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Temporal Patterns of Antibody-Secreting Cell Recovery Post-Allogeneic Hematopoietic Stem Cell Transplantation

Kenji Sato[1]*, Min Zhang[1], Hana T. Desta[1]

[1]Department of Medical and Clinical Sciences, Nihon University, Tokyo, Japan.

Abstract

Immune recovery after stem cell transplantation proceeds in distinct phases. While components of the innate immune system re-emerge quickly, adaptive lymphocytes—particularly T and B cells—require several months to fully reconstitute. In this study, we examined the dynamics and regeneration of newly formed B cells in patients undergoing CD3- and CD19-depleted haploidentical stem cell transplantation combined with additional in vivo T-cell depletion using a monoclonal anti-CD3 antibody. This transplantation strategy provides a unique opportunity to analyze the hierarchy and contribution of specific lymphocyte subsets in vivo without the confounding effects of mTOR or NFAT inhibitors. As anticipated, $CD3^+$ T cells and their subsets showed markedly delayed recovery, remaining below <100 cells/μL through day +90. Distinct naïve and memory $CD19^+$ B-cell populations became detectable around day +60. Unexpectedly, we identified a very early emergence of antibody-secreting cells (ASC) as early as day +14. These ASC expressed the donor's HLA haplotype and predominantly produced IgM and IgA, with lower levels of IgG. Their appearance was associated with a $CD19^-$ $CD27^-$ $CD38^{low/+}$ $CD138^-$ cell population.

Importantly, this ASC recovery occurred in the absence of circulating T cells and prior to any rise in BAFF or other B-cell–stimulating cytokines. In conclusion, we report a rapid restoration of peripheral ASC following CD3- and CD19-depleted haploidentical stem cell transplantation, well before the emergence of naïve or memory B cells. The early timing, independence from T-cell help, and spontaneous immunoglobulin secretion suggest that these ASC represent an innate-like population that may contribute to maintaining natural antibody levels during early immune reconstitution.

Keywords: Immune cells, Hematology, Allogenic stem cell transplantation, Reconstitution

Corresponding author: Kenji Sato
E-mail: kenji.sato@outlook.jp

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Introduction

Immune recovery following hematopoietic stem cell transplantation (HSCT) plays a critical role in controlling bacterial, viral, and fungal infections, influences the development of graft-versus-host disease (GvHD), and contributes to graft-versus-leukemia/lymphoma (GvL) activity [1, 2]. Moreover, patterns of immune reconstitution offer insight into lineage decisions made by hematopoietic stem cells during the restoration of innate and adaptive immunity. While innate immune components—such as granulocytes, monocytes, and NK cells—typically normalize within weeks [3, 4], the regeneration of adaptive immune subsets, including $CD4^+$ and $CD8^+$ T cells as well as B cells, may require up to two years [2, 4]. Numerous studies have examined T-cell recovery after HSCT [5–7]. Sequential phenotypic analyses of B-cell maturation markers have shown that

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transitional CD19⁺ CD24^high CD38^high B cells are among the earliest CD19⁺ subsets to appear, generally between six and eight weeks post-transplantation [8].

Innate-like B cells constitute a diverse group of unconventional B-cell populations that function independently of T-cell help, contribute to early innate immune responses, spontaneously produce immunoglobulins, display biased VDJ gene rearrangements, and have been proposed as a major source of natural polyreactive and autoreactive antibodies (NAbs) [9–11]. In mice, these cells correspond to the well-defined B1 B-cell subset. In humans, a small circulating population with similar attributes has been identified, characterized by the phenotype CD20⁺ CD27⁺ CD43⁺ CD70⁻ [12]. It remains unclear whether human lymphoid reconstitution follows a hierarchical pattern in which innate-type lymphocytes emerge before adaptive lymphocyte populations.

In this study, we performed longitudinal monitoring of the kinetics and regeneration of defined B-cell subsets in patients receiving T-cell– and B-cell–depleted haploidentical stem cell transplantation, combined with additional in vivo T-cell depletion using the monoclonal anti-CD3 antibody OKT3 [13, 14]. This transplantation platform allows an assessment of de novo B-cell development in vivo without the confounding effects of calcineurin inhibitors (CIs), other immunosuppressive drugs, or pre-existing donor-derived B cells within the graft.

Remarkably, by two weeks after HHCT, we detected the emergence of cells spontaneously secreting IgM, IgA, and IgG in all evaluated patients. These antibody-secreting cells (ASC) were confirmed to be donor-derived and were associated with a previously uncharacterized CD19⁻ CD27⁻ CD38^low/+ CD138⁻ cellular population. These ASC arose independently of T-cell recovery, prior to the appearance of transitional B cells, and in the absence of detectable B-cell-stimulating cytokines. Because they spontaneously produce immunoglobulins—predominantly IgM and IgA—they share features typical of innate-like B cells and may therefore be more closely aligned with innate rather than adaptive immunity.

Materials and Methods

Patients

From 2008 to 2009, this study examined ten successive patients (seven female, three male) participating in a prospective phase II trial of haploidentical hematopoietic cell transplantation (HHCT) employing CD3/CD19-depleted grafts (clinical trial registration NCT00202917) [13] at the Department of Haematology, Oncology and Immunology, University Hospital Tuebingen, Germany. Three individuals (one female, two male) were removed from the analysis because of graft failure, poor graft function, or unexpected death on day +2 following transplantation. Every patient gave written informed consent. Peripheral blood mononuclear cells (PBMCs) were obtained from the remaining patients immediately before transplantation (day 0) and then at weekly intervals starting from day +7 (week 1), day +14 (week 2), day +21 (week 3), continuing weekly until week 36 post-HHCT. All PBMC analyses were performed on fresh samples at the exact time of collection. Weekly serum specimens were stored and batch-analyzed after completion of the monitoring period. The research protocol was conducted according to the Declaration of Helsinki and received approval from the ethics committee of the Faculty of Medicine, Eberhard Karls University Tuebingen (IRB reference number 21/2004), University Hospital Tuebingen.

Conditioning regimen and haploidentical hematopoietic cell transplantation (HHCT)

Every patient was enrolled and treated under the protocol of a prospective phase II trial that utilized CD3/CD19-depleted grafts (registered as NCT00202917), exactly as detailed earlier [13, 14]. To summarize the treatment scheme:

A reduced-intensity conditioning regimen was applied, comprising fludarabine at 150–200 mg/m² from day −8 through day −4, thiotepa at 10 mg/kg on day −3, melphalan at 120 mg/m² on days −2 and −1, and OKT-3 at 5 mg daily from day −5 until day +14. On day 0, cryopreserved peripheral blood stem cells depleted of CD3 and CD19 were infused. Granulocyte colony-stimulating factor (G-CSF) was not administered to any patient following the haploidentical transplant. Immunosuppression after transplantation was short-term and consisted only of mycophenolate mofetil given from day 0 to day +30. On day +15, each patient received 15 g of intravenous immunoglobulin (IVIG) preparation that lacked IgA and IgM.

Flow-cytometry based analysis of immune reconstitution and chimerism

Donor hematopoietic cell chimerism analysis was carried out according to the method reported previously [15]. Monitoring of immune reconstitution was conducted on peripheral blood mononuclear cells (PBMCs) using multicolor flow cytometry from week 0 through week 38 post-transplant. PBMCs were separated from whole blood via density gradient centrifugation with Biocoll separating solution (Biochrom, Berlin, Germany).

For immunophenotyping, cells were directly stained with the following fluorochrome-conjugated monoclonal antibodies: CD38-PerCP (clone HB-7, BD Biosciences) - CD138-PE (clone MI15, BD Biosciences, Franklin Lakes,

NJ, USA) - CD27-FITC (clone M-T-271, BD Biosciences) - IgA-APC (clone 97924, R&D Systems, Minneapolis, MN, USA) - CD19-PacificBlue (clone SJ25C1, BD Biosciences) - IgM-APC (clone IL/41, ThermoFisher) - HLA-Class I B7-PE (clone BB7.1, abcam, Cambridge, UK) - HLA-A2-APC (clone BB7.2, ThermoFisher) - HLA-Class I B8-APC (Miltenyi Biotec) - IgG-APC (clone 97924, R&D Systems) - HLA-Class I BW6-PE (Miltenyi Biotec, Bergisch Gladbach, Germany)- CD138-PE (clone MI15, BD Biosciences, Franklin Lakes, NJ, USA)

Viable cells were distinguished by exclusion of dead cells with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher, Waltham, MA, USA). Acquisition was performed on either a FACS Canto II or a FACS Fortessa flow cytometer (BD Biosciences), and all data were subsequently analyzed with FlowJo version 10 software (FlowJo LLC, Ashland, OR, USA).

ELISpot assay to detect IgG, IgG and IgM antibody secreting cells

At specified intervals, PBMCs obtained from the patients were assayed in duplicate wells using a customized enzyme-linked immunospot (ELISPOT) method. The protocols for detecting IgM-, IgG-, and IgA-secreting cells were adapted from previously published methods [16, 17]. In short, the procedure was as follows: 96-well filtration plates (MAHA S4510, Millipore, Burlington, MA, USA) were incubated overnight at 4 °C with capture antibodies directed against human IgM, IgG, or IgA (Sigma Aldrich). After two PBS rinses, non-specific binding sites were blocked for 2 h at 37 °C using PBS supplemented with 1% BSA. Patient PBMCs were seeded into the wells and cultured overnight in a 5% CO_2, humidified incubator at 37 °C.

Next, wells were emptied and rinsed four times with PBS containing 0.25% Tween-20. Biotin-conjugated detection antibodies, diluted 1:2000 in PBS/1% BSA, were added and left for 2 h at room temperature. Wells were then washed four times with PBS/1% BSA, followed by a 1-hour incubation with streptavidin diluted 1:400. After sequential washes (twice with PBS/0.25% Tween-20 and twice with PBS only), color development was achieved by adding 100 µL per well of AEC substrate solution (0.3 mg/mL 3-amino-9-ethylcarbazole in 0.1 M sodium acetate, pH 5.0, plus 0.03% H_2O_2) and incubating 5 min protected from light. Plates were thoroughly rinsed with distilled water, allowed to dry overnight, and spots were quantified automatically with an ImmunoSpot Series 3B Analyzer running ImmunoSpot 3.0 software (CTL, Cleveland, OH, USA).

Nonspecific background was controlled by running parallel assays with a ready-to-use human IgM/IgG Double-Color ELISPOT kit (hIgMIgG-DCE-1M/2, ImmunoSpot®, Cleveland, OH, USA) exactly as recommended by the manufacturer.

Detection of immunoglobulin and cytokine levels using multiplex immunoassay system

Immunoglobulin concentrations and cytokine levels—including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, MCP-1 (MCAF), TNF-α, GM-CSF, MIP-1β, IFN-γ, G-CSF, as well as IgE, IgA, IgM, and IgG—were assessed in patients after HHCT using the Bio-Plex Multiplex Immunoassay platform (Bio-Plex™ Cytokine Assay and Bio-Plex Pro™ Immunoglobulin Hercules, Isotyping, Bio-Rad, CA, USA). All assays were conducted in strict accordance with the manufacturer's guidelines.

ELISA

BAFF protein levels were determined using a human BAFF ELISA kit (ab188391, Abcam) following the manufacturer's instructions. All measurements are presented as mean values ± SEM of triplicate samples.

Statistical analysis

For continuous variables, statistical analyses were performed using Student's t-test, the Mann–Whitney U test, one-way ANOVA, and the Friedman test. When ANOVA revealed significant differences, pairwise comparisons were conducted using Tukey's multiple comparison test. For significant results from the Friedman test, Dunn's multiple comparison test was applied. Data are presented as means with standard error of the mean (SEM) indicated by error bars. A p-value of less than 0.05 was considered statistically significant. All analyses were carried out using GraphPad Prism software (version 8.1.0).

Results and Discussion

Early elevation of serum immunoglobulins and antibody-secreting cells following HHCT with T- and B-cell–depleted grafts

To examine immune reconstitution after HHCT with CD3/CD19-depleted grafts, we monitored seven consecutive patients over a 36-week period **(Figure 1a)**. Neutrophil engraftment, defined as an absolute neutrophil count (ANC) exceeding 500 cells/µL, occurred at a median of day +10 (range: 9–13 days) **(Figure 1b)**. While total lymphocyte counts showed a significant rise by day +49 ($p = 0.02$, **(Figure 1c)**), recovery of $CD3^+$ T cells was substantially delayed, remaining below 100 cells/µL at day +90 **(Figure 1d)**.

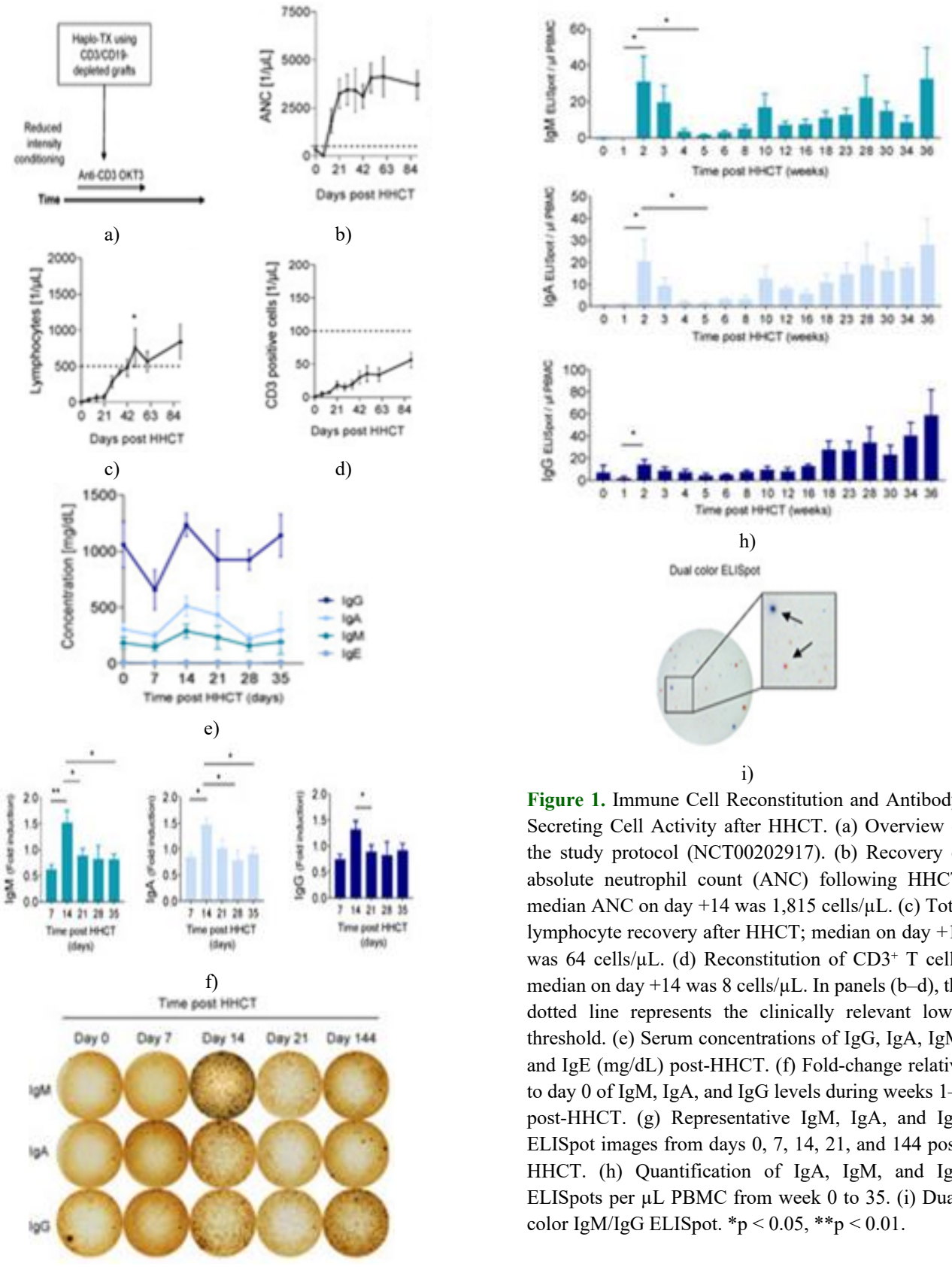

Figure 1. Immune Cell Reconstitution and Antibody-Secreting Cell Activity after HHCT. (a) Overview of the study protocol (NCT00202917). (b) Recovery of absolute neutrophil count (ANC) following HHCT; median ANC on day +14 was 1,815 cells/µL. (c) Total lymphocyte recovery after HHCT; median on day +14 was 64 cells/µL. (d) Reconstitution of $CD3^+$ T cells; median on day +14 was 8 cells/µL. In panels (b–d), the dotted line represents the clinically relevant lower threshold. (e) Serum concentrations of IgG, IgA, IgM, and IgE (mg/dL) post-HHCT. (f) Fold-change relative to day 0 of IgM, IgA, and IgG levels during weeks 1–5 post-HHCT. (g) Representative IgM, IgA, and IgG ELISpot images from days 0, 7, 14, 21, and 144 post-HHCT. (h) Quantification of IgA, IgM, and IgG ELISpots per µL PBMC from week 0 to 35. (i) Dual-color IgM/IgG ELISpot. *$p < 0.05$, **$p < 0.01$.

To evaluate the contribution of antibody-secreting cells (ASC) following HHCT with CD3/CD19-depleted grafts, we longitudinally measured serum levels of IgM, IgA, IgG, and IgE using a multiplex immunoassay. Notably, all patients exhibited a pronounced peak in IgM, IgA, and IgG levels approximately 14 days post-transplant **(Figure 1e)**, whereas IgE concentrations remained largely unchanged.

When normalized to baseline (day 0), the increases in IgA, IgG, and IgM were statistically significant **(Figure 1f)**. This early rise was transient, as all three immunoglobulin levels declined by day 21 and beyond **(Figure 1f)**. The observed increase in serum IgG likely reflects the administration of IVIGs, which do not contain IgM or IgA. To investigate whether this early surge in immunoglobulins corresponded to ASC activity, we applied a modified ELISpot assay to detect IgA-, IgM-, and IgG-secreting cells in circulating PBMCs **(Figure 1g)**. Consistent with serum measurements, we observed a transient peak of IgM, IgA, and IgG ELISpots per µL PBMC between days 14 and 21 post-HHCT **(Figure 1h)**. Among the three isotypes, IgA (20.5 ± 25.9) and IgM (31 ± 37.2) were more abundant than IgG (14 ± 12). To rule out nonspecific signals, an IgM/IgG double-color ELISpot was also performed **(Figure 1i)**.

In summary, these data indicate the presence of a previously uncharacterized population of early ASC following HHCT that secrete IgA, IgG, and IgM during the first weeks after transplantation.

Correlation of $CD27^-$ $CD19^-$ $CD138^-$ $CD38^{low/+}$ cells with early ASC after HHCT

To further explore the presence of early, previously uncharacterized antibody-secreting cells (ASC) after HHCT, we performed longitudinal monitoring of defined lymphocyte subsets in all patients. While the first naïve B cells were observed 5–6 weeks post-HHCT, plasma cells remained virtually undetectable during the initial 8 weeks. Consistent with prior observations, naive $CD19^+$ $CD27^-$ $CD38^+$ B cells were detected engrafting between 6 and 8 weeks after HHCT **(Figures 2a and 2b)** [8]. Interestingly, we also identified an early population of $CD19^-$ $CD38^{low/+}$ cells **(Figure 2a)**, which became detectable as early as 7–14 days post-transplant **(Figure 2b)**.

Further characterization revealed that these cells contained high levels of intracellular immunoglobulins, suggesting a potential role in the early surge of serum Ig. Approximately 3% of the intracellular Ig^high cells exhibited a phenotype resembling long-lived plasma cells ($CD27^+$ $CD38^{++}$ $CD138^+$). However, the majority of intracellular Ig^high cells displayed a previously undefined phenotype: $CD19^-$ $CD27^-$ $CD38^{low/+}$ $CD138^-$ **(Figure 2c)**. Notably, the proportion of $CD19^-$ $CD38^{low/+}$ cells correlated positively with the total number of ELISpot-detected ASC, supporting the idea that this population contributes substantially to the secretion of IgM, IgA, and IgG **(Figure 2d)**.

To confirm that this $CD19^-$ $CD27^-$ $CD38^{low/+}$ $CD138^-$ population was of donor origin, FACS analysis of HLA haplotypes was performed, which verified donor derivation in all patients **(Figure 2e)**. Additionally, donor–recipient chimerism analysis at week 2 showed that more than 99% of circulating cells carried donor alleles **(Figure 2f)**.

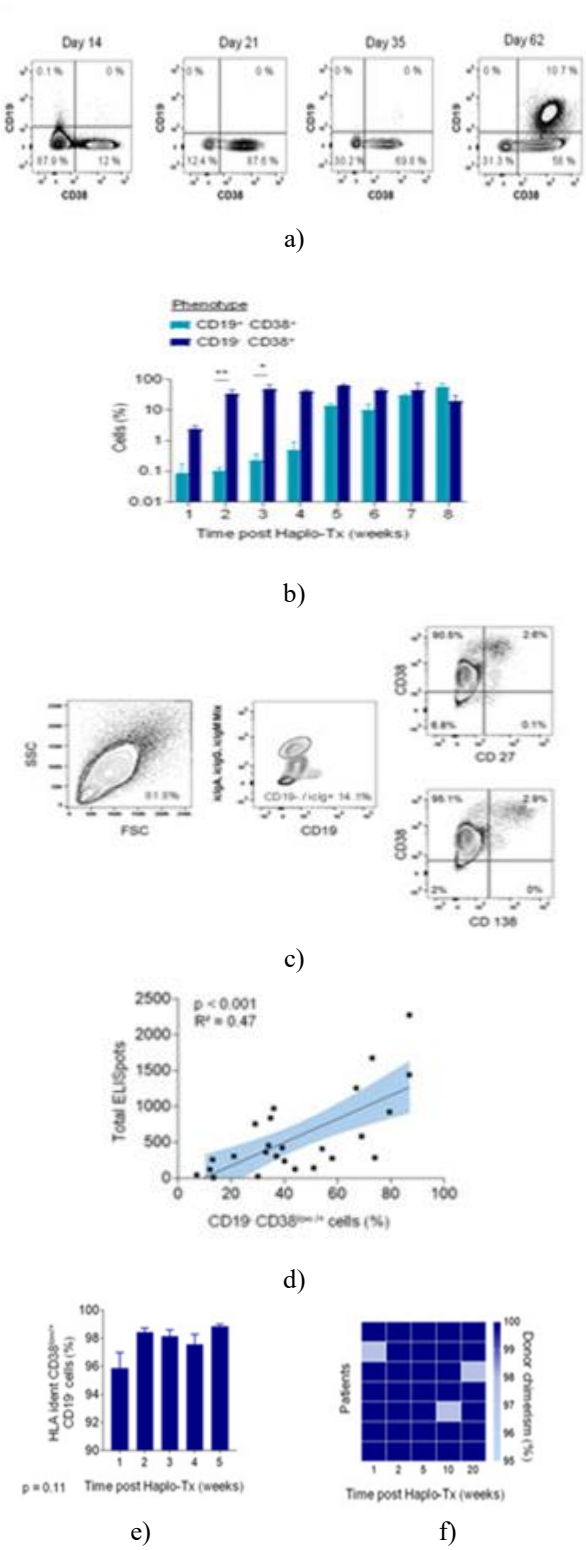

Figure 2. B Cell and Antibody-Secreting Cell (ASC) Reconstitution. (a) Representative flow cytometry gating illustrating the reconstitution of $CD19^+$ and $CD38^+$ immune cell populations. (b) Quantitative assessment of $CD19^+$ $CD38^+$ and $CD19^-$ $CD38^+$ populations during weeks 1–8 post-HHCT. (c) Identification of immature IgM-, IgA-, and IgG-

expressing CD38$^{low/+}$ CD19^{-} CD27^{-} CD138^{-} cells at two weeks after HHCT. (d) Linear regression analysis correlating the total number of ELISpots (IgG, IgA, and IgM) with the frequency of CD38$^{low/+}$ CD19^{-} cells as a percentage of total PBMCs ($p < 0.001$, $R^2 = 0.47$). (e) Frequency of CD38$^{low/+}$ CD19^{-} cells expressing donor-specific HLA alleles ($p = 0.11$). (f) Donor chimerism in all patients at weeks 1, 2, 5, 10, and 20 post-HHCT. *$p < 0.05$, **$p < 0.01$; low/+ indicates low expression.

Cytokine levels during immune cell reconstitution

B-cell reconstitution and maturation following HHCT and HSCT is a complex, multistep process influenced by treatment-, donor-, patient-, and disease-related factors, and regulated by multiple cytokines [2, 18, 19]. To identify cytokines potentially involved in the emergence of ASC in this context, we analyzed the cytokine profile during the first month after HHCT **(Figure 3a)**. While no significant changes were detected for IL-1β, IL-2, IL-4, IL-7, IL-10, IL-12, or IL-13, IL-5 levels were markedly elevated in all patients one week post-HHCT **(Figure 3b)**. In addition, we observed increased concentrations of inflammatory cytokines, including IL-6, IL-8, MIP-1β, MCAF, IFN-α, and TNF-α **(Figures 3a and 3b)**. Although most of these cytokine levels declined over time, MIP-1β and MCAF remained elevated throughout the first month post-transplant **(Figure 3a)**.

Given the established role of BAFF in B-cell maturation and its ability to support survival of plasmablasts derived from human memory B cells [20], we also measured serum BAFF concentrations in our patient cohort. Notably, BAFF levels remained low and did not reach relevant concentrations during the first 10 weeks post-HHCT. While BAFF plasma levels and CD3^{+} T-cell counts significantly correlated with plasma cell development in our cohort, the generation of early ASC appeared to occur independently of these two factors.

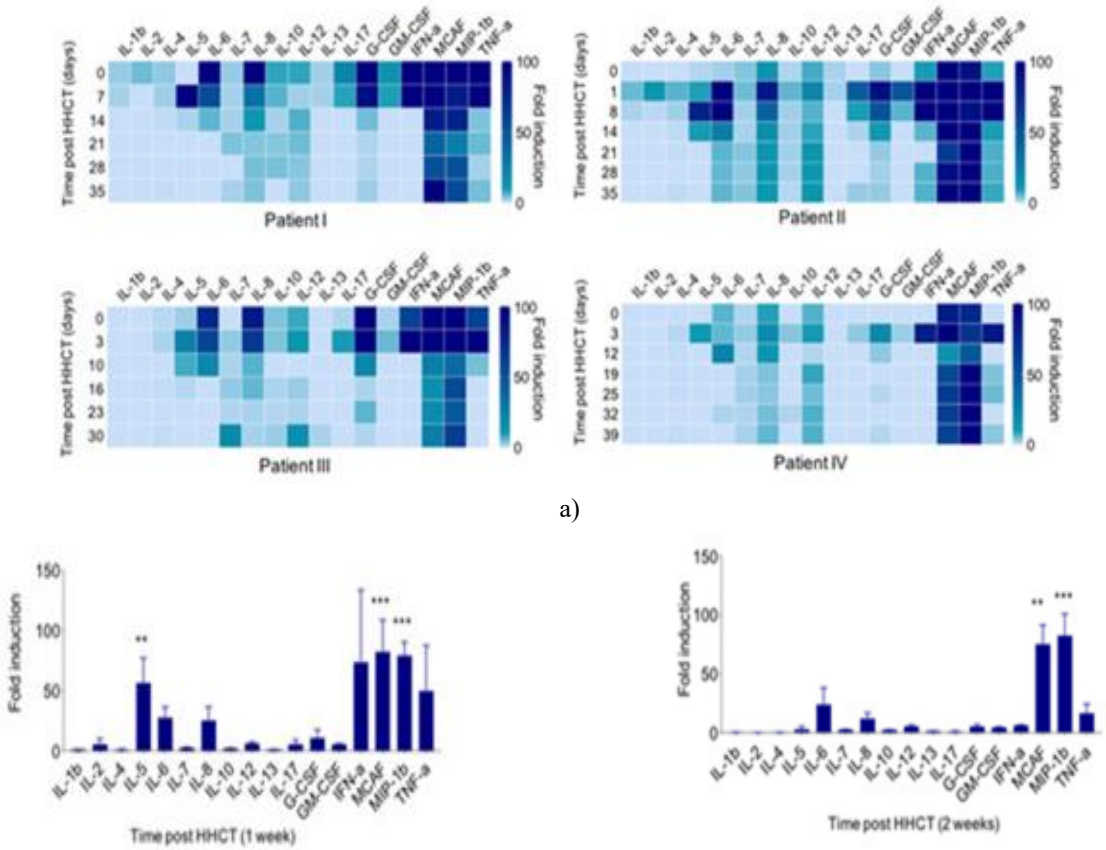

Figure 3. Serum Cytokine Profiles during Immune Reconstitution after HHCT. (a) Serum concentrations of IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, MIP-1β, MCAF, IFN-α, and TNF-α in four representative patients over the period of 0–39 days post-HHCT. (b) Quantitative assessment of cytokine levels at one week, and (c) at two weeks after HHCT. ***$p < 0.001$, **$p < 0.01$

Cells of the innate immune system, such as monocytes and NK cells, are detectable as early as two weeks following HSCT, whereas populations of antibody-secreting cells (ASC) are generally reported to appear six to twelve months post-transplant [1, 19]. Nevertheless, the first B-cell subsets begin to emerge 6–8 weeks after HSCT and are predominantly characterized as transitional or mature naïve CD19^{+} CD27^{-} CD38^{high} B cells [8]. In mice, innate-

like B cells—known as B1 B cells—are well characterized; they secrete immunoglobulins spontaneously, are activated through innate signaling without T-cell help, display a biased VDJ repertoire, and contribute to a pool of polyreactive and autoreactive natural antibodies [9, 10, 11]. In humans, a very small subset of circulating $CD19^+$ B cells exhibits similar characteristics, with a $CD20^+$ $CD27^+$ $CD43^+$ $CD70^-$ phenotype [12].

In the present study, we utilized CD3- and CD19-depleted haploidentical stem cell transplantation combined with in vivo T-cell depletion using a monoclonal anti-CD3 antibody [13, 14]. This approach allowed us to longitudinally study de novo B-cell development and ASC reconstitution in vivo. Consistent with previous reports, transitional B cells engrafted 6–8 weeks after HHCT [1, 8, 19]. Notably, we identified a previously uncharacterized population of $CD19^-$ $CD27^-$ $CD38^{low/+}$ $CD138^-$ cells with high intracellular levels of IgM, IgA, or IgG. These cells spontaneously secreted primarily IgM and IgA, with lower amounts of IgG. As they lack CD19 expression, they do not conform to the phenotype of conventional immature B cells [21].

Although IVIG administration may contribute to the observed IgG peak at day +14, we also observed peaks of IgM and IgA, which were confirmed by ELISpot assays. While a small fraction of plasma cells could theoretically contribute to the observed effect, the early ASC population developed independently of T cells and cytokines typically involved in B-cell or plasmablast development. This suggests that these cells may represent a previously undescribed subset of innate-like B cells. Supporting this notion, their emergence coincided with increased IL-5 levels, a cytokine implicated in T cell–independent development of innate-like B cells and Ig production [22]. Additionally, we observed elevated levels of IL-6, macrophage inflammatory protein-1β (MIP-1β), and monocyte chemotactic and activating factor (MCAF). Given that these cytokines are linked to macrophage homeostasis, and macrophages share developmental and effector characteristics with innate B cells [23], a potential functional crosstalk between these cytokines and early ASC can be hypothesized, although further studies are needed to confirm this interaction.

Our observation that the $CD19^-$ $CD27^-$ $CD38^{low/+}$ $CD138^-$ cells were of donor origin supports the idea of preferential engraftment of innate-like B cells following HCT. While this model allows the study of B-cell reconstitution without the confounding effects of calcineurin inhibitors or other immunosuppressive drugs, and in the absence of pre-existing donor-derived B cells, the findings may be specific to this type of transplantation. Additional studies in different HCT settings are necessary to validate the existence and function of this putative innate B-cell population. Nonetheless, the HHCT model used in our study provides a valuable system to gain further insights into immune cell reconstitution.

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Conflict of interest: None

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Ethics statement: This study was approved by the IRB (ethics committee of the Faculty of Medicine of the Eberhard Karls University Tuebingen) of the University Hospital Tuebingen and was conducted in accordance with the Declaration of Helsinki; reference number 21/2004. Written informed consent has been obtained from the patients.

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