNA3B	AVFDRKSDAK	A*11:01	LUMC	9-mer
BRLF1	DYCNVLNKEF	A*24:02	LUMC	9-mer
EBNA3A	RPPIFIRRL	B*07:02	LUMC	9-mer

Table S5 List of staining reagent and antibodies used for flow cytometry

Antigen	Fluorochrome	Clone ID	Company	Cat#
Zombie-Red			Biolegend	423110
CD8	APC-H7	SK1	BD Biosciences	560179
CD3	PE-Texas-Red	7D6	Invitrogen	MHCD0317
CD69	FITC	L78	BD Biosciences	347823
CD137	APC	4B4-1	BD Biosciences	550890
CD154	Pacific blue	24-31	Biolegend	310820
IL-2	PE	SCPL1362	BD Biosciences	130-091-646
IL-4	PERCPCY5.5	MP4-25D2	Biolegend	500822
FOXP3	AF700	PCH101	Thermo Fisher	56-4776-41
CXCR5	Pe-Vio770	REA103	Miltenyi	130-117-358
PD-1	BV786	EH12.1	BD Biosciences	563789
IL-17	BV650	N49-653	BD Biosciences	563746
IFN-γ	BV711	B27	BD Biosciences	564039
TNF-α	BV421	Mab11	BD Biosciences	562783
CD4	BV510	SK3	BD Biosciences	562970
CD45RA	PE-Texas-Red	MEM-56	Invitrogen	MHCD45RA17
CCR7	BV711	3D12	BD Biosciences	563712