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Mapping the pathogenesis of immune-mediated aplastic anemia: filling the gaps

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Imaging mass cytometry reveals the order of events in the pathogenesis of immune-mediated aplastic anemia

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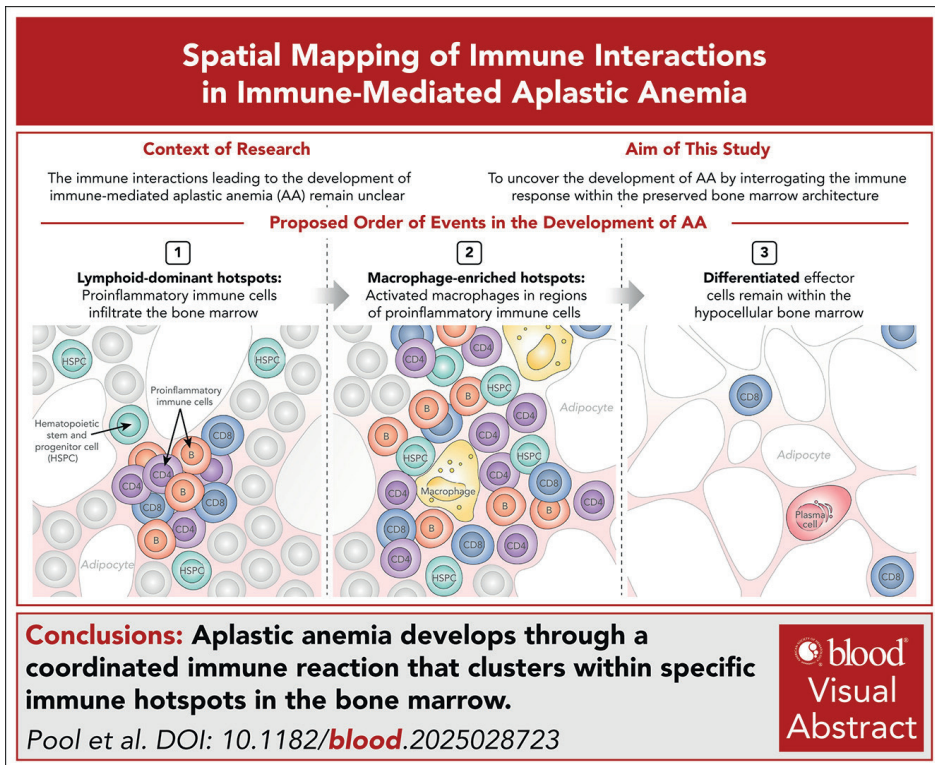
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Key points

- Development of AA is the result of a coordinated immune reaction that concentrates within immune hot spots in the bone marrow.
- During bone marrow destruction, the immune response gradually shifts from an activated to a more differentiated phenotype.

Visual abstract



Abstract

The autoimmune response driving hematopoietic stem and progenitor cell (HSPC) destruction in immune-mediated aplastic anemia (AA) remains incompletely understood. We previously identified a disease-specific immune cell network involving T-, B-, and myeloid cells. However, the interactions within this network, the interaction with the microenvironment and the chronological events in AA development, remain unclear. In this study, we aimed to characterize the changes occurring during disease development and to define the interactions between potential autoreactive cells and their target. Using imaging mass cytometry, we analyzed bone marrow biopsies from AA patients at diagnosis and after treatment with horse-derived anti-thymocyte globulin (hATG), and six controls. Within the hypocellular bone marrow architecture, we identified lymphoid-dominant 'immune hot spots' with high densities of pro-inflammatory lymphocytes, and macrophage-enriched hot spots that additionally contained activated macrophages in proximity to progenitors. These immune hot spots potentially represent sites where the active immune response resulting in HSPC destruction takes place. In bone marrow regions depleted of progenitors, effector cells with a differentiated phenotype remain. Our data indicate that HSPC destruction in AA is mediated by coordinated interactions among specific immune cell subpopulations. As the immune response progresses and HSPCs are depleted, the immune composition shifts, with activated T- and B-cells differentiating into terminally differentiated T-cells and plasma cells. In patients with normalizing bone marrow post-hATG treatment, most immune hot spots were depleted, underscoring their potential pathogenic role. Collectively, our study visualizes the complex interactions among immune cell subpopulations and, for the first time, reveals the order of events in the immune-mediated pathogenesis of AA.

Introduction

Immune-mediated acquired aplastic anemia (AA) is characterized by bone marrow hypocellularity due to a loss of hematopoietic stem and progenitor cells (HSPCs), leading to pancytopenia and life-threatening bleeding and infectious complications¹. Immunosuppressive therapy (IST) with horse-derived anti-thymocyte globulin (hATG) and long-term ciclosporin results in gradual improvement of peripheral blood counts within 6 months in approximately 70% of patients²⁻⁴. Response rates to IST can be increased by adding eltrombopag to first-line IST⁵ or as second-line treatment 6 months after start of IST. However, hematological recovery is partial in most patients, with few reaching complete normalization of blood counts. One-third of patients do not respond to IST and face prolonged pancytopenia with the accompanying risk of bleeding and infections⁴.

Better understanding of the pathogenesis of AA may lead to improved therapeutic strategies by identifying new treatment targets. Previously, by analyzing bone marrow aspirates from AA patients using mass cytometry, we identified an AA-specific immune cell network, containing CCR6⁺ B-cells, Th17-like CCR6⁺ memory CD4⁺ T-cells, and KLRG1⁺ terminally differentiated CD8⁺ T-cells⁶. This provided strong evidence that AA results from immune-mediated HSPC destruction involving specific immune cells associated with inflammation. These subpopulations were significantly increased in AA patients at diagnosis compared to healthy donors, and showed a trend toward normalization after successful IST. Additionally, we observed CD163⁺ macrophages in the bone marrow at diagnosis, despite depletion of the myeloid compartment, suggesting macrophage recruitment to clear apoptotic cells.

Although our previous work provides a strong indication of the cells involved, the precise interactions of these cells and their microenvironment cannot be deduced from studying bone marrow aspirates. Previous research demonstrated that bone marrow biopsies offer the unique possibility to analyze spatial cellular interactions in AA while overcoming issues of selective cell loss generally observed with bone marrow aspirates⁷. In this study, we aimed to define the interactions between potential autoreactive cells and their cellular target, and to reveal the order of events in AA development. To achieve this, we studied the spatial orientation of the immune response within its native microenvironment in bone marrow biopsies from AA patients at diagnosis (AA^{Dx}) and 6 months post-hATG (AA^{6M}) using imaging mass cytometry (IMC). Our study provides unique insight into the spatial biology of the bone marrow in AA. We confirmed the presence of the previously identified immune cell network *in situ*. Importantly, we identified immune 'hot spots' within the hypocellular bone marrow, where cells from the immune cell network and other activated immune cells colocalize with remaining progenitor cells. In bone marrow regions depleted of progenitors, these immune

hot spots are nearly absent and more differentiated cells remain. Collectively, our study sheds light on the possible chronological order of events in the pathogenesis of AA.

Methods

Patients and bone marrow biopsies

Bone marrow biopsies were collected from 16 AA patients at diagnosis (AA^{Dx}), with paired biopsies available for 12 of 16 patients 6 months after start of first-line IST (AA^{6M}) (Table 1, S1). As a proxy for normal bone marrow, bone marrow biopsies were collected from six age-, sex-, CMV- and EBV status-matched diffuse large B-cell lymphoma patients without bone marrow localization. Bone marrow collection was performed after given informed consent. All patients were treatment-naïve at diagnosis, did not have an additional underlying disease and were diagnosed and treated according to standard Dutch guidelines⁸. Patients <40 years were screened for Fanconi anemia to rule out a congenital form of bone marrow failure. IST consisted of 4 days of 40 mg/kg/day ATGAM® (hATG; Pfizer) and 5 mg/kg/day ciclosporin for ≥6 months. All biopsies collected 6M post-hATG were taken before the start of eltrombopag. This study was approved by the Medical Ethical Committee of the Leiden University Medical Center (protocol B20.037).

Table 1. Characteristics of patients and response to IST.

Characteristic	Dx	6M post-hATG	Controls
Patients (n)	16	12	6
Age (median, range in years)*	42 (23-79)	53 (24-79)	54 (44-83)
Sex (n, M:F)	9:7	6:6	5:1
Severity of disease at diagnosis (n)			
Non-severe AA	2	-	NA
Severe AA	8	7	NA
Very severe AA	6	5	NA
Response to IST (n) [#]			
≤ 6 months post-hATG	8	5	NA
≥ 6 months post-hATG	5	5	NA
Non-responder to IST	3	2	NA

AA indicates aplastic anemia; hATG: horse-derived anti-thymocyte globulin; F: female; M: male; NA: not applicable. *: at diagnosis. #: response to IST was defined as transfusion independence and a neutrophil count of $\geq 0.5 \cdot 10^9$ neutrophils/L. Response to IST ≥ 6 months post-hATG occurred either spontaneously or after eltrombopag (Revolade®, Novartis).

Acquisition and analysis of imaging mass cytometry images

Bone marrow tissue sections were stained with 37 metal isotope-tagged antibodies to detect both hematopoietic cells and non-hematopoietic cells of the bone marrow microenvironment (Table S2). For panel development and staining details, see supplementary methods⁹⁻¹⁴. High-dimensional images with 1 μm^2 resolution were acquired on the Hyperion™ Imaging

System (Standard BioTools) by laser-ablation at 200Hz. Data inspection showed clear cellular staining in bone marrow and control tonsil (Figures S1-2).

Bone marrow images were analyzed by quantifying tissue surface areas, cell segmentation and downstream clustering analyses¹⁵⁻²⁰. Detailed description is provided in the supplementary methods and is visualized in Figures S3-6.

Statistical analyses

Dunn's tests performed in R v4.3.0 were used for comparisons between groups, with *P*-values ≤ 0.05 considered statistically significant. Since this was an exploratory study, no adjustments for multiple testing were applied. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

Results

Bone marrow from AA patients at diagnosis features Ki-67⁺ regions with activated macrophages

The immune response in AA was studied using IMC (Figure 1A). Bone marrow biopsies from 16 AA patients at diagnosis (AA^{Dx}), 12 paired AA patients 6M post-hATG (AA^{6M}) and six age-matched controls were stained with a 37-marker IMC panel and imaged. 79 regions of interest (ROIs; 2-3 per biopsy; median 1.5mm²) were acquired across all sections.

AA is characterized by bone marrow hypocellularity at diagnosis, which can improve after IST (Figure 1B, upper row; 1C). The extent of hypocellularity varied between patients, suggesting the bone marrow was captured at different stages of bone marrow destruction. In line with previous observations²¹, the cellularity of the bone marrow did not significantly differ between disease severities (non-severe, severe and very severe AA median 1236, 1216 and 1010 cells/mm² respectively, Figure 1C; global bone marrow structure per patient in Figure S7). To enable quantitative comparisons between samples, we determined the surface areas of three major compartments in the bone marrow: 1) the hematopoietic compartment containing hematopoietic cells and their microenvironment, 2) adipocytes, and 3) bone (Figure 1B, lower row). The area of the hematopoietic compartment was determined based on pixels positive for DNA (nuclei), Vimentin (cytoplasm, cell membrane, microenvironment) and/or CD31 (cytoplasm of Vimentin⁺ CD31⁺ megakaryocytes) using Cytosplore¹⁸ Imaging (Figure S3). For the surface areas of bone and adipocytes, areas of CollagenI⁺ bone trabeculae and remaining surface areas (adipocytes) were quantified in ImageJ. Consistent with the observed bone marrow hypocellularity, the hematopoietic compartment was significantly decreased and replaced by adipocytes at diagnosis, while bone area was not affected (Figure 1D). 6M post-hATG, the area of the hematopoietic compartment increased in most patients, reflecting bone marrow recovery (Figure 1D).

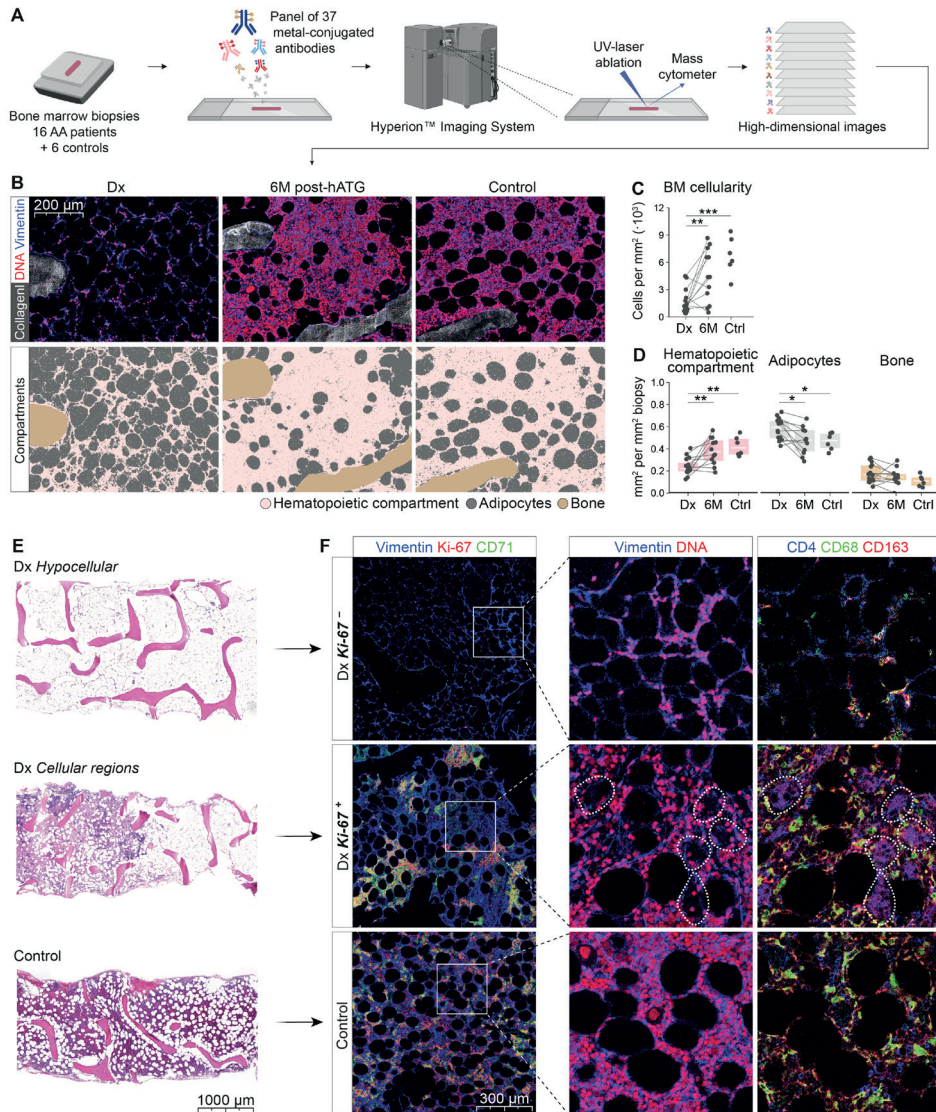


Figure 1. Histology of the bone marrow from AA patients and controls. [A] Experimental approach. Tissue sections of FFPE EDTA-decalcified bone marrow biopsies were cut, stained with a 37-marker IHC panel and measured on the Hyperion Imaging System, resulting in a dataset of 79 high-dimensional images. [B] Top row: global histology of the bone marrow, visualized for a representative AA^{Dx}, AA^{6M} and control ROI. Collagen (white) stains bone, Vimentin (blue) stains all hematopoietic cells and their microenvironment and DNA (red) stains all cellular nuclei. Bottom row: Defined tissue compartments visualized within the representative images: hematopoietic compartment (pink), adipocytes (gray) and bone (tan). [C] Bone marrow cellularity, quantified as the total number of cells per mm² of bone marrow biopsy. [D] Surface areas of the three compartments in AA^{Dx}, AA^{6M} and controls. [E] H&E stainings of 1) a representative completely hypocellular bone marrow biopsy lacking expression of Ki-67 at diagnosis, 2) a bone marrow biopsies containing cellular regions with islands of Ki-67⁺ progenitor cells at diagnosis and 3) a control bone marrow biopsy. [F] IHC images for a Ki-67⁻ region from a representative AA^{Dx}, a Ki-67⁺ region from a representative AA^{Dx} and a control ROI. Single cells, hematopoietic progenitor cells and tissue structure are visualized using DNA, CD71 and Ki-67, and Vimentin. Macrophages are visualized using the markers CD4, CD68 and CD163. Box plots present the median with the interquartile range (IQR), error bars present 1.5*IQR.

To capture the immune response, we assessed the hematopoietic compartment of all AA^{Dx} biopsies. The proliferation marker Ki-67 was used to detect sites of active hematopoiesis. This distinguished bone marrow regions in which all Ki-67⁺ progenitors were cleared (Ki-67⁻ regions) and regions that still contained pockets of hematopoiesis, including CD71⁺Ki-67⁺ erythroid precursors (Ki-67⁺ regions) (Figure 1E-F). Importantly, Ki-67⁺ and Ki-67⁻ bone marrow regions co-existed within bone marrow biopsies from several AA^{Dx}. Comparison of Ki-67⁻ and Ki-67⁺ regions from AA^{Dx} with control bone marrow revealed the presence of CD68⁺CD163⁺ macrophages in Ki-67⁺ regions from AA^{Dx} and controls, but not in Ki-67⁻ regions (Figure 1F, yellow cells). Strikingly, activated and enlarged CD68⁺CD163⁺CD4⁺ macrophages were exclusively found in Ki-67⁺ regions from AA^{Dx}, where they surrounded sites of active hematopoiesis (Figure 1F, purple cells within white circles). Notably, these macrophages were engulfing hematopoietic cells including a mixture of CD7⁺ T-cells and innate lymphoid cells, CD20⁺ B-cells, CD14⁺ monocytes and CD71⁺Ki-67⁺ erythroid precursors, as illustrated in Figure S8.

These data show that the differences in bone marrow cellularity between AA^{Dx} stem from the presence of Ki-67⁺ regions that still contain hematopoietic progenitors. Importantly, in AA^{Dx}, only these Ki-67⁺ regions contain enlarged, activated macrophages, suggesting the active immune response resulting in AA occurs in these regions.

Lymphoid cells predominate in the bone marrow from AA patients at diagnosis

To perform detailed analyses of the hematopoietic compartment in AA^{Dx} at the single-cell level, cell segmentation was applied to all ROIs (Figure S4). This generated a dataset of $0.5 \cdot 10^6$ cells with the spatial localization and the marker expression profile for each segmented cell. A median of 4,137 cells (range 2,028-19,622) were segmented for each AA^{Dx}, compared to a median of 12,913 cells (range 1,212-37,336) for each AA^{GM} and a median of 29,523 cells (range 11,094-54,334) for each control.

All segmented cells were visualized in an optSNE analysis (Figure 2A), which discriminated five major cell types based on marker expression: 1) lymphoid cells including CD7⁺ T-cells, CD7⁺ innate lymphoid cells, CD20⁺ B-cells, CD138⁺ plasma cells and CD7⁺CD20⁺CD127⁺ cells that could represent a population of early lymphoid cells due to their expression of CD127 in the absence of CD3, CD20 and HLA-DR^{22,23}; 2) mature myeloid cells including CD14⁺ monocytes, CD15⁺ neutrophils and CD68⁺ macrophages; 3) stroma including CD271⁺ stromal cells, osteoblasts and endothelium; 4) CD71⁺ erythroid precursors and 5) myeloid progenitors including CD31⁺CD34⁺ hematopoietic progenitor cells (HPCs) (Figure 2A). $0.04 \cdot 10^6$ cells divided across all ROIs were unclassifiable and thus were excluded from

downstream analyses. Visualization of all AA^{Dx} and controls in the optSNE analysis showed clear differences in the cellular composition of the hematopoietic compartment between groups, with lymphoid cells predominating in AA^{Dx} (Figure 2B). Visualization of each patient separately revealed heterogeneity within the hematopoietic compartment between all AA^{Dx} (Figure 2C, top row). 6M post-hATG, a trend towards normalization was observed for all patients, except for patients AA8 and AA17 whose hematopoietic composition remained comparable to diagnosis (Figure 2C, red box).

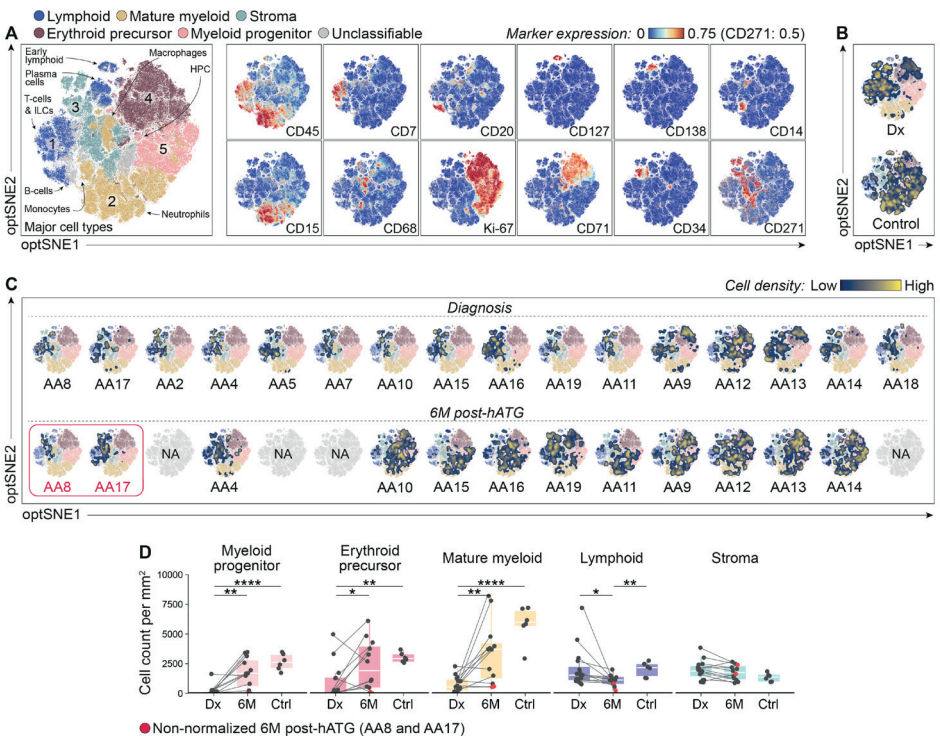


Figure 2. Global cellular composition of the hematopoietic compartment in bone marrow from AA patients and controls. Colors indicate major cell types (lymphoid cells, mature myeloid cells, myeloid progenitors, erythroid precursors, stroma and unclassifiable cells), groups (AA^{Dx}, AA^{6M} and controls) or marker expression values, where a marker expression value of 0.75 indicates that 75% of pixels of a segmented cell are positive for the corresponding marker. [A] 0.46-10⁶ cells segmented from 79 ROIs from AA^{Dx} (n=16 patients), AA^{6M} (n=12 patients) or controls (n=6 controls) visualized using an optSNE analysis. [B] Contour plots showing the cellular composition of the bone marrow in all ROIs collected from all AA^{Dx} (n=16) and controls (n=6). [C] Contour plots showing the cellular composition of the bone marrow in all ROIs collected from all AA^{Dx} and AA^{6M}, shown per patient per time point. The red box presents the patients with a non-normalized bone marrow 6M post-hATG (AA8 and AA17). [D] Abundance of the major cell types, shown as an absolute cell count per mm² of hematopoietic compartment. Each dot represents the mean of all ROIs collected from a patient or control. Box plots present the median with the interquartile range (IQR), error bars present 1.5*IQR.

To correct for myeloid cell depletion and bone marrow hypocellularity, we quantified the major cell types as an absolute count per mm² of hematopoietic compartment. As expected, we observed a profound decrease in precursor/progenitor and mature myeloid cells in all AA^{Dx} (Figure 2D). Consistent with the presence of Ki-67⁺ regions, precursor/progenitor cells were still present in the bone marrow from 7 AA^{Dx} (AA9, AA12-14, AA16-18), with most cells exhibiting a CD71⁺ erythroid precursor phenotype (Figure 2C). In contrast, absolute numbers of lymphoid cells and stroma did not differ between AA^{Dx} and controls, again indicating that they are the predominant cell types remaining in the bone marrow from AA^{Dx}.

Quantification of all major cell types 6M post-hATG confirmed a trend towards normalization of the precursor/progenitor, mature myeloid and lymphoid compartments in all patients, except for the two patients with a non-normalized bone marrow (Figure 2D).

Increase of pro-inflammatory lymphocytes in Ki-67⁺ regions in AA at diagnosis

Since activated CD68⁺CD163⁺CD4⁺ macrophages were exclusively present in Ki-67⁺ regions from AA^{Dx} (Figure 1F), we hypothesized that the active immune response resulting in HSPC destruction is best detectable in these Ki-67⁺ regions. Because patients can have both Ki-67⁺ and Ki-67⁻ regions at diagnosis, groups were defined by the presence of Ki-67⁺ cells, and the bone marrow was analyzed per ROI instead of per patient.

Clustering of cells from the hematopoietic compartment, excluding stroma, identified 40 distinct subpopulations of which the phenotypes were confirmed in the raw images (Figure S6). Identified phenotypes were clearly separable, except for CCR6⁺ B-cells (Figure 3A, magenta cells) and CCR6⁺CD45RO⁺CD4⁺ T-cells (Figure 3A, cyan cells) which were too close to be separated and thus were captured as one cluster.

15 progenitor and mature myeloid subpopulations (Figure 3B) and 25 lymphoid subpopulations were distinguished (Figure 3C). The absolute counts of all subpopulations (per mm² of hematopoietic compartment) across all ROIs were visualized as z-scores. ROIs were grouped according to disease status (patient versus control), treatment status (diagnosis or 6M post-hATG) and the presence of Ki-67. Consistent with the depletion of the progenitor compartment, CD31⁺CD34⁺ HPCs, CD31⁺CD34⁺ megakaryocytes, CD71⁺ erythroid precursors and other Ki-67⁺ myeloid progenitors were markedly reduced in AA^{Dx} compared to controls (Figure 3B, statistics in Figure S9). In line with reduced myelopoiesis in AA, all CD15⁺ neutrophils or CD14⁺ monocytes were decreased in AA^{Dx}. The presence of CD68⁺CD163⁺ macrophages in both Ki-67⁺ regions from AA^{Dx} and controls was confirmed in this analysis. Furthermore, a cluster of CD68^{dim}CD163⁺ macrophages was enriched in AA^{Dx} compared to controls. This cluster included the activated and enlarged CD68⁺CD163⁺CD4⁺ macrophages

exclusively found in Ki-67⁺ regions from AA^{Dx} (Figure 1F). Remarkably, CD11c⁺ mature myeloid cells fitting with a population of dendritic cells were abundant in the Ki-67⁺ regions from AA^{Dx}, a feature not observed in most Ki-67⁻ regions and controls. CD11b⁺ myeloid cells with differential expression of cKit were also abundant in the bone marrow from AA^{Dx}. CD11b⁺cKit⁻ cells resembling basophils²⁴ were prevalent in both Ki-67⁺ and Ki-67⁻ regions at diagnosis, whereas CD11b⁺cKit⁺ cells fitting with a population of mast cells²⁵ predominated across the Ki-67⁻ regions at diagnosis.

Within the lymphoid compartment (Figure 3C), we distinguished T- and B-cells in multiple differentiation stages. We observed a decrease of CD7⁻CD20⁻CD127⁺ early lymphoid-like cells in both Ki-67⁺ and Ki-67⁻ regions from AA^{Dx} compared to controls. The presence of other lymphoid subpopulations was heterogeneous among AA^{Dx}, with notable differences between Ki-67⁺ and Ki-67⁻ regions. All CD4⁺ T-cell, CD8a⁺ T-cell and CD56⁺ NK-cell populations were increased in Ki-67⁺ regions, whereas Ki-67⁻ regions mainly contained the expanded terminally differentiated (CD45RA⁺CD27⁻) CD8a⁺ T-cells from our previously identified immune cell network⁶. CCR6⁺ B-cells and CCR6⁺CD45RO⁺CD4⁺ T-cells, corresponding to the expanded CCR6⁺ lymphoid cells identified in our previous study⁶, were found in both Ki-67⁺ and Ki-67⁻ regions at diagnosis. B-cells with differential expression of the memory marker CD27²⁶ were increased in Ki-67⁺ regions but rarely observed in Ki-67⁻ regions at diagnosis. A cluster of CD20⁻CD138⁻CD27⁺CD38⁺ plasmablasts was decreased in all AA^{Dx}, with the exception of a few Ki-67⁺ regions. Plasma cells, reflecting a population of differentiated B-cells, were strongly increased in almost all AA^{Dx} and predominated in Ki-67⁻ regions at diagnosis. An exception to these patterns was observed for the Ki-67⁻ regions from AA8, which contained many T- and B-cell subpopulations across all differentiation stages.

These data indicate that the Ki-67⁺ regions are characterized by an influx of T-cells (green/cyan/yellow CD3⁺ T-cells in Figure 3D, top panel), ILCs and B-cells (red CD20⁺ B-cells in Figure 3D, lower panel) with a pro-inflammatory phenotype including the previously identified CCR6⁺ B-cells and CCR6⁺CD45RO⁺CD4⁺ T-cells. This observation fits with our hypothesis that Ki-67⁺ regions are the site of an active immune response in AA. In contrast, Ki-67⁻ regions from AA^{Dx} contain cells of a more terminally differentiated phenotype based on the presence of CD45RA⁺CD27⁻CD8a⁺ T-cells (cyan cells in Figure 3E, top panel) and plasma cells (green CD138⁺ cells in Figure 3E, lower panel) fitting with a more advanced stage of bone marrow destruction where the active immune response has waned. Collectively, this suggests a shift in the immune cell composition of the bone marrow during the development of AA. 6M post-hATG, a trend towards normalization of progenitors and immune cells was observed in patients with normalizing bone marrow, suggesting successful IST restores the balance in the bone marrow (Figure 3B-C).

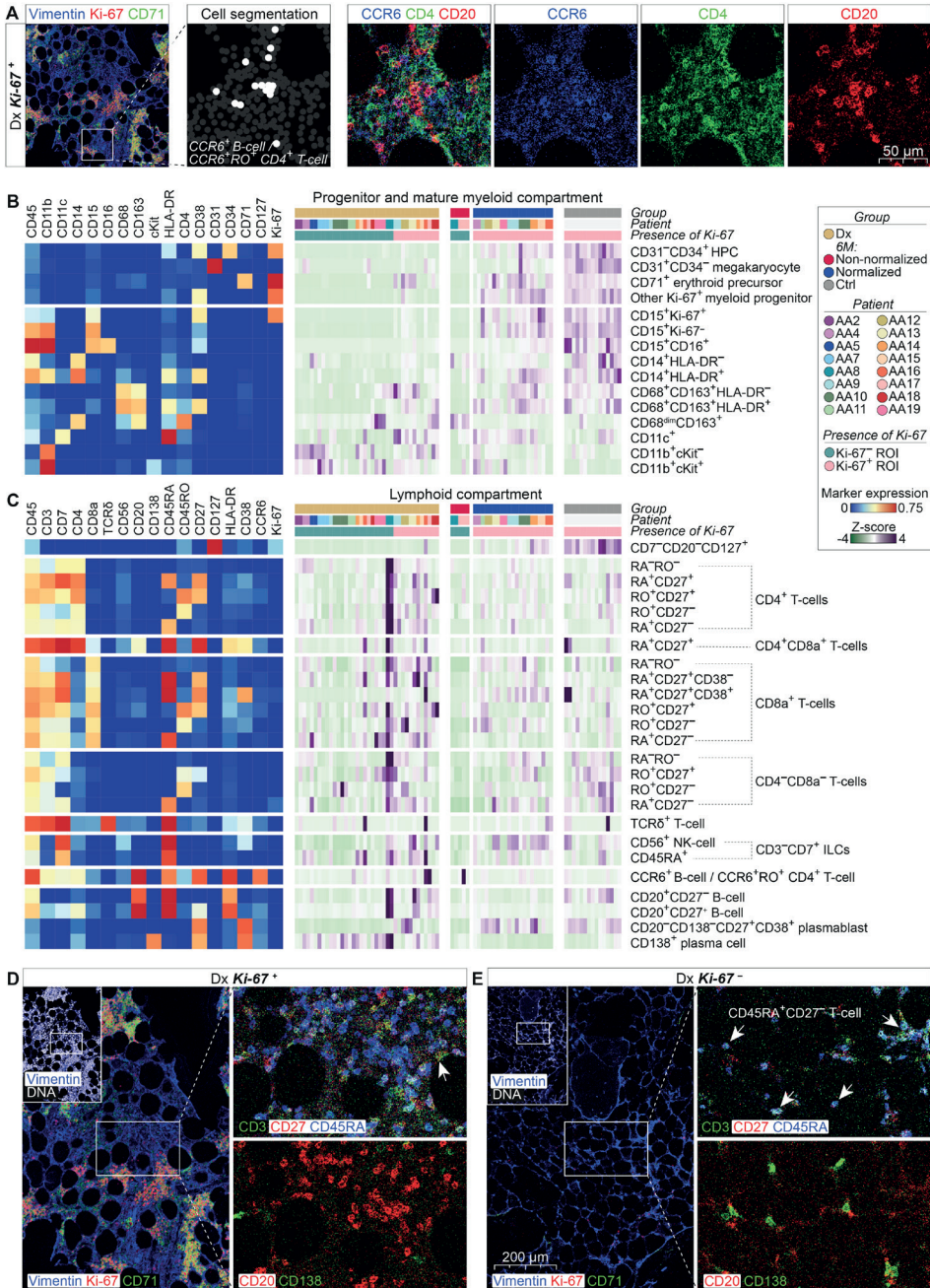


Figure 3. Subpopulation analysis of the myeloid and lymphoid compartments. [A] CCR6⁺ B-cells and CCR6⁺CD45RO⁺ CD4⁺ T-cells, visualized in a representative Ki-67⁺ ROI from an AA^{Dx}. CD4, CD20 visualize CD4⁺ T-cells and B-cells, respectively. [B-C] Heatmaps presenting the composition of the mature myeloid and progenitor compartments [B] or lymphoid compartment [C] across all AA^{Dx}, AA^{6M} and control ROIs at the subpopulation level. Left: heatmap showing the marker expression profiles of the identified

subpopulations, where a marker expression value of 0.75 indicates that 75% of pixels of a segmented cell are positive for the corresponding marker. Right: Heatmap visualizing the absolute cell counts of the identified cell subpopulations per mm² of hematopoietic compartment, shown as a z-score (green = less abundant; purple = more abundant compared to other ROIs). Colors indicate 1) groups, 2) patients or 3) the presence of Ki-67⁺ progenitor cells. Data are shown per ROI. [D] CD3⁺ T-cells in multiple differentiation stages; CD20⁺ B-cells and CD138⁺ plasma cells visualized in a representative Ki-67⁺ (left) and Ki-67⁻ (right) ROI from AA^{Dx}.

Pro-inflammatory lymphocytes colocalize with progenitors in the bone marrow in AA

To determine whether immune cells colocalize with precursors in Ki-67⁺ regions from AA^{Dx}, we examined the interactions of all identified subpopulations using cellular neighborhood analyses. This approach identified neighborhoods by clustering segmented cells from all groups based on the cellular phenotypes in their direct neighborhood (Figure 4A). Nine distinct neighborhoods were identified (Figure 4B). Neighborhoods 1 and 2, enriched for erythroid precursors, aligned with the CD71⁺ erythroblastic islands (Figure 4C). Neighborhoods 3-6, enriched for HPCs and/or mature myeloid cells, represented regions of progenitors and myelopoiesis. Neighborhood 7 and 8 were highly enriched for immune cells, with neighborhood 8 showing a more differentiated phenotype based on the presence of plasma cells. Neighborhood 9 mainly included stroma and aligned with blood vessels or osteoblasts lining the bone. Visualization of all neighborhoods in all AA^{Dx} and AA^{6M} is shown in Figure S10.

Neighborhoods 7 and 8, enriched for immune cells, were more abundant in the bone marrow from AA^{Dx} compared to controls (Figure 4D). 6M post-hATG, neighborhoods 7 and 8 decreased in patients with a normalizing bone marrow, suggesting a pathogenic role. Interestingly, multiple T-cell, ILC and B-cell subpopulations with a pro-inflammatory phenotype were also present in neighborhoods 3-6, suggesting a possible interaction between immune cells and progenitors in the bone marrow. In contrast, these activated immune subpopulations were absent in the erythroblastic islands (neighborhoods 1-2), indicating their interactions with erythroid precursors may be limited.

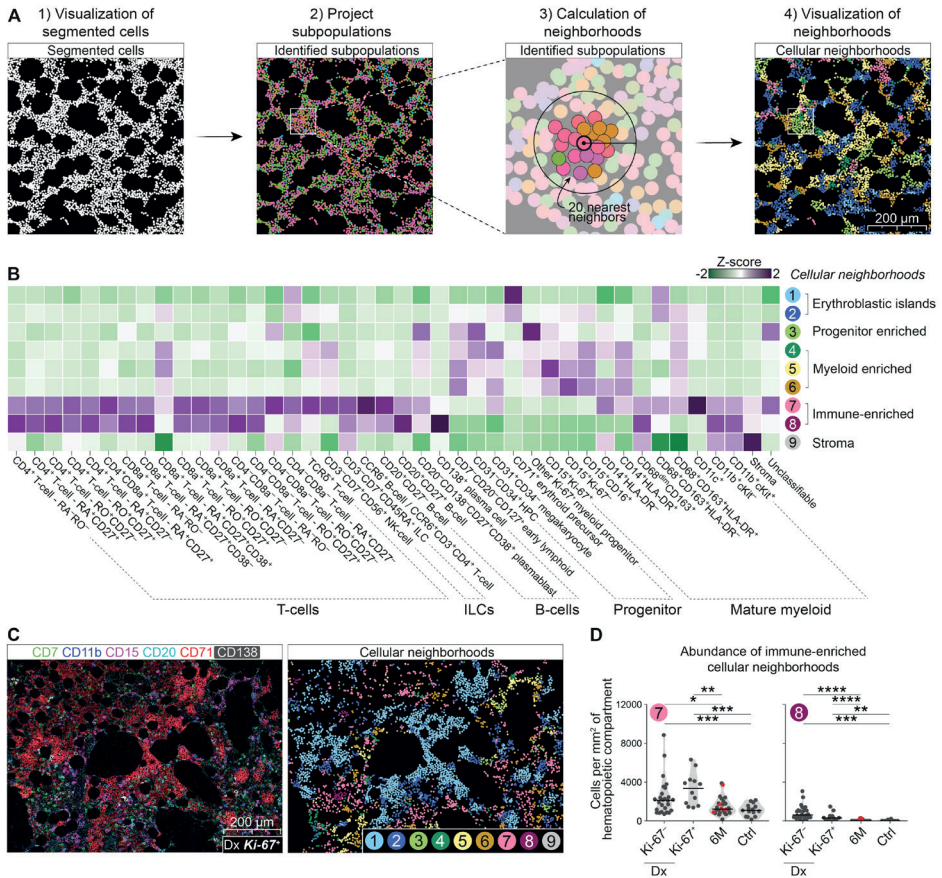


Figure 4. Identification of cellular neighborhoods in the bone marrow. Colors indicate identified subpopulations or cellular neighborhoods. [A] Approach used to identify neighborhoods. Step 1: The spatial location of each segmented cell was determined. Step 2: The phenotype of each segmented cell as identified in Figure 3 was visualized. Step 3: The neighborhoods were calculated. For each segmented cell in every ROI, the phenotypes of its 20 nearest neighbors within a 25-pixel distance were determined. Subsequently, all segmented cells were clustered into 9 neighborhoods based on the cellular composition of their direct microenvironment. [B] Cellular composition of the identified neighborhoods. Relative frequencies of all identified subpopulations within the neighborhoods are shown as a z-score (green = less frequent; purple = highly frequent compared to other neighborhoods). [C] IMC stainings and neighborhoods, visualized in a Ki-67⁺ region from an AA^{Bx} (patient AA9). CD7 stains T-cells and ILCs; CD11b stains myeloid cells; CD15 stains neutrophils; CD20 stains B-cells; CD71 stains erythroid precursors; CD138 stains plasma cells. [D] Abundance of neighborhoods 7 and 8 in the bone marrow across all groups, shown as an absolute cell count per mm² of the hematopoietic compartment. Data are shown per ROI. ROIs from patients with a non-normalized bone marrow 6M post-hATG are shown in red and were excluded from statistical analysis.

Development of AA can be visualized within the bone marrow

To investigate the interactions between immune cells and HSPCs, we examined the spatial distribution of the identified neighborhoods within the bone marrow. This discriminated four areas: 1) areas exclusively composed of erythroblastic islands (neighborhoods 1-2); 2) areas containing lymphoid cells from neighborhoods 7-8 along with progenitors; 3) areas where myeloid cells including progenitors (neighborhoods 3-6) were interspersed with lymphoid cells from neighborhoods 7-8 and 4) areas predominantly containing cells from neighborhood 8 (representative Ki-67⁺ region from AA^{Dx} in Figure 5A, all AA^{Dx} in Figure S10).

Each area represented a distinct stage in the development of AA. In the CD71⁺ erythroblastic islands (area 1), T- and B-cells were present in small numbers, similar to controls, indicating limited immune cell interactions with erythroid precursors in AA^{Dx} (Figure 5A-B, area 1, control in Figure S11). In contrast, area 2 included CD15⁺Ki-67⁺ neutrophil progenitors (green cells with blue nucleus in panel Bi) and was predominantly composed of pro-inflammatory T- and B-cell subpopulations in close proximity (Figure 5B, area 2). Therefore, we defined area 2 as a 'lymphoid-dominant' immune hot spot. This area included early effector T-cells (white CD3⁺CD45RA⁺CD27⁺ cells in panel Bii), memory T-cells (green/yellow CD3⁺CD45RA⁻CD45RO⁺ cells in panel Bii) and B-cells (red CD20⁺ cells in panel Biv), as well as the CCR6⁺ B-cells and CCR6⁺CD45RO⁺CD4⁺ T-cells (magenta CCR6⁺CD20⁺ / cyan CCR6⁺CD4⁺ cells in panel Biv) and terminally differentiated CD8a⁺ T-cells (cyan CD3⁺CD45RA⁺CD27⁻ in panel Bii) from our previously identified immune cell network⁶. The close proximity and highly activated state of the cells could represent the initial stage of immune activation in AA.

In area 3 (Figure 5A-B, area 3), the activated lymphoid cells and CD15⁺Ki-67⁺ neutrophil progenitors were accompanied by the activated and enlarged CD68⁺CD163⁺CD4⁺ macrophages (purple cells in panel Bv). Therefore, we defined these areas as 'macrophage-enriched immune hot spots', which may represent areas where the immune response has reached its maximum. Finally, in area 4 (Figure 5A-B, area 4), we observed high densities of terminally differentiated T-cells (cyan cells in panel Bii) and plasma cells (yellow cells in panel Biii), along with a few remaining CD68⁺CD163⁺CD4⁺ macrophages but no Ki-67⁺ progenitors.

We quantified the four identified tissue areas across all bone marrow regions using spatial context analyses previously described¹⁹. This confirmed the presence of the pro-inflammatory immune hot spots in all Ki-67⁺ regions from AA^{Dx} and demonstrated that Ki-67⁺ regions contained fewer immune-differentiated areas (area 4) compared to Ki-67⁻ regions (Figure 5C).

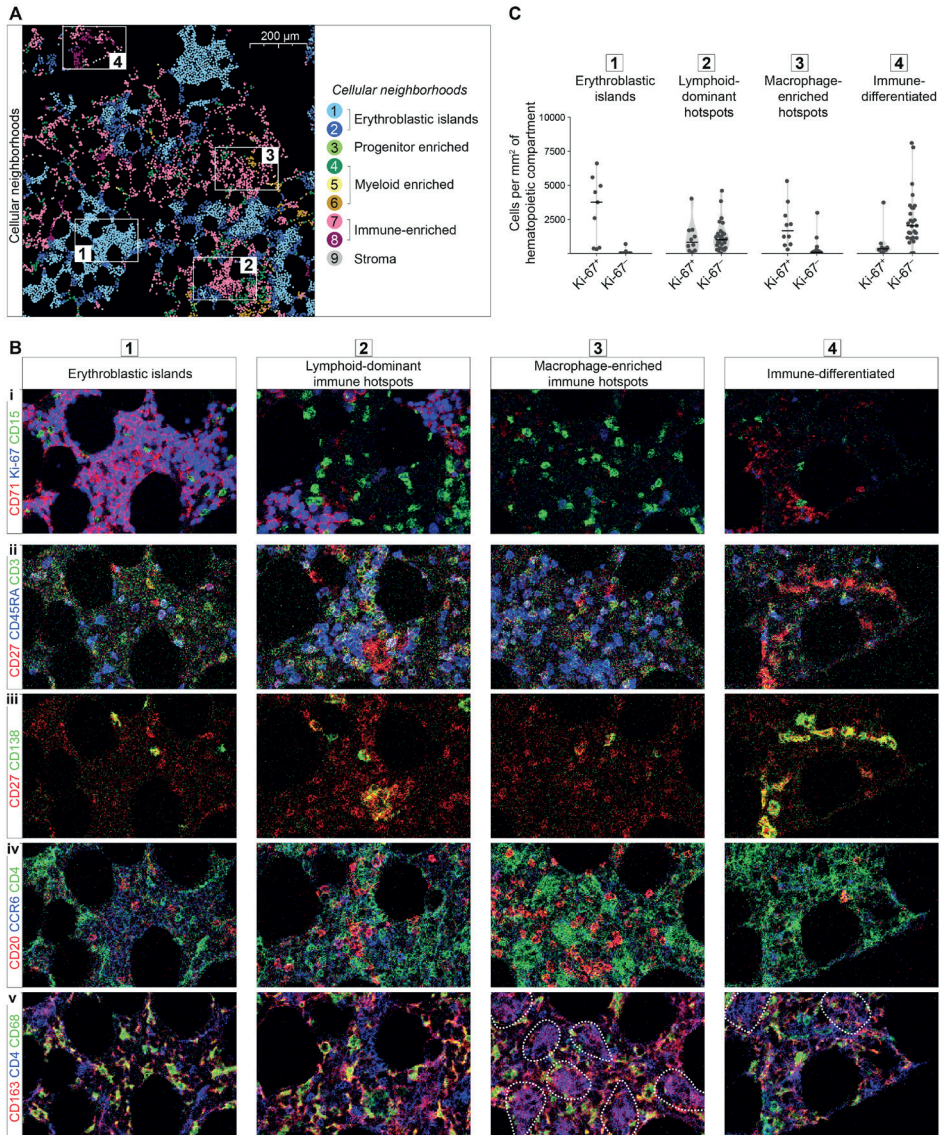


Figure 5. Cellular composition of four distinct microenvironments within the Ki-67⁺ regions from AA patients at diagnosis. [A] Visualization of cellular neighborhoods in a representative Ki-67⁺ region at diagnosis (patient AA13), revealing four distinct tissue areas: 1) erythroblastic islands; 2) lymphoid-dominant immune hot spots; 3) macrophage-enriched immune hot spots and 4) immune-differentiated tissue areas. [B] Presence of immune cell subpopulations, visualized in the four identified tissue areas. Panel Bi: Visualization of CD71⁺Ki-67⁺ erythroid precursors (red cells with blue nucleus) or CD15⁺Ki-67⁺ neutrophil progenitors (green cells with blue nucleus) in each tissue area. Panel Bii: Visualization of CD3⁺ T-cell populations in multiple differentiation stages in each tissue area. Panel Biii: Visualization of CD138⁺CD27⁺ plasma cells in each tissue area. Panel Biv: Visualization of (CCR6⁺) CD20⁺ B-cells and CD4⁺ T-cells in each tissue area. Panel Bv: Visualization of macrophages in each tissue area. CD4⁺CD68⁺CD163⁺ macrophages are circled. [C] Presence of the four identified tissue areas across all Ki-67⁺ and Ki-67⁻ ROIs from all AA^{dx}, shown as an absolute count per mm² of the hematopoietic compartment. Each dot represents a Ki-67⁺ or Ki-67⁻ ROI from an AA^{dx}. Horizontal bars present group median.

Bone marrow normalization is associated with a decrease in immune hot spots

Since IST can restore hematopoiesis, we investigated its effect on the immune response by studying the spatial distribution of the immune hot spots 6M post-hATG (Figure 6; all patients in Figure S10). In the two patients with a non-normalized bone marrow 6M post-hATG (AA8 and AA17), we observed a striking difference in the abundance of the hot spots. In AA8, the bone marrow was completely hypocellular and all hot spots were depleted, while in AA17 immune hot spots were still highly abundant (Figure 6A). Ten patients showed a trend toward normalization 6M post-hATG. In five of these patients, the hot spots were nearly absent (AA9, AA13: Figure 6B, top row; AA11, AA15, AA16: Figure S10). In the other five patients, hot spots persisted but were reduced in number compared to diagnosis, and activated $CD68^+CD163^+CD4^+$ macrophages were absent (AA10, AA14: Figure 6B, bottom row; AA4, AA12, AA19: Figure S10). No differences were observed between complete or partial responders to IST (Table S1). These findings indicate that bone marrow normalization 6M post-hATG is associated with changes in immune hot spots and can occur in all patients, regardless of the extent of hypocellularity of the bone marrow at diagnosis.

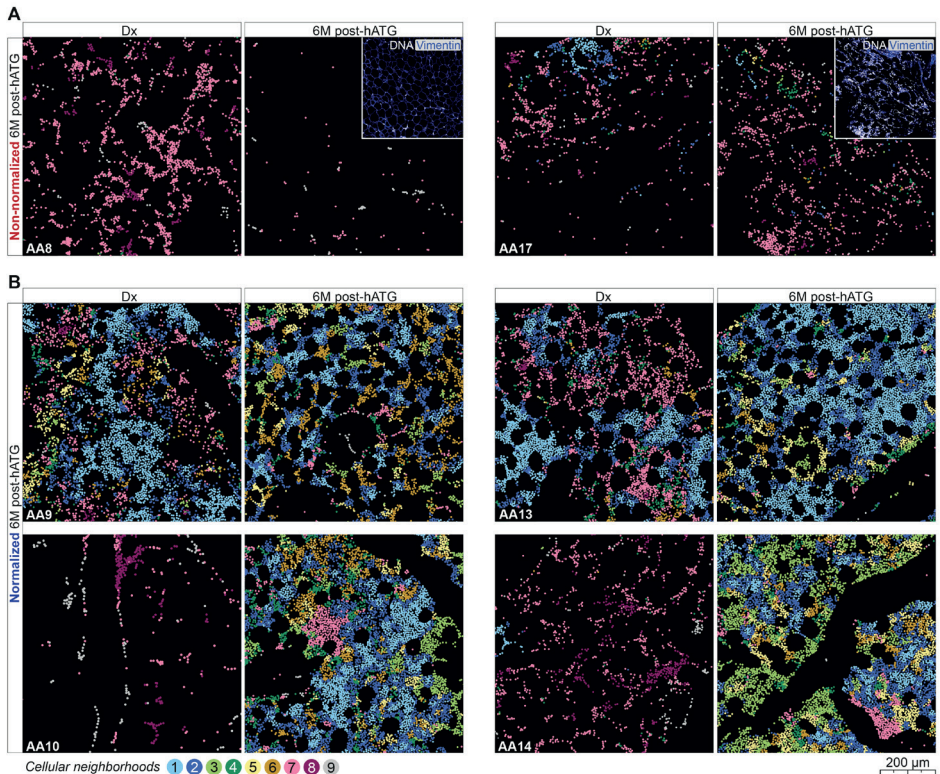


Figure 6. Effect of IST on the spatial cellular composition of the bone marrow. [A] Cellular neighborhoods, visualized in the bone marrow from the two patients with a non-normalized bone marrow 6M post-hATG (AA8 and AA17). [B] Cellular neighborhoods visualized in the bone marrow from four representative patients with a normalizing bone marrow 6M post-hATG (AA9, AA10, AA13, AA14). Colors indicate cellular neighborhoods.

Discussion

In this study, we aimed to interrogate the chronological order of events in the immune-mediated pathogenesis of AA by examining the spatial orientation of the immune response within the preserved bone marrow architecture. Previously, using suspension mass cytometry on bone marrow aspirates, we identified an AA-specific immune cell network enriched for CCR6⁺ B-cells, Th17-like²⁷⁻²⁹ CCR6⁺ memory CD4⁺ T-cells, and KLRG1⁺ terminally differentiated CD8⁺ T-cells⁶. We also observed CD163⁺ macrophages in the AA bone marrow, despite depletion of the myeloid compartment, suggesting macrophage recruitment to clear apoptotic cells. Building on these findings, we used imaging mass cytometry to study the spatial composition of the bone marrow microenvironment in AA. Our study expands upon early studies of the AA bone marrow⁷ and complements recent mappings of normal human and murine bone marrow using fluorescence-based imaging techniques^{23,30}. The present study extends our previous findings by confirming the presence of network populations in the AA bone marrow and demonstrating their spatial proximity. We show that CCR6⁺ B-cells and CCR6⁺CD45RO⁺CD4⁺ T-cells are organized in immune 'hot spots' in Ki-67⁺ regions where the active immune response in AA is concentrated. Remarkably, these CCR6⁺ cells are so closely located that they could not be separated in the clustering analysis, indicating strong pathogenic interactions of CCR6⁺ T- and B-cells in AA. Throughout both Ki-67⁻ and Ki-67⁺ regions from AA^{Dx} we observed terminally differentiated CD8⁺ T-cells representing the KLRG1⁺ terminally differentiated CD8⁺ T-cells with potential cytotoxic capacity³¹. These findings further support their involvement in AA and suggest these cells accumulate in the bone marrow following activation and differentiation due to repeated antigenic stimulation.

Building on our previous work, we exclusively observed CD68⁺CD163⁺CD4⁺ macrophages in Ki-67⁺ regions from AA^{Dx}, distinguishing them from other macrophage populations in AA^{Dx} and controls. These macrophages are activated, enlarged and are engulfing other hematopoietic cells, suggesting involvement in an active process within the AA bone marrow. CD163⁺ macrophages have been described in AA before, and have been associated with improved overall survival, particularly in non-severe AA patients²¹. It was suggested that these CD163⁺ macrophages could be beneficial through an immunosuppressive M2 macrophage-like state. Our results provide an alternative explanation. We found that CD68⁺CD163⁺CD4⁺ macrophages are organized in immune hot spots with high immune activity in Ki-67⁺ regions, where they are interspersed with activated lymphoid cells and progenitors. Whether CD68⁺CD163⁺CD4⁺ macrophages are direct contributors to HSPC destruction in AA or are mainly involved in the clearance of apoptotic cells cannot be deduced from our data and would be an important topic for future research.

Beyond the network populations, we demonstrate the presence of pro-inflammatory immune subpopulations in the Ki-67⁺ regions harboring the active immune response in AA. This underscores the importance of analyzing bone marrow biopsies, as relying solely on bone marrow aspirates may overlook potentially pathogenic populations and does not allow the analysis of cellular interactions within the bone marrow. Consistent with the view that AA is mediated by an autoreactive T-cell response¹, we identified various memory CD4⁺ and CD8⁺ T-cell subpopulations in the Ki-67⁺ regions, which could correspond to previous descriptions of expanded T-cell populations in peripheral blood and bone marrow aspirates^{28,32}. The presence of these activated and differentiated CD4⁺ and CD8⁺ T-cell subpopulations indicates ongoing T-cell responses and extends the previous observation demonstrating T-cell infiltration and clustering in the AA bone marrow, particularly around blood vessels⁷. In line with previous studies describing a profound decrease of regulatory T-cells in peripheral blood from AA patients^{28,29}, Tregs were not detected within the AA bone marrow. Beyond T-cells, we observed high numbers of ILCs including NK-cells in Ki-67⁺ regions from AA^{Dx}, contrasting with studies showing decreased NK-cell counts in peripheral blood³³⁻³⁵. Additionally, we detected high numbers of B-cells within Ki-67⁺ regions from AA^{Dx}, while B-cell counts were lower or comparable in peripheral blood between patients and controls^{33,35}. This suggests recruitment of specific immune subpopulations to the bone marrow in AA. This adds complexity to the immune response in AA, and suggests that the autoimmune response resulting in HSPC destruction is more diverse than previously recognized, and involves a broad spectrum of immune cell phenotypes rather than being only T-cell mediated.

Our findings raise important questions regarding the antigenic target(s) of the immune cells within the AA bone marrow. Despite advances in evaluating expanded T-cell clones in AA³⁶, the targeted antigen(s) and progenitor population(s) remain incompletely defined. Using IMC, we observed that Ki-67⁺ regions from AA^{Dx} contained fewer stem cells and myeloid progenitors, but similar numbers of erythroid precursors compared to controls. This could indicate the immune response in AA targets antigens confined to stem cells and myeloid progenitors. Erythroid precursors, further along in the hematopoietic tree, may evade depletion.

Our study provides a stepwise view of the immune response in AA development. Our data indicate the active immune response resulting in HSPC destruction in AA is mediated by a spectrum of T-, B- and myeloid cell subpopulations that interact within the bone marrow in a coordinated manner. We propose that the disease begins in 'lymphoid-dominant' immune hot spots within Ki-67⁺ regions, where interactions such as antigen presentation by B-cells and T-cell-mediated activation of B-cells activate the immune cascade. As the response progresses, 'macrophage-enriched' immune hot spots develop that are characterized by activated and enlarged CD68⁺CD163⁺CD4⁺ macrophages, which presumably are phagocytosing apoptotic

cells. In addition, activated T- and B-cells are still present that may orchestrate targeted immune responses through cytokine secretion and cytotoxic attack. After all HSPCs are depleted, only differentiated effector cells including terminally differentiated CD8⁺ T-cells and plasma cells remain. These terminally differentiated T-cells likely correspond to the clonally expanded³⁶ and cytotoxic^{32,36} T-cells identified in AA, contributing to continuous inflammation in the bone marrow. B-cell differentiation into plasma cells is also observed. Their differentiation and presence within the immune hot spots, alongside the detection of autoantibodies¹, suggests their active contribution to the immune response in AA, potentially through antigen presentation and antibody production. How the immune interactions lead to the actual bone marrow destruction would be an important topic for future *in vitro* studies.

To confirm the relevance of the immune hot spots in the pathogenesis of AA, we studied their presence 6M post-hATG and found a decrease in hot spots in all patients with a normalizing bone marrow. This suggests that IST works through a gradual process: hATG disrupts the coordinated immune cell interactions in the tissue, while ciclosporin remains needed to suppress remaining immune cells. Non-response to IST may result from AA not being immune-mediated, irreversible stem cell depletion pre-hATG or insufficient immunosuppression. In our study, two non-normalized patients showed distinct patterns: in AA8 immune hot spots were depleted, but bone marrow normalization may have been hindered by complete HSPC deletion due to the hyperactive immune response observed pre-hATG. In AA17, hot spots persisted 6M post-hATG, suggesting insufficient immunosuppression.

Collectively, our study provides the first visualization of the chronological order of events in the development of AA, mapping the complex interactions among immune cell subpopulations within the bone marrow microenvironment. These findings underscore the potential of both established and previously overlooked immune cell populations as possible therapeutic targets, and pave the way for research on more precise and effective treatment strategies for AA.

Acknowledgements

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Authorship contributions

E.S.P. designed the experimental set-up, performed experiments, analyzed results, interpreted the data, made the figures and wrote the manuscript. S.J.L. designed the experimental set-up, performed experiments and assisted with data analysis. M.E.IJ. designed the experimental set-up. V.vU. and J.H.F.F. interpreted the data. N.F.dM. designed the experimental set-up. F.K. and M.H.M.H. interpreted the data and supervised the project. J.M-L.T. conceptualized the study, recruited patients, interpreted the data, supervised the project and wrote the manuscript. All authors critically edited and reviewed the manuscript. All authors approved the final manuscript.

Conflicts of interest

The authors declare no competing financial interests.

Data availability statement

Imaging mass cytometry data are available from the corresponding author on reasonable request. Contact: Jennifer M-L Tjon, MD PhD; j.m.l.tjon@lumc.nl.

Supplemental methods

IMC panel design, antibody conjugation and antibody validation

A 37-marker IMC antibody panel for formalin-fixed paraffin-embedded (FFPE) EDTA-decalcified bone marrow biopsies was designed and optimized to capture both hematopoietic cells and non-hematopoietic cells of the bone marrow microenvironment. Antigens were selected based on publicly available RNA sequencing¹³ and our published suspension mass cytometry data of bone marrow aspirates from AA patients⁶. 31 of 37 antibodies of the IMC panel were selected from validated IMC panels published by our institute⁹⁻¹¹ and applied on FFPE EDTA-decalcified bone marrow in screening experiments with FFPE non-decalcified tonsil and FFPE EDTA-decalcified tonsil as a control to confirm antibody performance after tissue decalcification. For the other 6 antibodies of the IMC panel (CD34, CD59, CD71, CD271, CXCL12 and cKit), BSA and carrier-free formulated antibodies were purchased from multiple vendors and tested for performance on control FFPE non-decalcified tonsil, control FFPE EDTA-decalcified tonsil and FFPE EDTA-decalcified bone marrow using immunohistochemistry (IHC). All IHC stainings showed adequate signal-to-noise ratios and matched the staining patterns of publicly available tissue protein expression data in both non-decalcified and decalcified tissue¹⁴. The 6 antibodies were then conjugated in-house to heavy metal isotopes using Maxpar® X8 Antibody Labeling Kits (Standard BioTools) according to the manufacturer's instructions. After conjugation, the antibodies were eluted in 200mL of antibody stabilizer PBS (Candor Biosciences, cat 131125) containing 0.05% sodium azide. Subsequently, the metal-conjugated IMC antibodies were titrated for performance on FFPE EDTA-decalcified bone marrow and tonsil at 1:50 or 1:100 at 5 hours at room temperature (RT) or overnight at 4°C. All 37 IMC antibodies were stored at 4°C at all times. Since KLRG1⁺ EMRA CD8⁺ T-cells were part of our previously identified disease-specific immune cell network in AA⁶, we evaluated several KLRG1 antibodies but did not find a suitable clone and therefore did not add KLRG1 to our IMC panel.

Tissue staining

All bone marrow biopsies were fixed in 4% formalin, decalcified overnight in 10% EDTA and embedded in paraffin after collection. To perform IMC, the FFPE EDTA-decalcified bone marrow biopsies were cut at a thickness of 5µm and mounted on FLEX IHC Microscope Slides (Dako, cat K8020). Subsequently, slides were baked overnight at 37°C and stored at 4°C until further use. Slides were sealed in paraffin to reduce oxidation if stored at 4°C for longer than four weeks.

Bone marrow tissue sections were stained with the 37-marker IMC panel over three days as previously described⁹. Briefly, tissue sections were deparaffinized in xylol, rinsed in ethanol and boiled for 10 minutes in IHC Antigen Retrieval Solution 10x Low Ph (Invitrogen, cat 00495558). Subsequently, slides were cooled to RT and blocked for 30 minutes at RT with Superblock Blocking Buffer (ThermoFisher, cat 37515). Next, excess blocking solution was removed and slides were stained overnight at 4°C with the unconjugated primary antibodies for CD4 and TCR δ in a volume of 100 μ L. The following morning, slides were washed and stained with the two secondary antibodies conjugated to 145Nd and 148Nd in a volume of 100 μ L in PBS with 1% BSA and 0.05% Tween. All washes were performed 3 times for 5 minutes in PBS with 1% BSA and 0.05% Tween. After a one hour incubation at RT, slides were washed and stained with 17 of 35 primary IMC antibodies for 5 hours at RT in a volume of 100 μ L (Table S2). Subsequently, slides were washed and stained overnight with the remaining 18 primary IMC antibodies at 4°C in a volume of 100 μ L. On the morning of the third day, slides were washed and stained with DNA intercalator-Ir (Standard BioTools, cat 201192A) for 5 minutes at RT in a volume of 100 μ L. Lastly, slides were washed and dried under an air flow. Slides were stored at 4°C until measurement.

Definition of three histological compartments in the bone marrow tissue

AA is characterized by severe damage to the bone marrow, leading to an 'empty' bone marrow. Therefore, large differences in bone marrow histology were observed between AA^{Dx}, AA^{6M} and controls. To enable quantitative comparison between biopsies we quantified the surface areas of three major compartments in the bone marrow: the hematopoietic compartment, adipocytes and bone.

The hematopoietic compartment was defined as hematopoietic cells including their direct microenvironment. To determine the surface area of the hematopoietic compartment, for each ROI, multi-page .tiff images were exported from MCD viewer, hyperbolic arcsinh transformed with a cofactor of 5 and loaded into Cytosplore Imaging software v3.2.6 (unpublished, adapted from Cytosplore¹⁸) for dimensionality reduction. Subsequently, a 2-level hierarchical stochastic neighbor embedding (HSNE) analysis was performed for all pixels (1 pixel = 1 μ m²) in each ROI with default settings and 500 iterations. All markers of the IMC panel and the DNA stain were used to generate the HSNE. At the overview level of each HSNE analysis, all pixels in an ROI were summarized into landmarks based on similarities in marker expression intensities and the spatial localization of each landmark in each ROI could be evaluated. Next, we selected all landmarks corresponding to the hematopoietic compartment. DNA⁺ landmarks were selected to capture the nucleus of all cells within the hematopoietic compartment. Vimentin⁺ landmarks were selected to capture the cytoplasm

and cell membrane of all cells and their microenvironment. Vimentin was chosen over CD45 in this process to capture all pixels corresponding to both non-hematopoietic cells and hematopoietic cells (visualized in Figure S3). Additionally, CD31⁺ landmarks were selected to capture the cytoplasm of Vimentin⁺CD31⁺ megakaryocytes. The combined selection of these DNA⁺, Vimentin⁺ and/or CD31⁺ pixels enabled us to determine the total number of pixels within the hematopoietic compartment (where 1 pixel = 1μm²). A few ROIs in the IMC dataset included unrepresentative regions with few cells and poor tissue quality due to blood clots, which therefore were subtracted from the surface area of the hematopoietic compartment. To determine the surface areas of these unrepresentative regions, .tiff images of these ROIs were exported from MCD Viewer and loaded into ImageJ v1.53q (Fiji). Subsequently, the surface area of the blood clots was manually selected, calculated and subtracted from the total surface area of the hematopoietic compartment using the Vimentin and DNA stains. A visualization of the approach used to determine the surface area of the hematopoietic compartment in Cytosplore Imaging is shown in Figure S3.

Bone was defined as the part of the bone marrow section that consists of Collagen I⁺ bone trabeculae. To determine the surface area of bone, for each ROI, .tiff images were exported from MCD Viewer and loaded into ImageJ v1.53q (Fiji). Subsequently, surface areas corresponding to bone were identified, selected and calculated using the Collagen I, Vimentin and DNA stains.

The surface area of adipocytes was defined by subtracting the surface areas of the hematopoietic compartment, blood clots and bone from the total ROI surface area. A few ROIs were ablated at the edge of a bone marrow biopsy and therefore included 'empty' regions without bone marrow tissue. If present, the surface areas of these empty regions were calculated by manual selection in ImageJ and also subtracted from the total ROI surface area to obtain the surface area of adipocytes.

Cell segmentation and background removal

To enable detailed interrogations of single cells in the images we applied a cell segmentation pipeline as shown in Figure S4 to all acquired ROIs. First, a single-cell mask was generated for each acquired ROI as previously described¹⁶. To ensure proper segmentation of cells with different shapes, namely megakaryocytes and stromal cells, a multi-layer cell segmentation mask was applied.

To generate the cell segmentation mask, Ilastik v1.3.2³⁷ was used to generate three probability maps based on the signal of the DNA stain, the stromal cell marker CD271 and megakaryocytes (CD31⁺Vimentin⁺) for each ROI. Subsequently, the three probability maps

were loaded into CellProfiler v4.2.1¹⁷, where each respective map was used to identify primary objects in each ROI. The size of each DNA object was increased by 1 pixel (1 pixel = $1\mu\text{m}^2$) to accurately capture the marker expression on the cells' membrane in downstream analyses. The holes in all identified megakaryocyte objects, corresponding to the megakaryocyte nuclei, were filled using the fill holes strategy in CellProfiler to avoid separate segmentation of the megakaryocyte cytoplasm and nucleus.

Next, the three sets of primary objects (DNA⁺, CD271⁺ and CD31⁺Vimentin⁻ objects) were combined using the preservation strategy for overlapping objects in CellProfiler. Since the primary aim of this study was to interrogate the immune response in the bone marrow in AA, DNA⁺ objects were favored over stromal cell objects if these objects overlapped. Megakaryocyte objects were placed over DNA objects to avoid the separate segmentation of the megakaryocyte cytoplasm and nucleus (described above). Finally, the 3-layer single-cell mask with DNA⁺, CD271⁺ and CD31⁺Vimentin⁻ objects was exported from CellProfiler for each ROI and compared to the raw IMC images. Since CD271⁺ objects were placed under DNA⁺ and CD31⁺Vimentin⁻ objects in the cell segmentation mask, stromal cells were regularly split into multiple objects in the final 3-layer mask.

To optimize the quality of the recovered data and to reduce noise, semi-automated background removal by an interactive machine learning algorithm in Ilastik v1.3.2 was used to remove background signals from true signals and to create binary signal masks for each marker in the IMC panel. Background removal was performed as previously described¹⁶ and returned binary signal masks for each marker in the IMC panel for every acquired ROI. To ensure proper noise removal, exported binary signal masks were compared to the original IMC images (unthresholded stainings) before proceeding to the next step in the segmentation pipeline.

Finally, to retrieve single-cell information from the IMC images, the 3-layer segmentation masks and corresponding binary signal masks were loaded into ImaCyte v1.0 and aligned for each ROI. Subsequently, a .fcs file with marker expression values for all identified cells was generated and exported for each ROI.

Cell clustering analysis

.fcs files of all $0.50 \cdot 10^6$ segmented cells from all acquired ROIs were loaded into OMIQ (Dotmatics) for data inspection. $0.04 \cdot 10^6$ cells were positive or negative for all markers of the IMC panel and/or were positive for the background channel ²⁰⁸Pb and thus were excluded from the analysis. Subsequently, .fcs files of all remaining $0.46 \cdot 10^6$ cells were exported, ROI-tagged and imported in Cytosplore¹⁸ for dimensionality reduction. A three-level hierarchical stochastic neighbor embedding (HSNE) clustering analysis was performed with default

perplexity and iterations (30 and 1000, respectively; see Figure S5 for HSNE analysis pipeline). Three of 37 markers in the IMC panel (CD19, CD57 and CD59) were not used in the HSNE clustering analysis; CD19 and CD59 stainings were suboptimal and CD57 staining was not performed on all bone marrow sections. Three major groups of cells of $\leq 0.5 \cdot 10^6$ cells were distinguished at the overview level of the HSNE analysis by Gaussian mean shift (GMS) clustering. Group 1 included cells expressing Collagen I, CXCL12, CD31, CD71 and/or CD271. Group 2 consisted of cells expressing CD3, CD7, CD20, CD27, CD127 and/or CD138. Group 3 included all remaining cells. Subsequently, each group of cells was selected for detailed analysis at the data level using t-distributed stochastic neighbor embedding (tSNE) analyses with up to $0.5 \cdot 10^6$ landmarks. A total of 236 clusters with distinct marker expression were identified within the three tSNEs by Gaussian mean shift (GMS) clustering. Next, .fcs files of all 236 phenotypes were exported and projected back onto the cell segmentation masks in ImaCytE to verify their marker expression profile in the unthresholded IMC stainings. All clusters except for a cluster of Collagen I⁺ bone cells were then manually merged to 5 major cell types or to 42 cell subpopulations based on similarities in marker expression profile using Cytofast¹⁵ in R v4.3.0 for analyses at the major cell type or cell subpopulation level, respectively. The phenotypes of all clusters of hematopoietic cells were confirmed in the raw images (Figure S6). To compare marker expression profiles and abundancies of all cell types between groups, .fcs files of major cell types or cell subpopulations were loaded into R and studied using the Cytofast pipeline¹⁵. Identified cell clusters were visualized using optSNE analyses performed in OMIQ (Dotmatics).

Identification of cellular neighborhoods and spatial context analyses

To evaluate the spatial distribution of cell subpopulations in the bone marrow from AA^{Dx}, AA^{6M} and controls, we performed cellular neighborhood analyses. XY coordinates of all segmented cells were extracted from CellProfiler and added to the .fcs files of the cell clusters identified in the HSNE analysis by aligning the data in R. Cellular neighborhood analyses were then performed for all acquired ROIs using the R package imcRtools¹⁹. Cells were grouped into 10 cellular neighborhoods by unbiased k-means clustering based on the phenotypes of their 20 nearest neighbors within a 25-pixel distance. Two of the identified neighborhoods were comparable in cellular composition and therefore were merged, resulting in a total of 9 neighborhoods. To interrogate the interactions of distinct neighborhoods, we performed spatial context analyses downstream of the cellular neighborhood analyses using default settings and a 50-nearest neighbor graph for spatial context detection, also with imcRtools. All further analyses and visualization of the neighborhoods were performed in R.

Supplemental tables

Table S1. Studied time points and patient characteristics.

Patient	Studied time points		Peripheral blood counts at diagnosis			Days between marrow sampling and start hATG	Response to IST	
	Dx	Disease severity	Hb (mmol/L)	Reticulocytes ($\cdot 10^9/L$)	Thrombocytes ($\cdot 10^9/L$)	Neutrophils ($\cdot 10^9/L$)	6M post-hATG	>6M post-hATG if NR at 6M
AA2	NA	VSAA	4.2	5.3	20	0.02	7	PR
AA4		VSAA	4.9	4.6	15	0.01	17	NR
AA5	NA	NSAA	5.9	39.2	31	2.73	30	PR
AA7	NA	SAA	5.7	13.6	24	0.37	49	NR
AA8		VSAA	5.8	2.2	23	0.00	33	NR
AA9		SAA	4.1	38.0	26	0.32	33	PR
AA10		VSAA	4.0	4.7	10	0.03	16	PR
AA11		VSAA	5.8	6.7	15	0.16	19	NR
AA12		SAA	6.4	20.7	22	0.81	13	NR
AA13		SAA	5.2	37.3	15	0.50	30	NR
AA14		SAA	4.4	11.9	38	0.23	16	PR
AA15		SAA	5.3	10.0	15	0.35	6	CR
AA16		SAA	5.7	4.7	132	0.61	20	CR
AA17		SAA	4.8	4.2	38	0.02	133	NR
AA18	NA	NSAA	3.9	60.0	14	0.81	52	PR
AA19		VSAA	5.4	5.8	12	0.04	56	NR

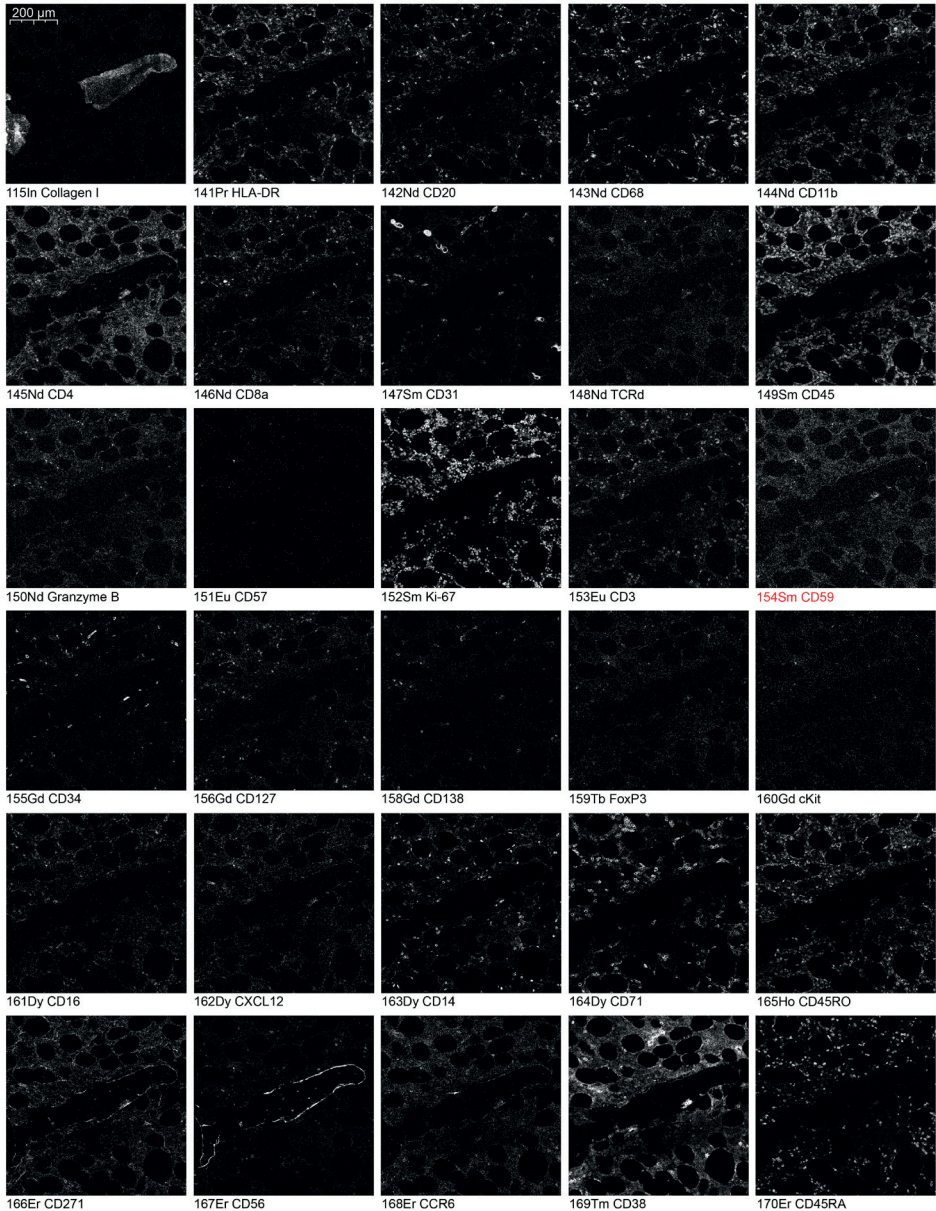
AA indicates aplastic anemia; CR: complete response; hATG: horse-derived anti-thymocyte globulin; Hb: hemoglobin; IST: immunosuppressive therapy; M: months; NA: not available; NR: no response; NSAA: non-severe AA; PR: partial response; SAA: severe AA; VSAA: very severe AA. Response to IST was defined as transfusion independence and a neutrophil count of $\geq 0.5 \cdot 10^9$ neutrophils/L. The distinction between partial and complete response to IST was made in accordance with the Dutch treatment guidelines. Response to IST ≥ 6 months post-hATG occurred either spontaneously or after eltrombopag (Revolade®, Novartis).

Table S2. 37-marker imaging mass cytometry panel.

Metal	Marker	Clone	Vendor	Dilution	Condition
115In	CollagenI	EPR7785	Abcam	1:100	ON 4°C
141Pr	HLA-DR	TAL-1B5	ThermoFisher	1:100	5H RT
142Nd	CD20	H1	BD Biosciences	1:100	ON 4°C
143Nd	CD68	D4B9C	Cell Signaling Technology	1:100	ON 4°C
144Nd	CD11b	D6X1N	Cell Signaling Technology	1:100	5H RT
145Nd	CD4	EPR6855	Abcam	1:200	Indirect
146Nd	CD8a	D8A8Y	Cell Signaling Technology	1:50	5H RT
147Sm	CD31	89C2	Cell Signaling Technology	1:100	ON 4°C
148Nd	TCRd	H-41	Santa Cruz	1:50	Indirect
149Sm	CD45	D9M8I	Cell Signaling Technology	1:50	ON 4°C
150Nd	Granzyme B	D6E9W	Cell Signaling Technology	1:100	5H RT
151Eu	CD57	HNK-1/Leu-7	Abcam	1:100	ON 4°C
152Sm	Ki-67	8D5	Cell Signaling Technology	1:100	ON 4°C
153Eu	CD3	D7A6E	Cell Signaling Technology	1:50	ON 4°C
154Sm	CD59	MEM-43/5	Exbio	1:50	ON 4°C
155Gd	CD34	QBEND/10	ThermoFisher	1:100	5H RT
156Gd	CD127	EPR23747-333	Abcam	1:50	5H RT
158Gd	CD138	MI15	BioLegend	1:100	5H RT
159Tb	FoxP3	D6O8R	Cell Signaling Technology	1:50	ON 4°C
160Gd	cKit (CD117)	D3W6Y	Cell Signaling Technology	1:50	5H RT
161Dy	CD16	D1N9L	Cell Signaling Technology	1:100	ON 4°C
162Dy	CXCL12 (SDF-1)	79018	R&D Systems	1:50	ON 4°C
163Dy	CD14	D7A2T	Cell Signaling Technology	1:100	5H RT
164Dy	CD71	H68.4	Invitrogen	1:100	5H RT
165Ho	CD45RO	UCHL1	Cell Signaling Technology	1:100	ON 4°C
166Er	CD271 (NGFR)	NGFR5	Abcam	1:50	ON 4°C
167Er	CD56	E7X9M	Cell Signaling Technology	1:100	5H RT
168Er	CCR6	EPR22259	Abcam	1:50	5H RT
169Tm	CD38	EPR4106	Abcam	1:100	ON 4°C
170Er	CD45RA	HI100	BioLegend	1:100	5H RT
171Yb	CD15	BRA-4F1	Cell Signaling Technology	1:100	ON 4°C
172Yb	CD19	D4V4B	Cell Signaling Technology	1:100	5H RT
173Yb	CD163	EPR14643-36	Abcam	1:50	5H RT
174Yb	CD7	EPR4242	Abcam	1:100	5H RT
175Lu	CD27	EPR8569	Abcam	1:100	ON 4°C
176Yb	CD11c	EP1347Y	Abcam	1:100	5H RT
194Pt	Vimentin	D21H3	Cell Signaling Technology	1:50	ON 4°C
191-193Ir	DNA	NA	Standard BioTools	1:100	5min RT

CCR indicates chemokine receptor; CD: cluster of differentiation; CXCL: C-X-C motif chemokine; HLA: Human Leukocyte Antigen; ON 4°C: overnight at 4°C; RT: room temperature; TCR: T-cell receptor; 5H RT: 5 hours at room temperature.

Supplemental figures



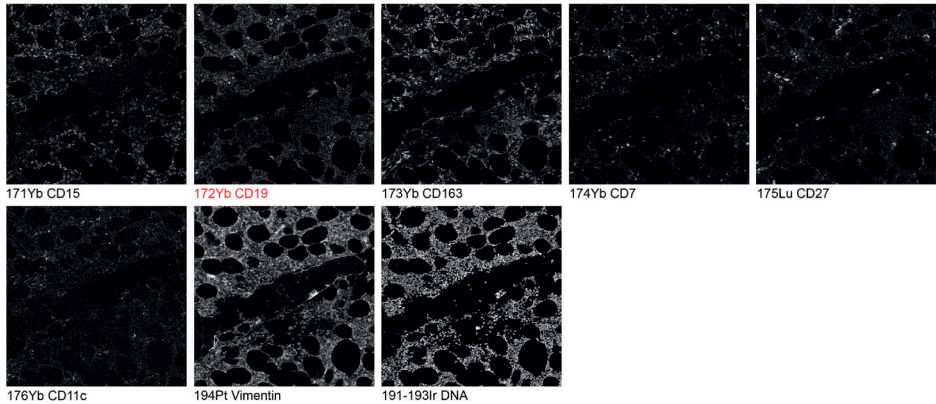
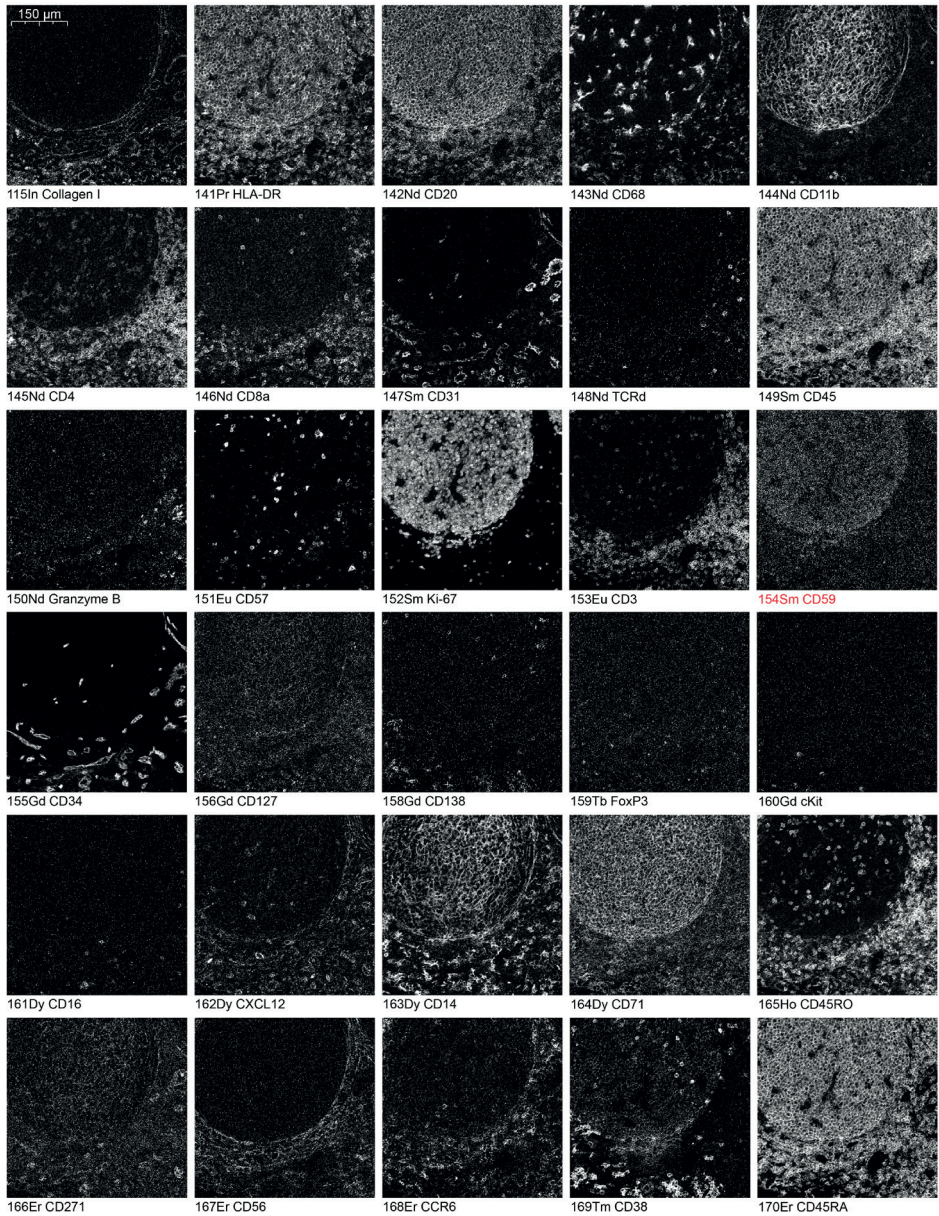


Figure S1. Performance of the 37-marker IMC panel for bone marrow on FFPE EDTA-decalcified bone marrow. Thresholded images of the staining results are shown for a representative control bone marrow ROI (Control5) with a surface area of 0.56mm². Suboptimal stainings excluded from downstream analyses are highlighted in red. CD59 stainings were weak and could not distinguish between CD59⁺ and CD59⁻ cells, as evaluated on a FFPE EDTA-decalcified bone marrow biopsy from a PNH patient with 98% GPI-negative clones in peripheral blood. CD19 stainings showed co-expression of CD19 and CD20 in FFPE EDTA-decalcified tonsil, but did not produce a similar result in FFPE EDTA-decalcified bone marrow.



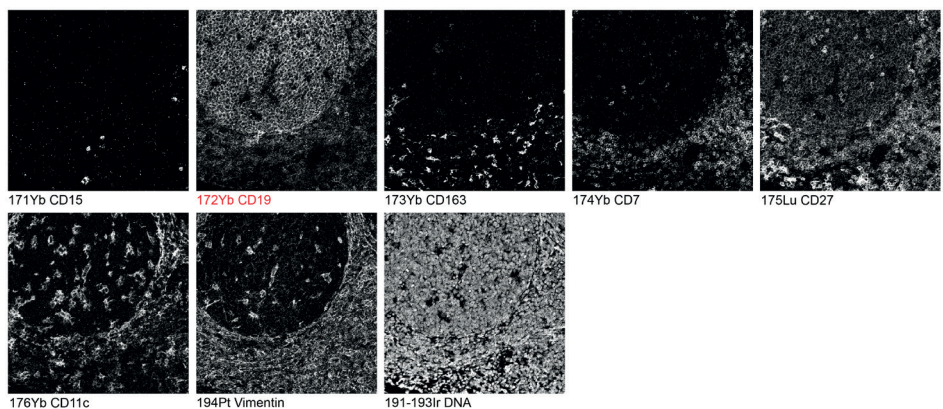


Figure S2. Performance of the 37-marker IMC panel for bone marrow on FFPE EDTA-decalcified tonsil. Thresholded images of the staining results are shown for FFPE EDTA-decalcified human tonsil with a surface area of 0.25mm², which was used as a positive control to confirm panel performance on decalcified tissue. Suboptimal stainings excluded from downstream analyses are highlighted in red. CD59 stainings were weak and could not distinguish between CD59⁺ and CD59⁻ cells, as evaluated on a FFPE EDTA-decalcified bone marrow biopsy from a PNH patient with 98% GPI-negative clones in peripheral blood. CD19 stainings showed co-expression of CD19 and CD20 in FFPE EDTA-decalcified tonsil, but did not produce a similar result in FFPE EDTA-decalcified bone marrow.

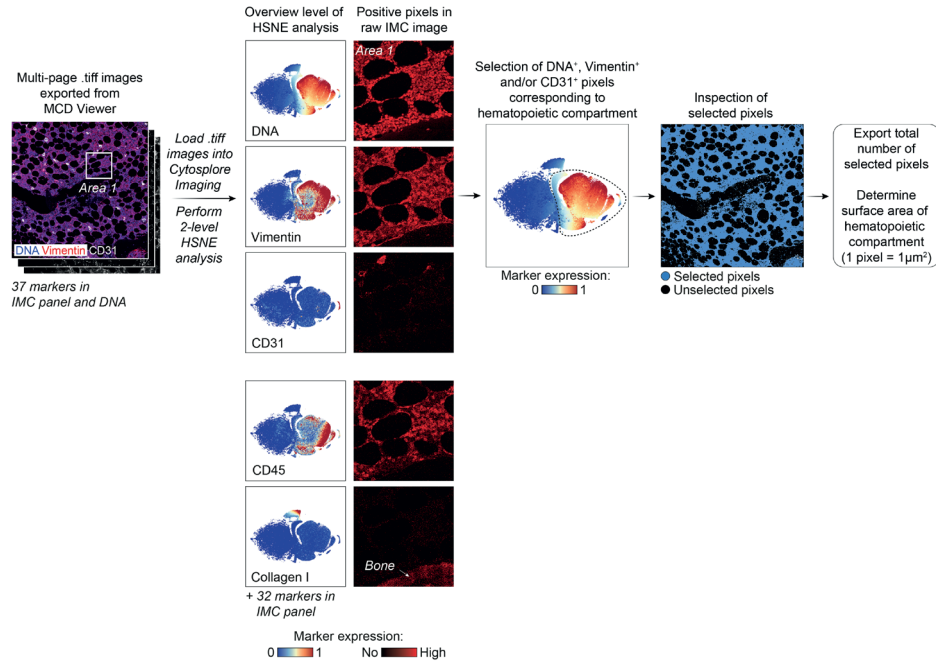


Figure S3. Approach used to determine the surface area of the hematopoietic compartment in Cytospore Imaging. The analysis pipeline is illustrated for a representative control ROI (Control5). For each ROI, multi-page .tiff files were exported from MCD Viewer and loaded into Cytospore Imaging for dimensionality reduction. A 2-level HSNE analysis was run for all pixels in each ROI. At the overview level of the 2-level HSNE analysis, all pixels in the ROI were summarized into landmarks based on similarities in marker expression intensities. Visualization of all markers of the 37-marker IMC panel and DNA in the HSNE showed the presence of DNA⁺, Vimentin⁺, CD31⁺, CD45⁺ and/or Collagen I⁺ pixels in each ROI image. DNA⁺ pixels captured cellular nuclei; Vimentin⁺ pixels captured cytoplasm and cell membrane; CD31⁺ pixels captured the Vimentin⁺ cytoplasm of megakaryocytes. Vimentin stained both non-hematopoietic and hematopoietic cells and therefore captured a broader range of cells than the hematopoietic cell marker CD45. Next, DNA⁺, Vimentin⁺ and/or CD31⁺ pixels were selected and inspected by projecting all selected pixels back on to the ROI image. Finally, the total number of selected pixels was determined and the surface area of the hematopoietic compartment was calculated (where 1 pixel = 1 μm²).

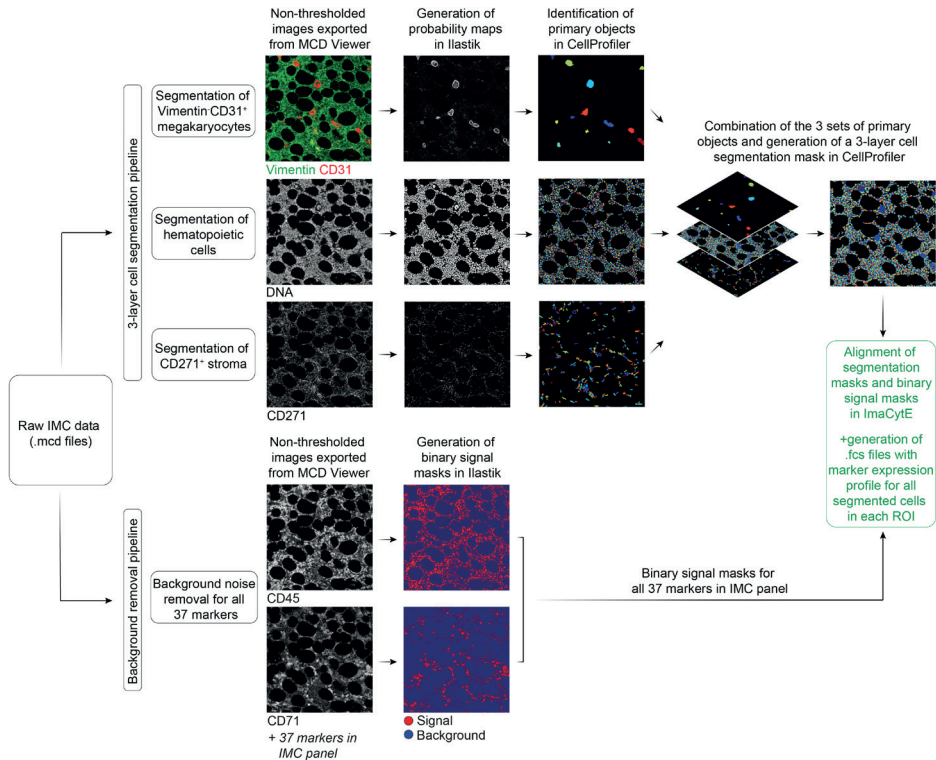


Figure S4. Cell segmentation pipeline. To extract single-cell information from the high-dimensional images, a 3-step cell segmentation pipeline was applied on all acquired ROIs. The pipeline is shown for a representative control ROI (Control5). Step 1) Generation of a cell segmentation mask. A 3-layer cell segmentation mask was generated for each ROI in the IMC dataset. For proper segmentation of megakaryocytes, hematopoietic cells and stromal cells separate cell segmentation masks were generated for each of these cell types. First, raw (non-thresholded) images of the Vimentin and CD31, DNA and CD271 stainings were exported from MCD Viewer and imported in Ilastik to generate probability maps by pixel classification. Subsequently, all probability maps were loaded into CellProfiler to create a segmentation mask for based on each probability map, which were then stacked to generate the final 3-layer cell segmentation mask. Step 2) Generation of binary signal masks for background noise removal. Raw (non-thresholded) images were exported from MCD Viewer for all 37 markers in the IMC panel and all ROIs in the IMC dataset. Next, true marker signals were distinguished from background noise by performing pixel classification in Ilastik. Subsequently, the generated thresholded binary signal masks were exported. Step 3) Alignment of segmentation masks and binary signal masks. Finally, to generate .fcs files with marker expression values for every segmented cell in each ROI, 3-layer segmentation masks and their corresponding thresholded binary signal masks were imported in ImaCyte and aligned. .fcs files were exported for each acquired ROI separately.

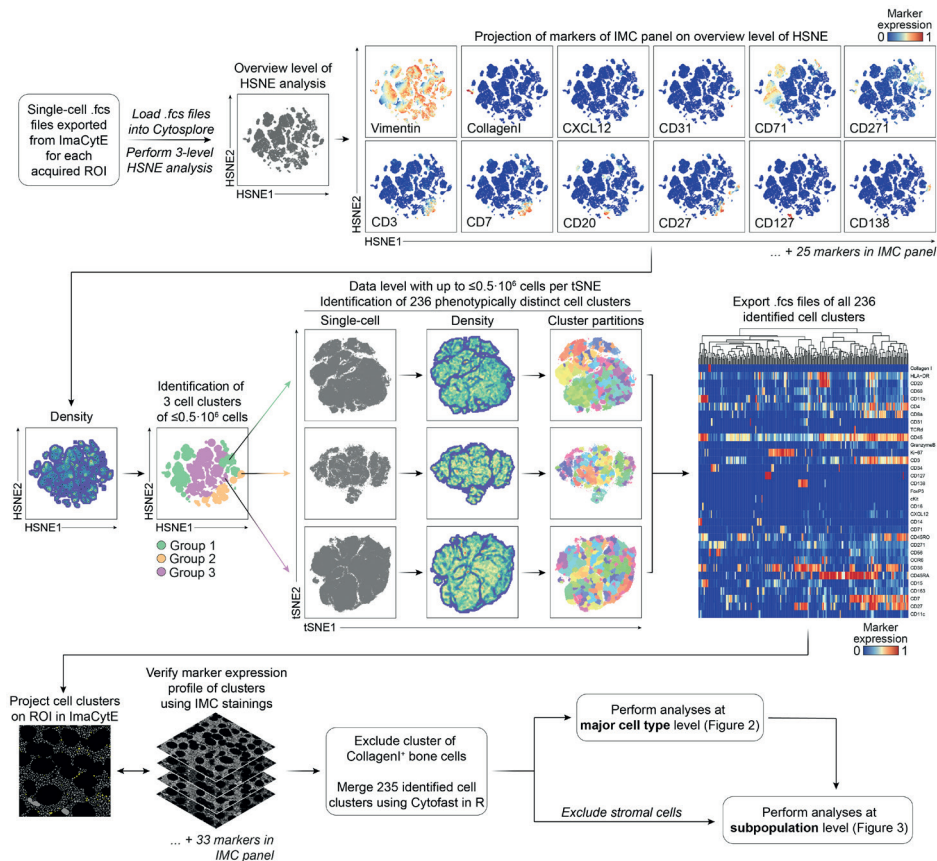
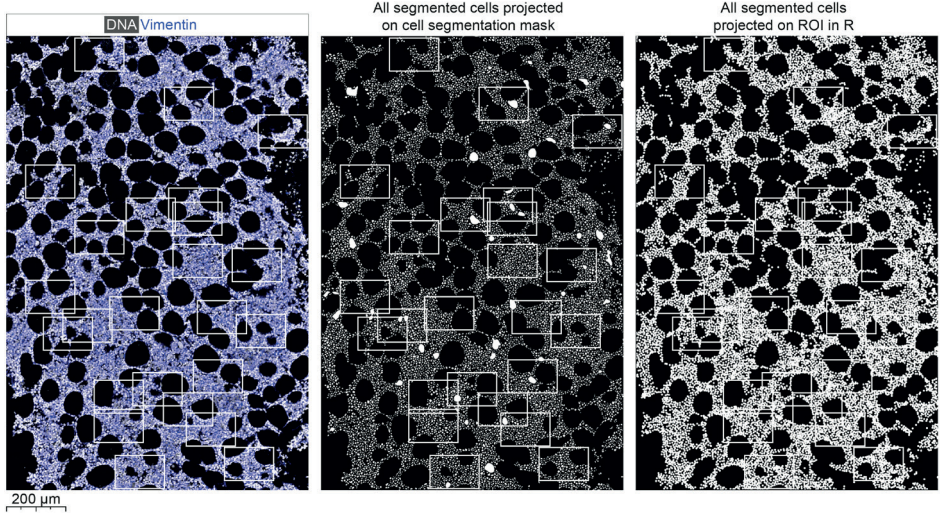
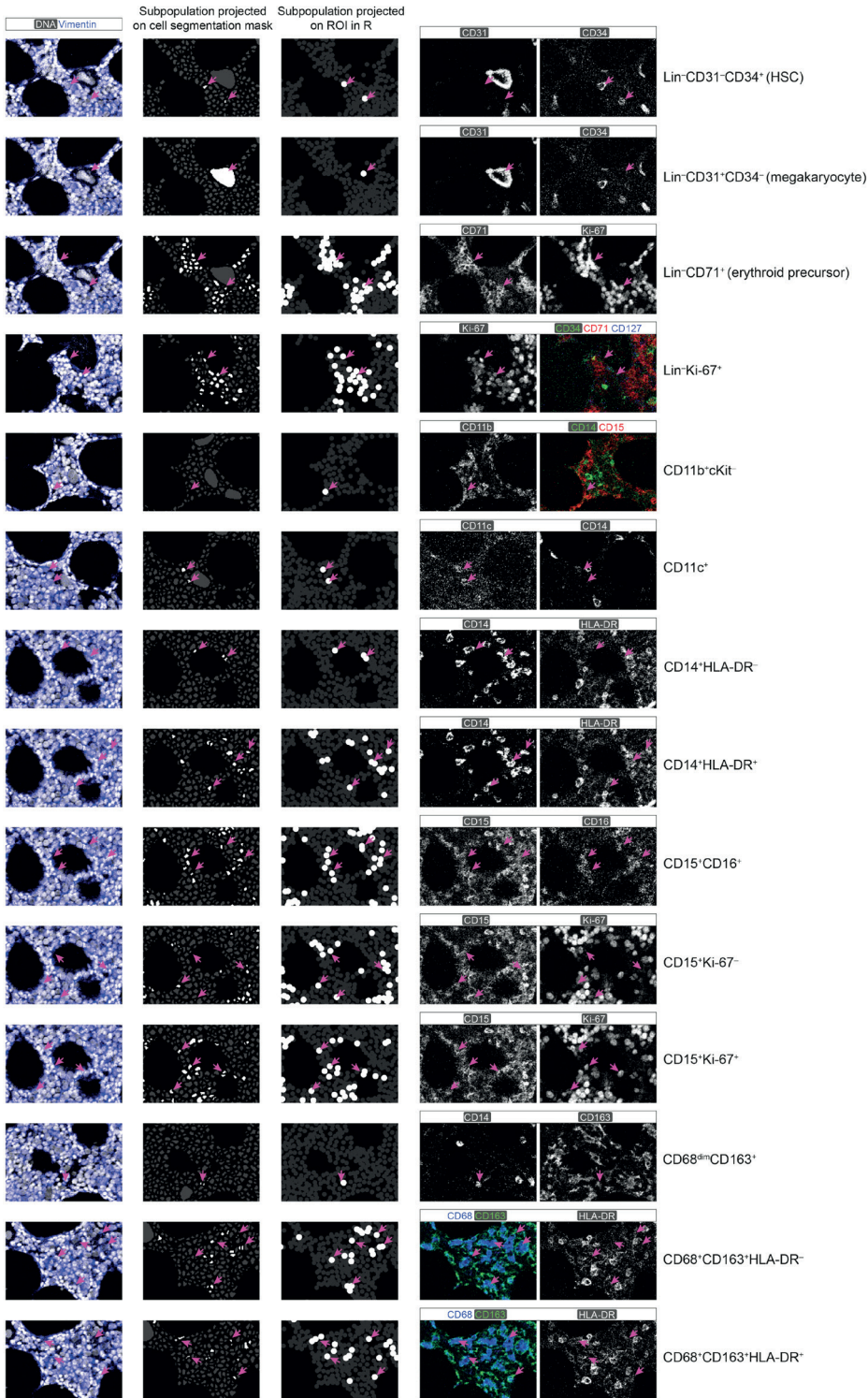
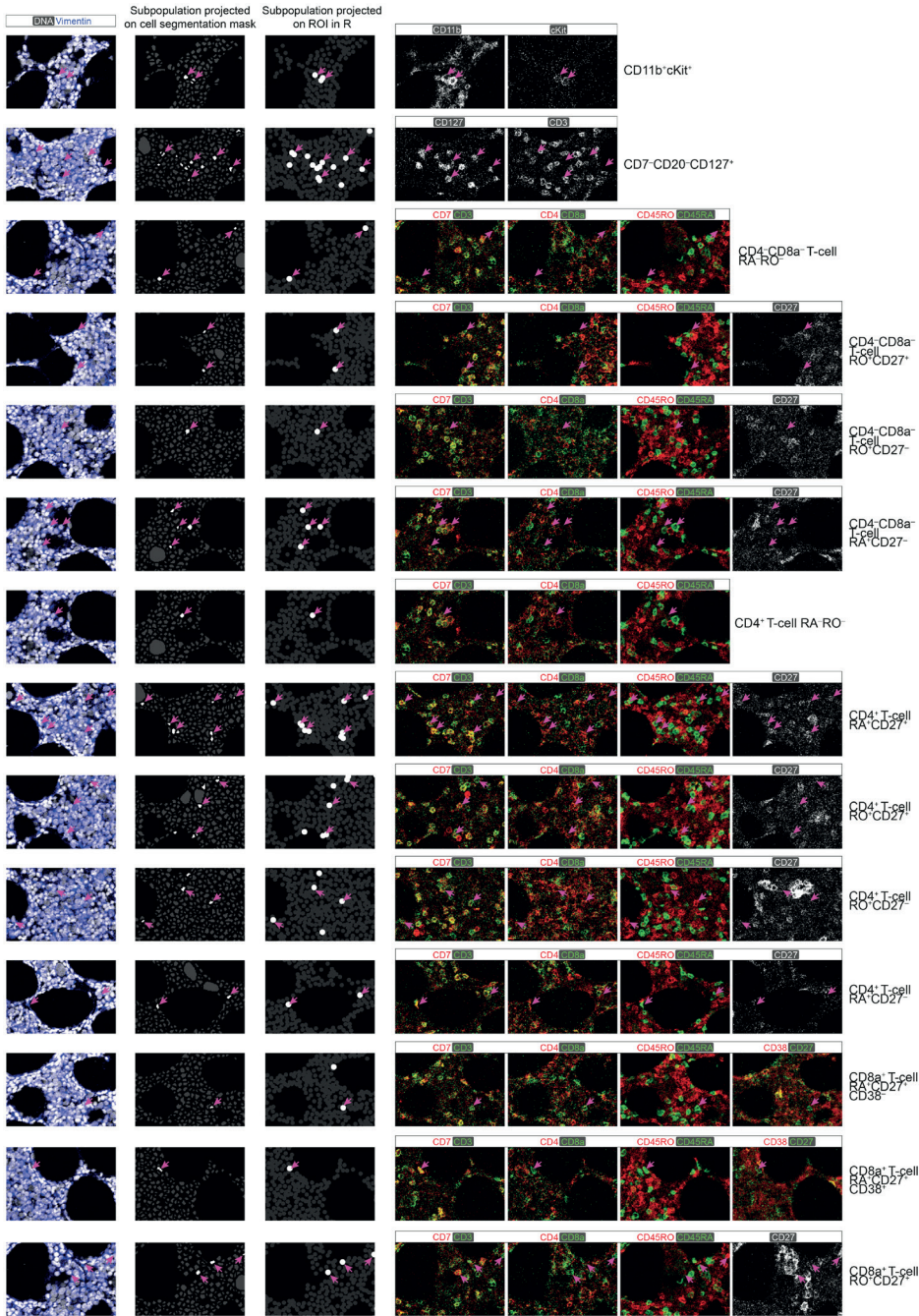


Figure S5. Cell clustering analysis pipeline. To compare the cellular composition of the bone marrow from AA patients at diagnosis, AA patients post-hATG and controls, the $0.52 \cdot 10^6$ cells segmented from all ROIs were analyzed using a 3-level HSNE analysis in Cytoscore. At the overview level of the HSNE analysis, the $0.52 \cdot 10^6$ cells were clustered into three groups of cells. Group 1 included cells expressing Collagen I, CXCL12, CD31, CD71 and/or CD271. Group 2 consisted of cells expressing CD3, CD7, CD20, CD27, CD127 and/or CD138. Group 3 included all remaining cells. Next, each group of cells was studied and clustered at the data level of the 3-level HSNE analysis. 236 phenotypically distinct cell clusters were identified. To verify the marker expression profile of each identified cell cluster, all clusters were projected back onto the cell segmentation mask in ImaCytE. Subsequently, the ImaCytE images were compared to the unthresholded IMC stainings. Finally, the 236 identified cell clusters were merged in R for downstream analysis at the major cell type or subpopulation level.







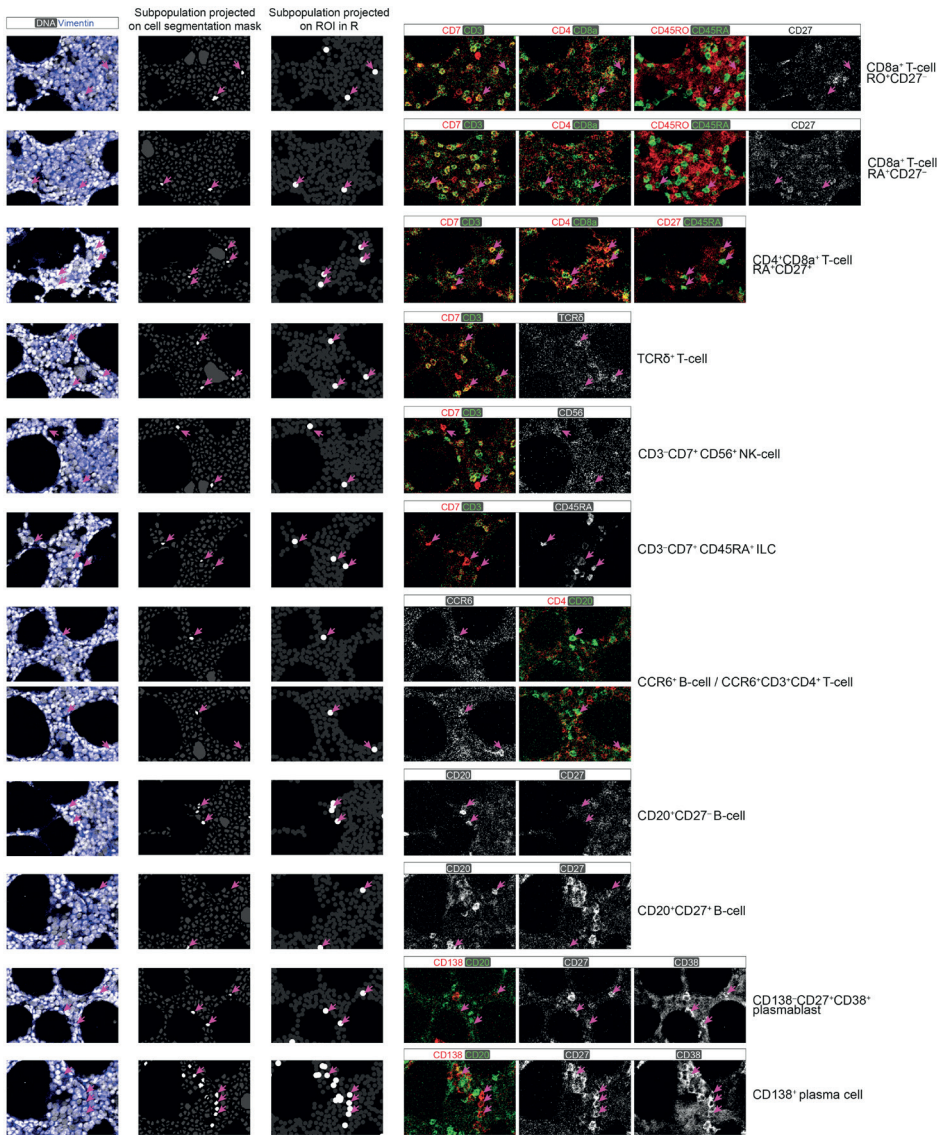


Figure S6. Confirmation of identified phenotypes in raw IMC stainings. Raw IMC stainings and identified hematopoietic cell subpopulations are shown throughout different regions of a representative control ROI (Control5). Identified subpopulations are visualized in both the cell segmentation mask and the images acquired during the data analysis pipeline in R.

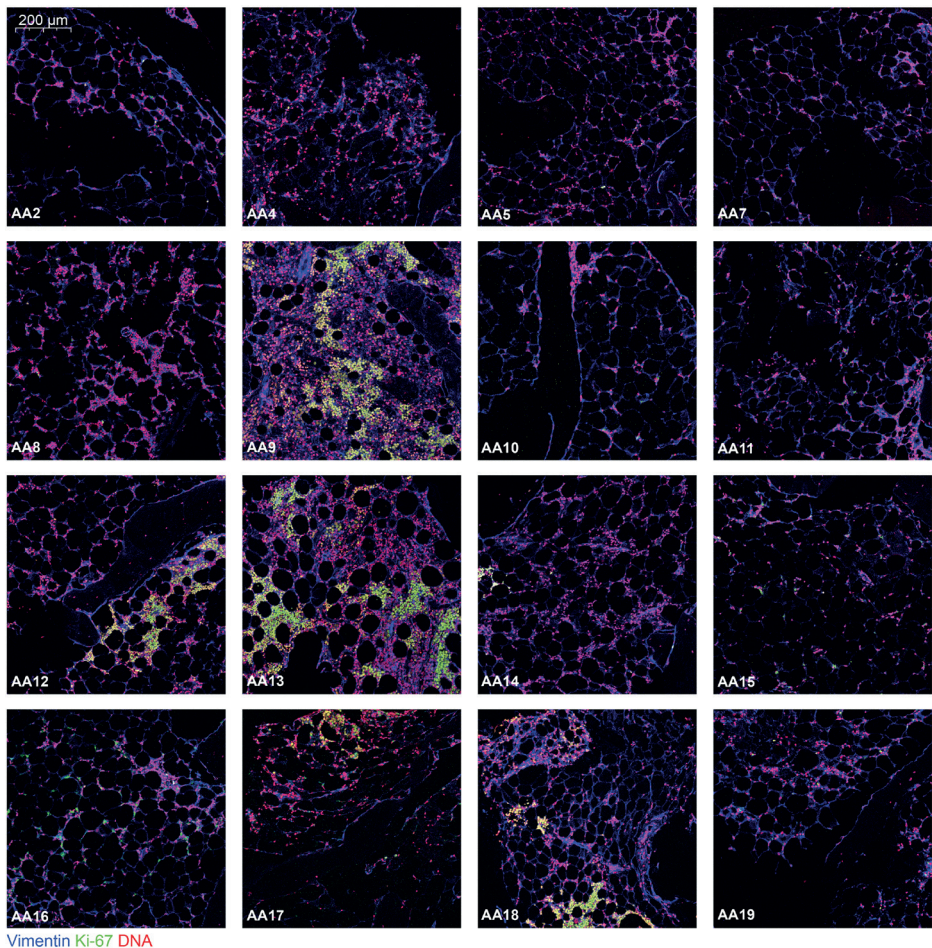


Figure S7. Global architecture of the bone marrow from all AA patients at diagnosis. Vimentin (blue) stains all hematopoietic cells and their microenvironment, DNA (red) stains all cellular nuclei, Ki-67 stains all proliferating cells. One imaged region of interest (ROI) is shown for each patient.

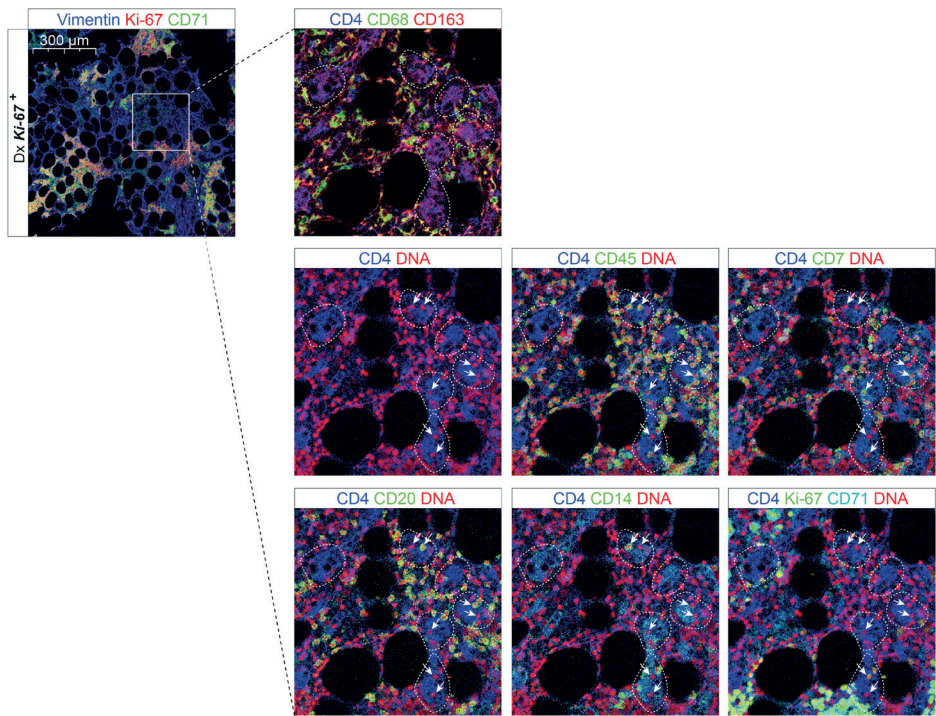
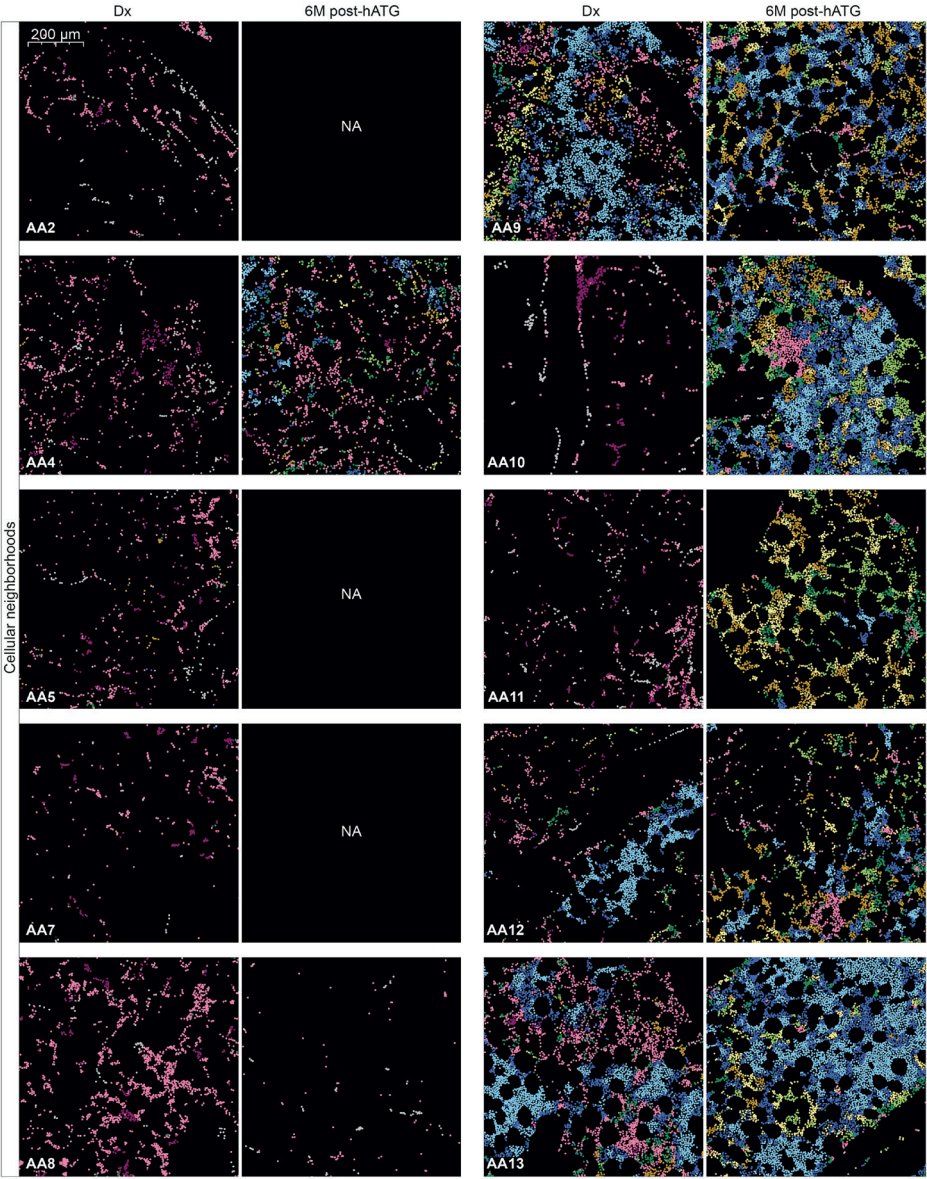


Figure S8. Hematopoietic cell populations within the CD4⁺CD68⁺CD163⁺ macrophages in Ki-67⁺ regions from AA^{Dx}. CD4⁺CD68⁺CD163⁺ macrophages in the bone marrow from a representative AA^{Dx} are indicated with white dotted circles. Cells within the CD4⁺CD68⁺CD163⁺ macrophages are indicated by arrows. DNA stains cellular nuclei; CD45 stains all hematopoietic cells; CD7 stains T-cells and innate lymphoid cells; CD20 stains B-cells; CD14 stains monocytes; Ki-67 and CD71 stain erythroid precursors.



Figure S9. Abundances of identified phenotypes in ROIs. Abundances of identified progenitor, mature myeloid, CD4⁺ T-cell, CD8⁺ T-cell, ILC, B-cell and plasma cell subpopulations are shown as an absolute count per mm² of hematopoietic compartment. Each dot represents one ROI; red dots represent patients with a non-normalized bone marrow 6M post-hATG. Horizontal bars present group median.



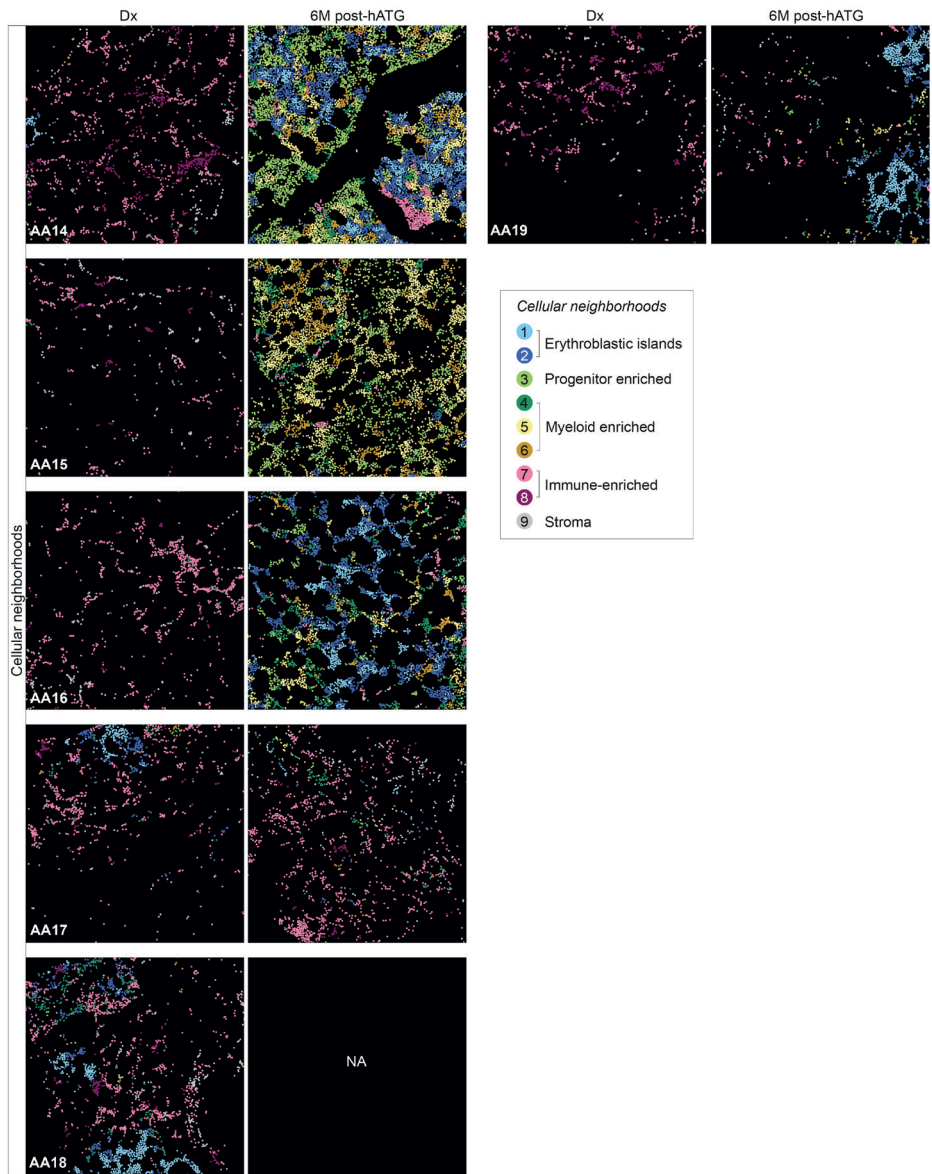


Figure S10. Visualization of all identified cellular neighborhoods in all AA patients at diagnosis and 6M post-hATG. Cellular neighborhoods visualized in a representative ROI from each AA^{Dx} (n=16) and AA^{6M} (n=12). Bone marrow tissue samples were not studied for 4 of 16 AA patients 6M post-hATG. Colors indicate cellular neighborhoods, each dot represents one segmented cell.

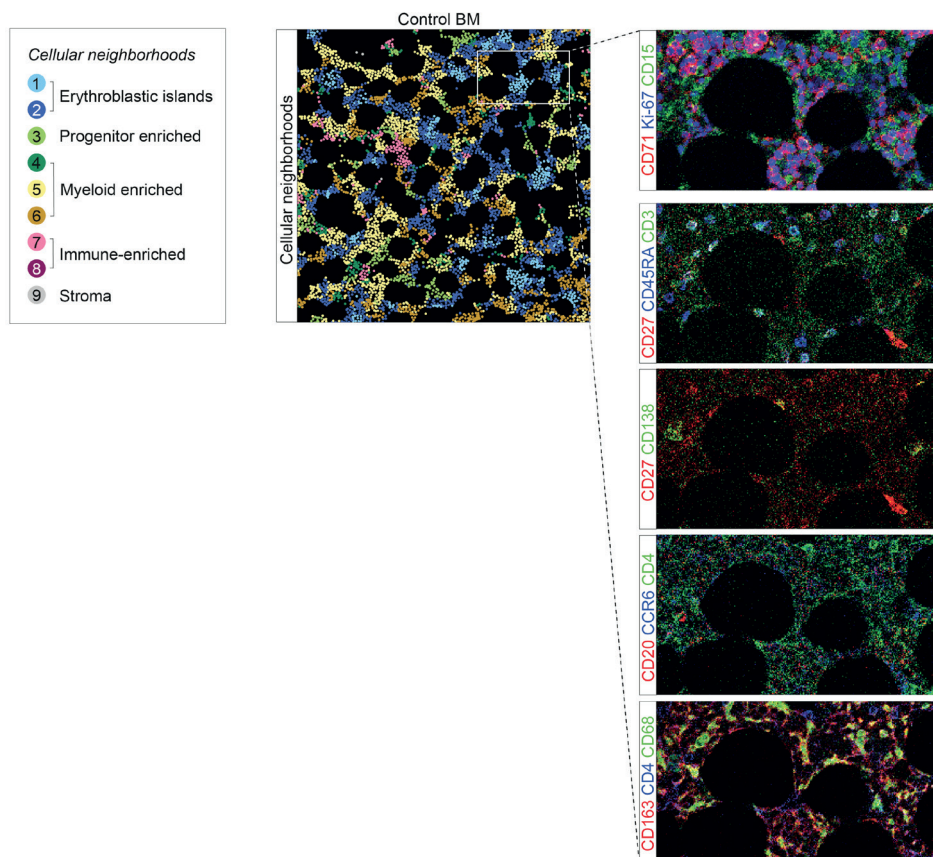


Figure S11. Immune cells subpopulations within the erythroblastic islands in control bone marrow. Left: Cellular neighborhoods visualized in a representative control ROI. Colors indicate cellular neighborhoods, each dot represents one segmented cell. Right: Cell subpopulations visualized within the erythroblastic islands in the control ROI. Top panel: Visualization of CD71⁺Ki-67⁺ erythroid precursors (red cells with blue nucleus) or CD15⁺Ki-67⁺ neutrophil progenitors (green cells with blue nucleus). Second panel: Visualization of CD3⁺ T-cell populations in multiple differentiation stages. Third panel: Visualization of CD138⁺CD27⁺ plasma cells. Fourth panel: Visualization of (CCR6⁺) CD20⁺ B-cells and CD4⁺ T-cells. Fifth panel: Visualization of macrophages using CD4, CD68 and CD163.

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