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

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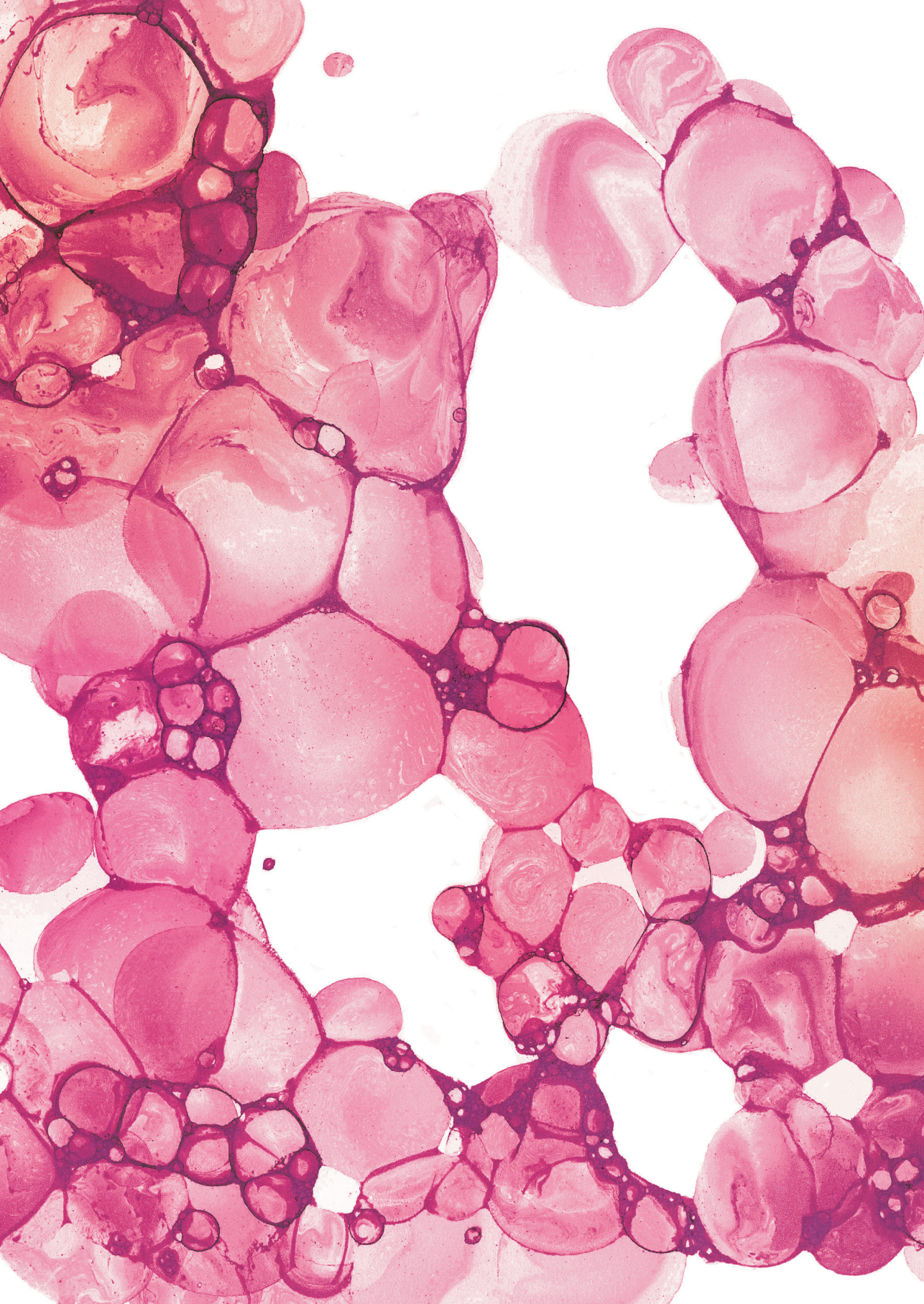

Summary and general discussion

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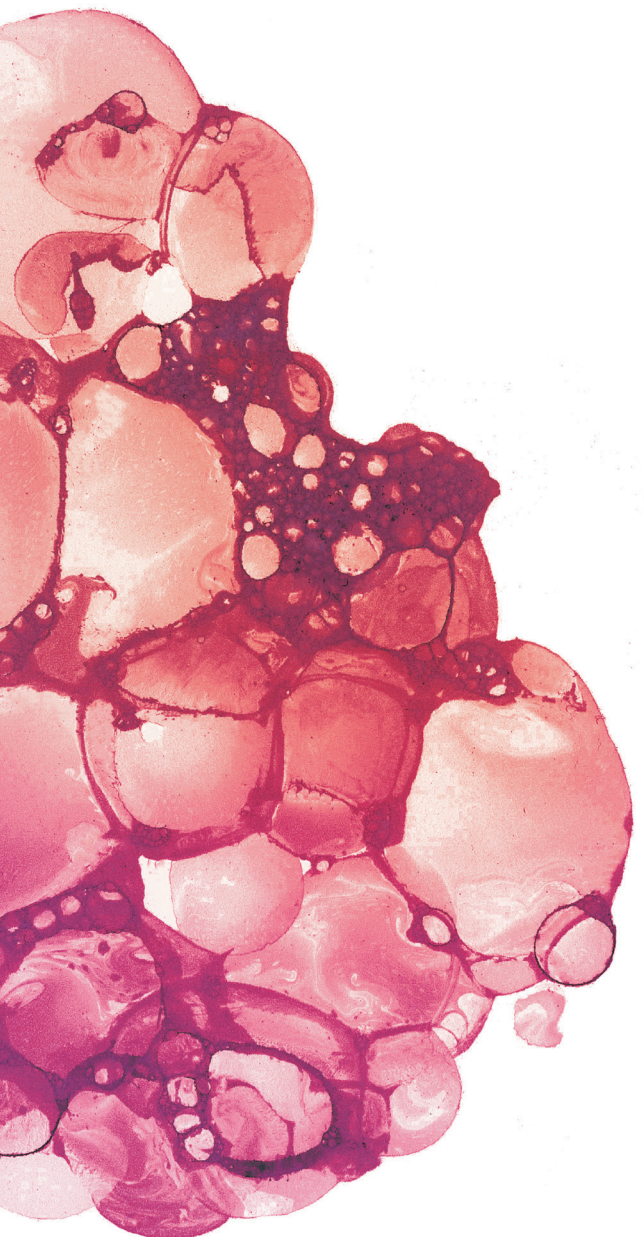


Summary

Aplastic anemia (AA) is a rare bone marrow failure syndrome that is most often classified as 'idiopathic', despite a long history with the first case described in the late 19th century by Paul Ehrlich[1]. In the 1980s it was discovered that AA patients can respond to immunosuppressive therapy (IST)[2-4]. Therefore, AA is typically viewed as an immune-mediated disease, with T-cells playing a central role[5]. However, IST remains a suboptimal treatment since only 70% of patients respond to first-line IST with horse-derived anti-thymocyte globulin (hATG, ATGAM®) and long-term ciclosporin[6,7]. Therefore, there is a need for improved therapies that specifically target the pathogenic immune cell populations driving stem cell destruction in the bone marrow. However, a comprehensive understanding of the immune response in AA has been hampered by limitations in the number of cellular phenotypes that could be studied simultaneously. Furthermore, spatial analysis of the bone marrow microenvironment has not been possible due to technical limitations caused by tissue decalcification and the lack of spatially-resolved imaging platforms that can map the full complexity of its cellular communities. Therefore, the behavior of potentially pathogenic immune cells within the affected bone marrow has remained unclear.

In this thesis we provide the first atlas of the immune landscape in AA with the aim to unravel the immune response underlying the pathogenesis of AA. We profiled bone marrow and peripheral blood samples collected from AA patients using high-dimensional immunophenotyping techniques. This enabled detailed analyses of all major immune lineages at the immune subpopulation level. We identified potentially pathogenic immune cell subpopulations associated with chronic inflammation, and deciphered their spatial context within the affected bone marrow across different stages of bone marrow tissue destruction. To confirm their relevance, we elucidated the impact of hATG-based IST and demonstrated that these subpopulations were selectively depleted with successful treatment while other memory immune cell subpopulations persisted. Building on these findings, this thesis also includes a case study that explores the long-term biology within the bone marrow following initial AA diagnosis. We demonstrated the added value of analyzing bone marrow tissue samples over bone marrow aspirates, and gained insight into the mechanisms underlying the development of secondary clonal disorders.

Mass cytometry is a high-dimensional phenotyping technique that has pushed the analysis of both common and rarer cell populations forward. Consequently, it has deepened the understanding of immunology in health and disease[8-13]. In the setting of AA, mass cytometry was used to perform detailed analyses of the T-cell compartment in peripheral blood[14,15] but was not used to interrogate the presence of all immune populations within the bone

marrow simultaneously. In **Chapter 2**, we applied mass cytometry on cryopreserved bone marrow aspirates samples from AA patients before and six months after start of IST, and healthy controls with the aim to delineate which immune cell populations may be involved in the pathogenesis of AA. An antibody panel for 39 cell surface markers enabled broad immunophenotyping of all major immune lineages and hematopoietic stem and progenitor cells for the first time. Using dimensionality reduction analyses, we demonstrate that the bone marrow from AA patients pre-IST features a distinct immune cell composition compared to healthy controls, and is characterized by significantly decreased frequencies of hematopoietic stem and progenitor cells, myeloid cells and B-cells. In contrast, $CD4^+$ and $CD8^+$ T-cell frequencies are increased. Further analysis at the immune subpopulation level demonstrated the differential presence of both innate and adaptive immune clusters in AA patients pre-IST. Using correlation network analyses of these cell clusters, we identified a disease-specific immune cell network including $CD16^+$ myeloid cells, $CCR6^{++}$ B-cells, Th17-like $CCR6^+$ memory $CD4^+$ T-cells and $KLRG1^+$ terminally differentiated effector memory $CD8^+$ T-cells, compatible with a state of chronic inflammation. This network is more abundant in AA patients pre-IST compared to healthy controls, and its presence shows a trend towards normalization with successful IST. This suggests a potential pathogenic role. Collectively, our results provide a unique, single-cell view of the immune landscape in the bone marrow in AA that contributes to our understanding of AA as an immune-mediated disease. These findings reveal possible new players in the pathogenesis of AA and identify the chemokine receptor CCR6 as a potential therapeutic target.

In **Chapter 3**, we studied the immune response underlying the pathogenesis of AA in further detail within its native microenvironment in the bone marrow. Because the precise interactions between potentially pathogenic immune cells as well as the chronological order of events leading to AA development cannot be deducted from studying bone marrow aspirates, we performed an analysis of bone marrow tissue biopsies using imaging mass cytometry. Our results reveal that the active immune response in AA is concentrated in immune 'hotspots' within bone marrow tissue regions that still harbor pockets of $Ki\text{-}67^+$ hematopoietic progenitor cells. We propose that AA development begins in 'lymphoid-dominant' immune hotspots, where interactions between pro-inflammatory T- and B-cells, including the previously identified $CCR6^+$ B- and $CD4^+$ T-cells from **Chapter 2**, initiate the immune cascade. As the disease progresses, we observed the development of 'macrophage-enriched' immune hotspots. These hotspots are characterized by the presence of activated and enlarged $CD68^-CD163^+CD4^+$ macrophages along with activated T- and B-cells in close proximity to $Ki\text{-}67^+$ hematopoietic progenitor cells. These findings suggest that macrophages are involved in the pathogenesis of AA through the phagocytosis of dead and apoptotic

cells, while T- and B-cells orchestrate a targeted immune response by cytokine secretion and a cytotoxic attack. After the depletion of all Ki-67$^+$ hematopoietic progenitor cells, terminally differentiated effector memory T-cells and plasma cells remain in the hypocellular bone marrow, fitting with a more advanced stage of the disease where the active immune response has waned. These data, together with the findings from **Chapter 2**, underscore that the autoimmune response resulting in stem cell destruction is more diverse than previously recognized, and likely involves a broad spectrum of innate and adaptive immune cells rather than being only T-cell mediated. In addition, we generate a hypothesis on how the immune response evolves during disease development that illustrates which pro-inflammatory immune cells may contribute to each stage of bone marrow destruction.

To improve the treatment of AA by targeted therapy, more knowledge is needed about the immunomodulatory mechanisms underlying successful treatment. In **Chapter 4**, we investigated the effects of first-line IST with four days of ATGAM and long-term ciclosporin. We focused on active ATGAM, the fraction of this polyclonal antibody preparation capable of binding human lymphocytes and mediating its immunomodulatory effects. We demonstrate there is substantial variability in active ATGAM exposure among AA patients, caused by weight-based dosing, and reveal that higher exposure associates with earlier hematologic recovery. At high plasma levels, ATGAM binds all major lymphoid lineages and profoundly reduces CD4$^+$ T-, CD8$^+$ T-, and NK-cell counts. Remarkably, although ATGAM also binds B-cells, it does not induce B-cell depletion but instead promotes an increase of CD27$^+$ B-cells in peripheral blood, potentially reflecting redistribution of memory B-cells from the lymphoid organs into the circulation post-ATGAM. Deep immunophenotyping on series of peripheral blood samples collected up to three years after start of IST demonstrated that ATGAM induced rapid depletion of memory T-cells, including the potentially pathogenic KLRG1$^+$ terminally differentiated CD8$^+$ T-cells and Th17-like CCR6$^+$CD4$^+$ T-cells identified in **Chapter 2**. Although naïve and virus-specific T-cells were also depleted, they recovered quickly, indicating preservation of protective immunity post-ATGAM. Notably, CCR6^{++} B-cells from **Chapter 2** escaped ATGAM depletion but reduced gradually over time along with residual potentially pathogenic T-cells, including the CCR6$^+$CD4$^+$ T-cells. This could explain the crucial contribution of long-term ciclosporin to successful IST. Collectively, our results identify ATGAM exposure as a factor influencing hematologic recovery, and indicate that the therapeutic effect of IST goes beyond total lymphodepletion but is rather the result of selective depletion and suppression of key lymphocyte subpopulations including the T- and B-cell subpopulations from **Chapter 2**. This underscores the pathogenicity of these lymphocyte populations in the development of AA and highlights their potential as possible therapeutic targets.

In 10-15% of patients who survive AA, the development of secondary clonal disorders including acute myeloid leukemia (AML) remains a concerning long-term complication post-IST. Prediction and prevention remain challenging since the underlying mechanism driving clonal evolution to acute myeloid leukemia is unknown. It has been postulated that clonal evolution to AML may be the result of environmental selection, where mutant clones are more resistant to the autoimmune attack in the bone marrow and therefore expand as dominant clone in size and time[16,17]. Alternatively and additionally, stem cell clones may acquire somatic mutations that confer a proliferative advantage as compared to other remaining stem cell clones in the hypocellular bone marrow post-hATG. Evidence supporting these models is, however, indirect. To address this knowledge gap, in **Chapter 5**, we performed a multi-modal analysis of bone marrow samples from an AA-to-AML patient to track the development of secondary AML following the initial diagnosis of AA at the site of its development. Using imaging mass cytometry, we demonstrate that, in our described patient, the clinical diagnosis of AML was preceded by a shift in the bone marrow architecture. This shift was characterized by the expansion of Ki-67$^-$CD45^{dim}CD38$^+$ preleukemic clones post-hATG. Although the expansion of these Ki-67$^-$CD45^{dim}CD38$^+$ cells was also observable in non-evolving AA patients post-hATG, their continued expansion was unique to our AA-to-AML patient. These data suggest that the initial bone marrow failure could create a microenvironment that both favors and triggers preleukemic clones that survive the autoimmune attack, but that an additional cell-intrinsic event may be required for their continued expansion. By performing targeted next generation sequencing on sorted bone marrow aspirates from the AA-to-AML patient, we further investigated the genetic factors that possibly contributed to clonal evolution to AML. We found that leukemic driver mutations were only detectable at AML diagnosis 29 months post-hATG. Strikingly, we further observed a somatic mutation in the splicing factor *ZRSR2*, which was present in all hematopoietic cells in the bone marrow from AA diagnosis onwards. Overall, these data provide support to the close link between clonal hematopoiesis and the development of clonal disorders. In addition, our results support a model in which clonal evolution to AML is determined by both microenvironmental and genetic factors, and highlight a possible role for the identified *ZRSR2* mutation in this process.

General discussion

Paradigm of immune-mediated AA

AA is generally perceived as an immune-mediated form of bone marrow failure if no other causes of pancytopenia are found[5,18]. The widely accepted paradigm suggests that AA is the result of a T cell-mediated autoimmune response[19]. The disease is thought to develop when an antigen(s) expressed on the surface of hematopoietic stem and progenitor cells triggers

the activation and expansion of T-cells, which subsequently acquire a cytotoxic effector profile and cause damage to the bone marrow[19]. Evidence supporting this paradigm is mostly indirect. Bone marrow recovery after IST with hATG plus long-term ciclosporin suggests T-cell involvement[5]. Furthermore, there are reports of oligoclonal T-cell expansion and T-cell infiltration into the bone marrow in AA[20-24]. More recently, studies have identified patient-derived T-cells capable of killing stem cells *in vitro*[25,26]. Despite these findings, however, the precise mechanisms underlying AA development remain unknown, and two main elements require further exploration. The first relates to the identity of the targeted antigen(s) on the surface of the hematopoietic stem and progenitor cells, which has not yet been determined. The second element concerns the identity of the pathogenic immune cells involved in the autoimmune response, which remains largely elusive. Although there are indications that macrophages[27-29] and NK-cells[26,30,31] may contribute, a potential role of other immune cells besides T-cells has largely been neglected. In addition, in the absence of biomarkers to reliably diagnose the disease, it remains uncertain whether AA is immune-mediated in all patients, and if so, whether the same immune cell subpopulations are involved in all cases. Non-response to IST, defects in the function of the bone marrow niche[32], and frequent detection of somatic mutations[33] could indicate an alternative, non-immune mediated pathogenesis in some patients.

This thesis aimed to decipher the autoimmune response leading to AA development by using high-dimensional single-cell technologies to map the immune landscape in AA patients. By identifying potentially pathogenic immune cell populations, our results advance our understanding of AA as an immune-mediated form of bone marrow failure. Importantly, our findings further challenge the existing paradigm by revealing that AA is driven by an immune response involving a broad spectrum of innate and adaptive immune cells, instead of just T-cells alone. In **Chapter 2**, we propose that AA may be mediated by a disease-specific immune cell network of potentially pathogenic $CCR6^{++}$ B-cells, Th17-like $CCR6^{+}$ memory $CD4^{+}$ T-cells, $KLRG1^{+}$ terminally differentiated effector memory $CD8^{+}$ T-cells, and $CD16^{+}$ myeloid cells, all compatible with a state of chronic inflammation. In **Chapter 3**, we extend the findings from **Chapter 2** by visualizing a phenotypically diverse immune response involving all major immune lineages across different stages of bone marrow destruction. Finally, in **Chapter 4**, we show that bone marrow recovery after successful IST depends on the selective removal of both the pro-inflammatory T- and B-cell subpopulations from our immune cell network identified in **Chapter 2**. It is noteworthy that the immune landscape in all studied patients showed strong similarities, and was characterized by the expansion of the same pro-inflammatory immune cell subpopulations that show a trend toward normalization with successful IST. This implies that all studied patients had immune-mediated AA with involvement of the same

immune cell subpopulations, even though our analyses were performed on a heterogeneous patient population that ranges in age, sex, clinical disease severity, cytomegalovirus (CMV) status and response to IST. These findings are reassuring, as they suggest that the current treatment guidelines are successful in distinguishing patients with immune-mediated AA, despite the fact that AA remains a diagnosis of exclusion[18]. These novel insights into the pathogenesis of AA were made possible by our distinct approach, which differs from seminal papers in the field by 1) studying the immune response at the site of stem cell destruction in the bone marrow instead of in peripheral blood; 2) enabling a simultaneous analysis of all major immune lineages by broad immunophenotyping; 3) visualizing the interactions of in-depth phenotyped cells within the bone marrow architecture; 4) capturing the morphology of immune cells within the bone marrow tissue and 5) delineating the immunomodulatory effects associated with successful IST.

Proposed immunological model of AA

The most important contribution of this thesis is the generation of a hypothesis on the chronological order of events in AA development. By identifying 'lymphoid-dominant' immune hotspots with high densities of pro-inflammatory lymphocytes within the bone marrow tissue in **Chapter 3**, we believe to have captured the initial phase of immune activation in AA for the first time. Our data confirm involvement of ($CCR6^+$) $CD4^+$ and $CD8^+$ T-cells and additionally highlight a previously unappreciated role for ($CCR6^+$) B-cells in this process. As the immune response progresses, 'macrophage-enriched' immune hotspots develop, where a spectrum of pro-inflammatory immune cells including T-cells, B-cells and activated $CD68^-$ $CD163^+CD4^+$ macrophages is colocalized with progenitor cells. We interpreted these tissue areas as sites of peak immune activity, where T- and B-cells may coordinate targeted immune responses through antigen-specific T-cell receptor and B-cell receptor signaling, cytokine secretion, antigen presentation and cytotoxic attack. In contrast, activated $CD68^-CD163^+CD4^+$ macrophages may phagocytose dead and apoptotic cells. In the hypocellular bone marrow regions depleted of progenitor cells, terminally differentiated $CD8^+$ T-cells and plasma cells remain after the immune response has waned, suggesting accumulation of these cells in the bone marrow after repeated antigenic stimulation.

Distinguishing autoreactive immune cells from bystander cells in AA

The findings from this thesis together provide an immunological model of AA development that, for the first time, unravels the distinct roles of immune cell subpopulations during each phase of bone marrow destruction. This enables us to distinguish potentially autoreactive immune cells from bystander cells in AA. As illustrated in Figure 1, we propose that the early

immune environment leading to AA development is created by interactions between ($CCR6^+$) T- and B-cell within the 'lymphoid-dominant' immune hotspots. This develops following T- and/or B-cell activation by recognition of an autoantigen presented by the hematopoietic stem cell (Figure 1, Steps 1-3). We believe these T- and B-cell interactions sustain an antigen-specific immune response. The observation that pro-inflammatory innate immune cells including activated $CD68^-CD163^+CD4^+$ macrophages or NK-cells only come in later, with the development of the 'macrophage-enriched' immune hotspots, suggests that these cells play a secondary role in the pathogenesis of AA. Our findings thereby place the presence of macrophages and NK-cells in a new perspective. In previous studies, it was postulated that macrophages and NK-cells are recruited to the bone marrow in AA to enhance the killing of stem cells by stimulating effector cell function via the production of pro-inflammatory cytokines[26-28]. In this thesis, we demonstrate that macrophages and NK-cells are present in similar numbers between control bone marrow and the macrophage-enriched hotspots in AA. Importantly, we further show that macrophages are already observable within the lymphoid-dominant immune hotspots, but have not yet obtained an activated state. We therefore do not believe that the $CD68^-CD163^+CD4^+$ macrophages and NK-cells are actively recruited to the bone marrow in AA. Instead, it is more likely that these cells are pre-existing tissue-resident populations that are activated in response to the inflammatory signals within the bone marrow microenvironment (Figure 1, Step 4). This interpretation downplays the presumed importance of NK-cells in AA, which could still secrete pro-inflammatory cytokines and/or kill stressed cells within the macrophage-enriched hotspots, but are unlikely to be one of the main effector cell populations.

In contrast, for $CD68^-CD163^+CD4^+$ macrophages we consider a distinct role in phagocytosing the dead and apoptotic cells in the bone marrow (Figure 1, Step 5). Based on the findings from **Chapter 2**, we speculate that these macrophages are activated in the lymphoid-dominant immune hotspots, and subsequently start to engulf debris and dead hematopoietic cells targeted by the pathogenic T- and B-cells. Interestingly, we also found that these macrophages contained activated T- and B-cells, indicating that these lymphocytes possibly are phagocytosed after they have exerted their effector function. This uptake likely leads to the enlarged morphology of the macrophages observed in the macrophage-enriched immune hotspots. Once the active immune response has waned and their phagocytic function is no longer needed, the activated $CD68^-CD163^+CD4^+$ macrophages return to a resting state and recover to their normal size (Figure 1, Steps 6-7). Whether these $CD68^-CD163^+CD4^+$ macrophages are pro- or anti-inflammatory in AA will require further functional studies. Their expression of the scavenger receptor CD163, typically associated with anti-inflammatory M2 phenotype[34,35], strongly supports an immunosuppressive role in AA. However, various studies show that CD163 is not exclusive to an anti-inflammatory state[36,37]. Therefore, both scenarios are plausible: the $CD68^-CD163^+CD4^+$ macrophages could attempt to dampen inflammation

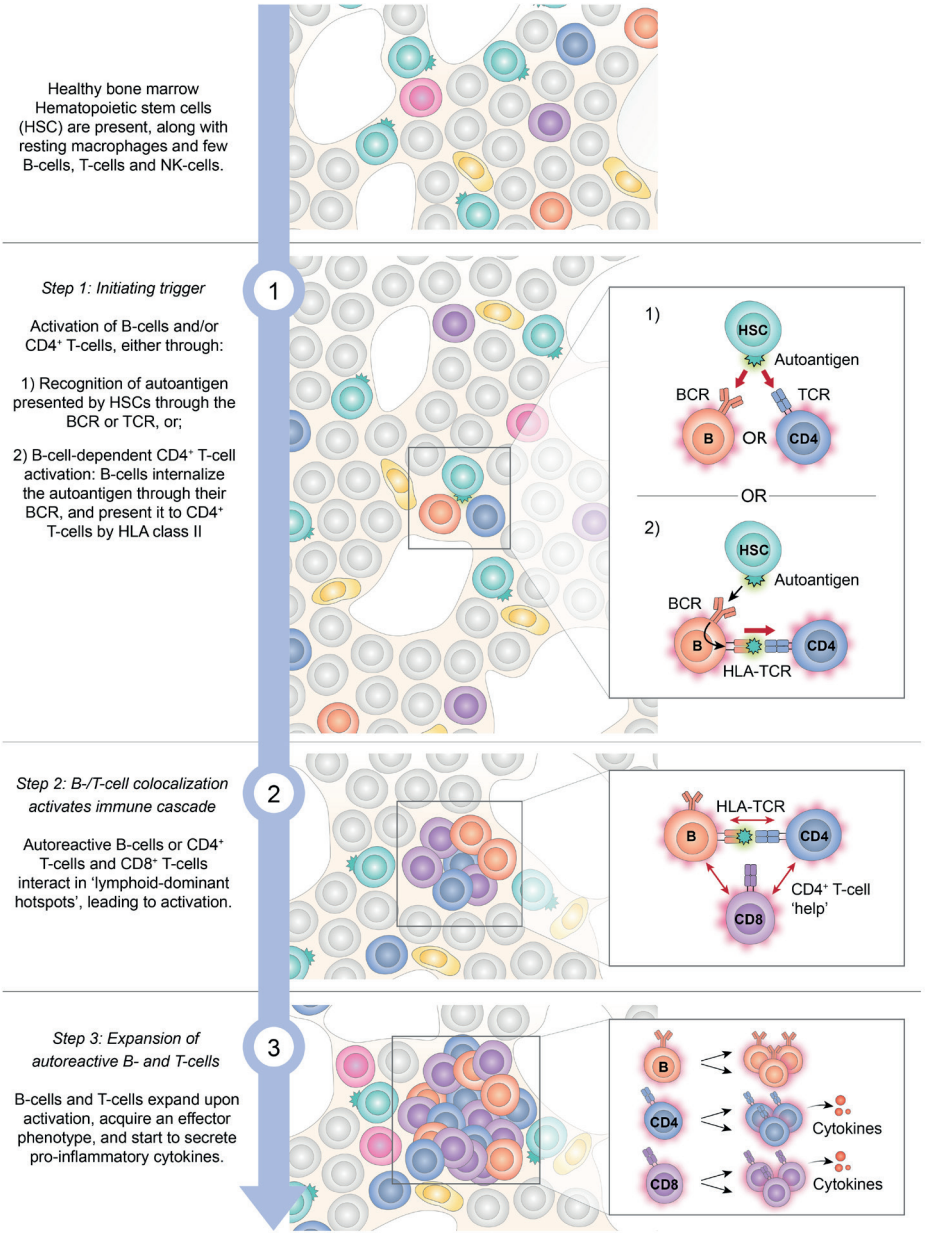
and damage induced by T- and B-cells. Alternatively, they might support inflammation by the production of reactive oxygen species and secretion of pro-inflammatory cytokines that support effector cell function.

Understanding the central role of T- and B-cells in AA

The central role of T- and B-cells and their interactions in our proposed immunological model of AA development calls for further discussion of their potential function. We focus on CCR6$^+$ B-cells, Th17-like CCR6$^+$ CD4$^+$ T-cells and KLRG1$^+$ terminally differentiated CD8$^+$ T-cells, as we consider these cells to be the most important players. This is based on their presence in the identified AA-specific immune cell network from **Chapter 2**, their detection in the affected bone marrow tissue in **Chapter 3**, and their selective removal following successful IST in **Chapter 4**. It is interesting that these subpopulations are frequently described in autoimmunity, and that the combined involvement of T- and B-cells drives many autoimmune diseases, including multiple sclerosis and rheumatoid arthritis[38-40]. This suggests that the observed immune response in AA may not be 'disease-specific', but may in fact be similar to the inflammatory state broadly observed in autoimmunity. Our findings, therefore, add direct evidence that AA shares pathophysiological features with other autoimmune diseases, corroborating previous observations[41-44]. These parallels support the idea that we have identified pathogenic immune cell subpopulations, even though further research is needed to elucidate their functional role and antigenic specificity. Based on prior reports, we speculate that CCR6$^+$ B-cells, Th17-like CCR6$^+$ CD4$^+$ T-cells and KLRG1$^+$ terminally differentiated CD8$^+$ T-cells may contribute to AA development through two distinct mechanisms.

CCR6$^+$ T- and B-cells in the pathogenesis of AA

For CCR6$^+$ B-cells and Th17-like CCR6$^+$ CD4$^+$ T-cells, we consider a role early in the immune response leading to bone marrow destruction. CCR6 has become a well-described marker in autoimmunity[45,46]. This chemokine receptor is found on the membrane of several immune cell types, with the highest expression observed on T- and B-cells[47], and levels increasing on B-cells after activation[48]. In line with its broad expression, CCR6 has been implicated as an important marker in mediating immune cell migration, mostly through the CCR6/CCL20 axis[45]. In this axis, CCL20, secreted in response to inflammatory signals, can attract pro-inflammatory CCR6$^+$ lymphocytes including CCR6$^+$ Th17 cells. This shifts the ratio between Th17 cells and regulatory T-cells to a pro-inflammatory state[49,50]. Numerous studies have highlighted the importance of the CCR6/CCL20 axis in autoimmunity, particularly in sustaining the autoimmune response by mediating immune cell recruitment to the inflamed tissues[45]. This includes renal inflammation, where this axis facilitates the development of lymphoid infiltrates that function as sites of T- and B-cell activation[51].


Healthy bone marrow
Hematopoietic stem cells (HSC) are present, along with resting macrophages and few B-cells, T-cells and NK-cells.
Step 1: Initiating trigger
Activation of B-cells and/or CD4+ T-cells, either through:
1) Recognition of autoantigen presented by HSCs through the BCR or TCR, or;
2) B-cell-dependent CD4+ T-cell activation: B-cells internalize the autoantigen through their BCR, and present it to CD4+ T-cells by HLA class II
1
1)
HSC
Autoantigen
BCR
TCR
B
OR
CD4
OR
2)
HSC
Autoantigen
BCR
B
CD4
HLA-TCR
Step 2: B-/T-cell colocalization activates immune cascade
Autoreactive B-cells or CD4+ T-cells and CD8+ T-cells interact in 'lymphoid-dominant hotspots', leading to activation.
2
HLA-TCR
B
CD4
CD4+ T-cell 'help'
CD8
Step 3: Expansion of autoreactive B- and T-cells
B-cells and T-cells expand upon activation, acquire an effector phenotype, and start to secrete pro-inflammatory cytokines.
3
B
CD4
Cytokines
CD8
Cytokines

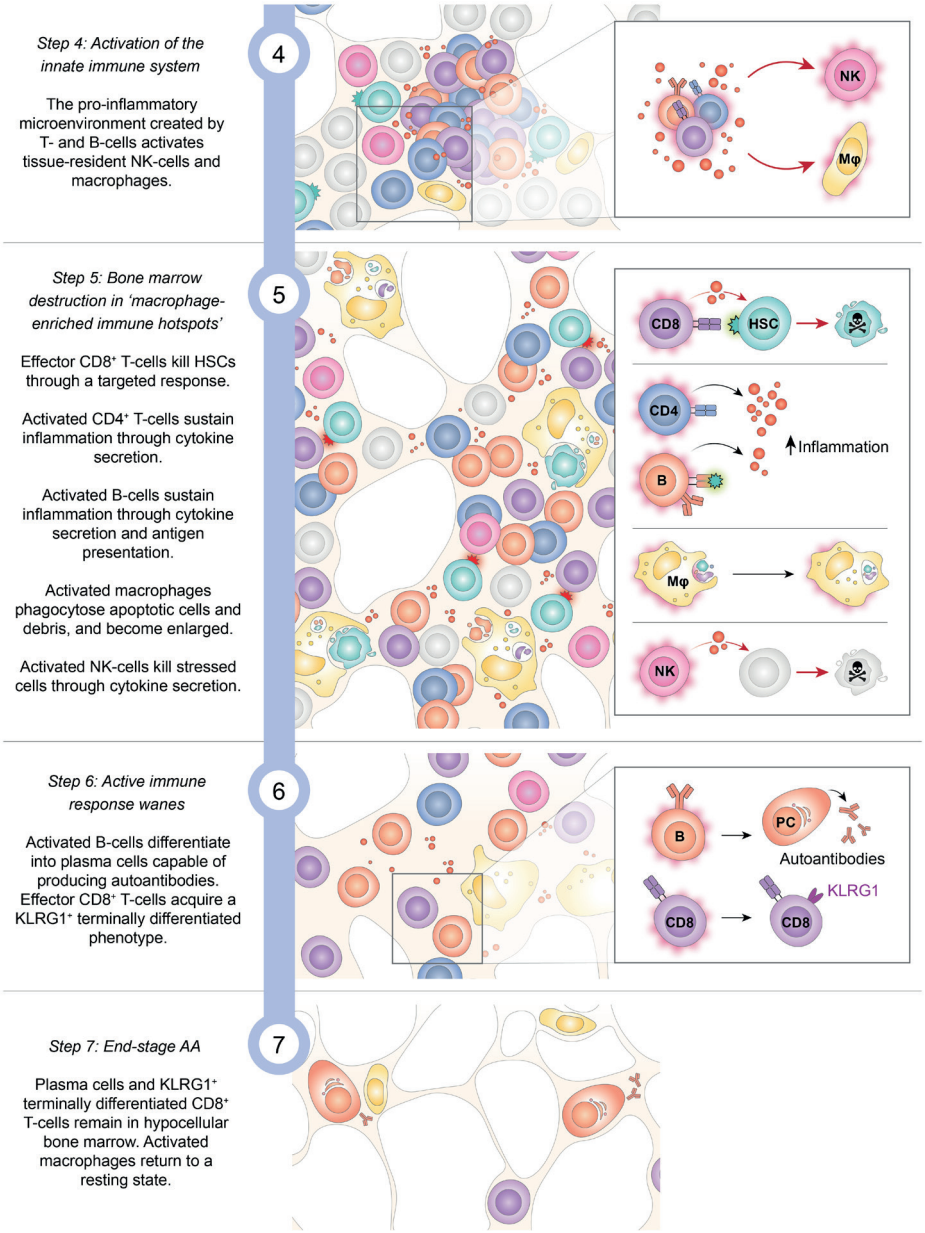


Figure 1. Visualization of the proposed immunological model of AA. BCR: B-cell receptor; HSC: hematopoietic stem cell; Mφ: macrophage; PC: plasma cell; TCR: T-cell receptor.

In the context of AA, we are the first to report on the importance of CCR6. We found that CCR6$^+$ B-cells and Th17-like CCR6$^+$ CD4$^+$ T-cells are prominent populations within the 'lymphoid-dominant' immune hotspots in the bone marrow, where these cells may interact through engagement of their T- and B-cell receptors. The fact that these CCR6$^+$ B-cells and Th17-like CCR6$^+$ CD4$^+$ T-cells were so closely colocalized that they could not be separated by cell segmentation supports that these cells interact within the bone marrow. We believe that these CCR6$^+$ B- and/or T-cells are the autoreactive immune cells that initiate the immune cascade in AA. The activation of these CCR6$^+$ B- and/or T-cells could either occur 1) directly, through recognition of an autoantigen presented by hematopoietic stem cells by the B- or T-cell receptor, or 2) through B-cell dependent T-cell activation, where B-cells take up the autoantigen through the B-cell receptor and activate the CD4$^+$ T-cells by presenting the antigen in HLA class II (e.g. HLA-DR) (Figure 1, Step 1). Following their activation, the interactions of these B- and CD4$^+$ T-cells may amplify the immune response: CCR6$^+$ CD4$^+$ T-cell 'help' to CD8$^+$ T-cells could drive CD8$^+$ T-cell activation, while interactions between CCR6$^+$ B-cells and CCR6$^+$ CD4$^+$ T-cells may promote their local expansion in the bone marrow (Figure 1, Steps 2-3).

These possible mechanisms of the CCR6$^+$ B-cells and CCR6$^+$ CD4$^+$ T-cells are supported by unpublished data on a tertiary lymphoid structure (TLS) from our group, which therefore warrant further discussion. This TLS was detected within a hypocellular bone marrow region from one of our AA patients at diagnosis using imaging mass cytometry (Figure 2). Because of its singular detection, the TLS was examined separately from the cohort-wide analysis presented in **Chapter 3**. Interestingly, the analysis revealed the TLS contained high densities of CCR6$^+$ CD4$^+$ T-cells in close proximity to zones of CCR6$^+$ B-cells, all located between CD11c$^+$HLA-DR$^+$ dendritic cells and/or CD8$^+$ T-cells. Given that TLS are defined as local hubs where autoreactive T- and B-cells are concentrated[52], this provides strong support for identifying the CCR6$^+$ B-cells and CCR6$^+$ CD4$^+$ T-cells as the autoreactive immune cells driving inflammation in AA. Furthermore, the observation that CCR6$^+$ CD4$^+$ T-cells were colocalized with CD8$^+$ T-cells in the TLS illustrates that these CD4$^+$ T-cells may indeed provide the "help" needed to activate cytotoxic CD8$^+$ T-cells in AA. In contrast, CCR6$^+$ B-cells may form germinal center-like structures with follicular helper T-cells, where they undergo clonal expansion, class switching and differentiation to autoantibody-secreting plasma cells. This could expand the autoreactive B-cell pool. It is interesting that TLS serve as sites of T- and B-cell differentiation and expansion, since this implies that CCR6$^+$ B-cells and CCR6$^+$ CD4$^+$ T-cells are not (only) recruited to the bone marrow in AA following clonal expansion in the draining lymph node, but (also) expand locally within it. In this context, the role of CCR6 may not be to facilitate CCL20-dependent immune cell recruitment as described in other autoimmune disorders[45], but to facilitate the retention of autoreactive T- and B-cells within the AA bone marrow microenvironment, where CCL20 levels are high[53].

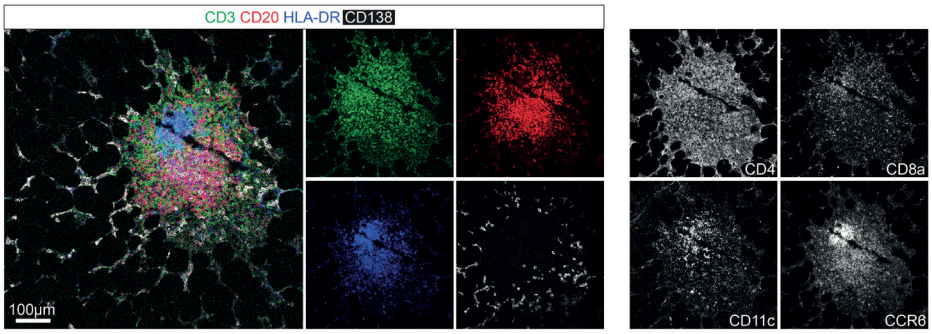


Figure 2. Tertiary lymphoid structure detected within the hypocellular bone marrow from an AA patient (AA13 from Chapter 2) at diagnosis by imaging mass cytometry. Stainings demonstrate high densities of CCR6$^+$ B- and CCR6$^+$ CD4$^+$ T-cells, located in close proximity to CD11c$^+$ dendritic cells, CD8$^+$ T-cells and CD138$^+$ plasma cells.

KLRG1$^+$ terminally differentiated CD8$^+$ T-cells in AA

For terminally differentiated CD8$^+$ T-cells we hypothesize a distinct role in maintaining chronic inflammation. CD8$^+$ T-cells are the most thoroughly studied immune cell population in AA, and have been described to be clonally expanded[22,25,54], express cytotoxic molecules[24-26], and kill hematopoietic stem cells *in vitro*[25,26]. This thesis adds important insight by demonstrating that terminally differentiated CD8$^+$ T-cells are present in the immune hotspots of peak immune activity. In addition, we show that these CD8$^+$ T-cells accumulate in the hypocellular bone marrow after all progenitors have been cleared (Figure 1, Step 6-7). We therefore believe that these cells represent the highly cytotoxic and terminally differentiated state of the activated CD8$^+$ T-cells that are capable of killing the hematopoietic stem cells in the bone marrow. Interestingly, we found that these cells express KLRG1 in **Chapter 2**. This could provide further insight into their cellular state. KLRG1 is a cell surface marker broadly expressed T- and NK-cells that was long considered a marker of T-cell senescence, but has more recently been recognized as a marker for highly activated and short-lived effector cells[55,56]. As an immune checkpoint molecule, KLRG1 can regulate immune responses by interacting with cadherins on the surface of antigen-presenting cells to elevate the activation threshold of T-cells. This process involves dephosphorylation of signaling molecules downstream of the T-cell receptor[55,57]. Therefore, KLRG1$^+$ terminally differentiated CD8$^+$ T-cells are reported to be in a balanced state between cytotoxicity and reduced functionality, which provides a protective mechanism against excessive immune activity[55].

The inhibitory role of KLRG1 is well-described in aging and infection with viruses including CMV, where KLRG1$^+$ terminally differentiated T-cells accumulate in the bone marrow and KLRG1 function increases after repeated antigenic stimulation[55,58]. In this thesis, the antigenic

specificity of the KLRG1$^+$ terminally differentiated CD8$^+$ T-cells was not evaluated, and this should be topic of future studies. However, our data strongly suggest that the expansion of KLRG1$^+$ terminally differentiated CD8$^+$ T-cells in AA is more likely to be the result of the autoimmune response driving stem cell destruction, than to aging or CMV infection. In **Chapter 2**, KLRG1$^+$ terminally differentiated CD8$^+$ T-cells were identified in CMV-negative healthy donors, illustrating that their antigenic specificities go beyond CMV. Furthermore, in **Chapter 3**, we showed that terminally differentiated CD8$^+$ T-cells are more abundant in the bone marrow from AA patients compared to age- and CMV status-matched controls. As a result, we speculate that the KLRG1$^+$ terminally differentiated CD8$^+$ T-cells accumulate in the hypocellular bone marrow to sustain immune memory of the targeted autoantigen in AA. The fact that KLRG1$^+$ terminally differentiated CD8$^+$ T-cells are highly abundant in the most severely damaged tissue regions and correlate with disease severity[55,59-61] in other autoimmune diseases supports this idea.

Identity of the progenitor-specific effector cell in AA

Given that our model implies a central role for both T- and B-cells, it is reasonable to question whether the immune response in AA is initiated by autoreactive T-cells or B-cells. Addressing this subject is incredibly complex, since T- and B-cells can activate each other, and AA is only diagnosed after severe immune damage to the bone marrow has occurred. For now, we can only speculate. Our findings provide no evidence that refutes the widely accepted view that AA is the result of a T-cell mediated immune response[5]. We still consider this a possibility, also because previous reports 1) have identified patient-derived T-cells capable of eliminating hematopoietic progenitor cells *in vitro*[25,26], and 2) have demonstrated loss of HLA expression on hematopoietic stem cells, suggesting their escape from T-cell mediated destruction[62,63]. Our model does support a more prominent role for CD4$^+$ T-cells, in addition to CD8$^+$ T-cells, than has been appreciated to date. At the same time, the recent success of B-cell depleting cellular therapies (e.g. CD19 CAR-T) in other autoimmune diseases traditionally considered T-cell mediated cannot be overlooked, since it suggests a more central role for B-cells than just a supportive function[64]. Whether B-cells could also drive AA development through antigen presentation, autoantibody production and/or cytokine secretion remains an open question that warrants further investigation. Given the close proximity of B- and T-cells within the bone marrow (e.g. lymphoid-dominant immune hotspots and the identified TLS), it is imaginable that B-cells present antigens to CD4$^+$ T-cells, leading to CD4$^+$ T-cell activation and an amplification of the immune response by cytokine production which could support the B-cells as well as the CD8$^+$ T-cells. In support of a possible involvement of autoantibodies, we discovered that bone marrow destruction is accompanied by B-cell differentiation into plasma cells, corroborating previous reports that identified potential autoantibodies[65,66] and

demonstrated deposition of IgG antibodies and complement factors within the bone marrow tissue in AA[67].

To fully elucidate the roles of T- and B-cells in AA development, it would be essential to isolate the CCR6$^+$ B-cells, CCR6$^+$ CD4$^+$ T-cells and KLRG1$^+$ EMRA CD8$^+$ T-cells from bone marrow samples for in-depth analyses of their functional state and clonality. Co-culture experiments of these T- and B-cells with autologous hematopoietic stem cells could shed light on potential autoreactivity by measuring T- and B-cell activation and cytokine secretion. On the other hand, analysis of their T- and B-cell receptor repertoires could offer more direct evidence of whether AA is T- and/or B-cell mediated, through the detection of oligoclonal expansion and the identification of their antigenic target(s). As part of the follow-up research on this thesis, we have already isolated CCR6$^+$ B-cells, CCR6$^+$ CD4$^+$ T-cells and KLRG1$^+$ EMRA CD8$^+$ T-cells from bone marrow aspirates collected from AA patients at diagnosis through flow cytometry-based cell sorting. This illustrates that such approaches are feasible. Preliminary data further show that downstream T- and B-cell receptor sequencing can be conducted successfully. Although this strategy is accompanied by several advantages including the large number of cells that can be assessed simultaneously to detect clonally expanded T- or B-cells, it precludes analyses of the entire B-cell compartment due to the loss of plasma cells during aspiration and sample processing. In addition, it hampers immune repertoire analyses within specific bone marrow tissue regions due to the loss of spatial context. Therefore, T- and B-cell receptor sequencing on bone marrow biopsies could be of added value. Bulk T-cell receptor beta sequencing is a currently available option. A pilot experiment from our group demonstrates that this technique can successfully be applied on technically challenging formalin-fixed paraffin embedded bone marrow biopsies to detect expanded T-cell clones within pre-specified tissue regions. However, joint T- and B-cell receptor sequencing on such bone marrow biopsies remains impossible at a single-cell resolution. Furthermore, combined T-cell receptor alpha and beta chain analysis cannot be done, but is necessary to identify the targeted antigen. Looking ahead, the further development of single-cell T- and B-cell receptor sequencing at a subcellular resolution *in situ* is therefore promising[68].

Clinical implications of the proposed immunological model of AA

It should be underscored that our proposed immunological model of AA also has important clinical implications. hATG-based IST, the first-line treatment for most AA patients, is limited by several issues, including the fact that it is a non-specific therapy with low response rates of approximately 70%[18]. Therefore, the overarching aim of this thesis was to decipher the immune-mediated pathogenesis of AA to identify new therapeutic targets for improved therapy. Based on **Chapters 3 and 4** we conclude that successful treatment for AA depends on

two major processes: 1) the disruption of the coordinated immune cell interactions driving AA development in the bone marrow and 2) the selective depletion of pro-inflammatory immune cell subpopulations including the CCR6$^+$ B-cells, Th17-like CCR6$^+$ CD4$^+$ T-cells and KLRG1$^+$ terminally differentiated CD8$^+$ T-cells discussed above. This presents two distinct therapeutic approaches, each with its own advantages and limitations, that may be applied to tackle both aspects.

First, the use of receptor-targeted therapies more broadly applied in the setting of autoimmunity could be successful since the immune response in AA seems to share pathophysiological features with other autoimmune diseases. This is interesting since AA is such a rare disorder and the development of orphan drugs remains a challenge. CCR6 antagonists have already been reported to be effective in reducing inflammation in autoimmunity in a preclinical setting[45,55]. Furthermore, targeting of KLRG1$^+$ lymphocytes has become a topic of interest in the field of tumor immunology[55], and may be adapted in order to permanently remove KLRG1$^+$ terminally differentiated CD8$^+$ T-cells.

A disadvantage of such receptor-targeted therapies is the inevitable depletion of healthy, non-autoreactive CCR6$^+$ or KLRG1$^+$ immune cells besides the potentially pathogenic populations. The second, and preferred, therapeutic approach would therefore be to specifically deplete the autoreactive T- and B-cells based on their antigenic specificity. This underscores the need of future studies on the T- and B-cell receptor repertoires of the CCR6$^+$ B-cells, Th17-like CCR6$^+$ CD4$^+$ T-cells, and KLRG1$^+$ terminally differentiated CD8$^+$ T-cells in order to identify the targeted antigen in AA. Whether successful treatment of AA depends on the elimination of autoreactive T- or B-cells, or relies on a combination of both, is a relevant question that warrants further investigation. Cellular therapies mediating either approach are emerging. For example, engineered T-cells expressing a chimeric autoantigen receptor (CAAR), containing the targeted autoantigen, hold promise in specifically killing autoreactive B-cells[69].

High-dimensional techniques to study the bone marrow at a single-cell resolution

Beyond these new insights into the pathogenesis of AA, this thesis provides an overview of the experimental and computational workflows that can be used when applying high-dimensional techniques to study the bone marrow at a single-cell resolution. **Chapters 2 and 3** offer an important comparison of two proteomics-based approaches, which demonstrates that suspension-based and spatially-resolved techniques provide complementary data and are most powerful when performed side by side. In **Chapter 2**, suspension-based mass cytometry was useful in screening the large numbers of cells needed to detect potentially pathogenic phenotypes in both common and rarer populations based on differential abundance. This technique therefore is useful for the generation of new hypotheses on the

mechanisms of disease. In **Chapters 3 and 5**, imaging mass cytometry offered insight into the biological processes occurring within the bone marrow tissue. Although this technique is not suitable for analyzing large numbers of cells or rare populations, it preserves spatial context to unravel the cellular interactions and morphologies that were lost in suspension.

It is worth discussing two discrepancies between our data from bone marrow aspirates (**Chapter 2**) and biopsies (**Chapter 3**), as they provide important considerations for future studies. The first discrepancy concerns the activated $CD68^{-}CD163^{+}CD4^{+}$ macrophages, which were found to be involved in AA development based on their distinct morphology in **Chapter 3**, but were not enriched in the bone marrow in absolute numbers. This indicates that cellular abundance is not always an indication of importance, and that relevant subpopulations can be missed when cells are studied outside of their native spatial context. The second discrepancy relates to the stark differences in the composition of the myeloid compartment between bone marrow biopsies and aspirates. For example, $CD16^{+}$ myeloid cells were not identified within bone marrow biopsies in **Chapter 3**, but accounted for a median 30% of myeloid cells in bone marrow aspirates from AA patients in **Chapter 2**. In contrast, myeloid cell subpopulations including macrophages, mast cells and granulocytes were identified in biopsies, but hardly found in aspirates. These data illustrate that analyses of bone marrow aspirates can be biased through selective cell loss: granulocytes and mast cells can be lost with cryopreservation[70,71], while macrophages are underrepresented in aspirates due to their "stickiness" during collection. This could explain why $CD16^{+}$ myeloid cells were the predominant myeloid cell population in bone marrow aspirates from AA patients. Together, these two discrepancies support broader implementation of spatially-resolved tissue profiling techniques for a more accurate analysis of the cellular processes within the bone marrow, either independently, or to validate results found in suspension. This does not only apply to AA, but also to other hematological disorders where myeloid, stromal and progenitor cells are a prominent feature and cannot be studied in their full complexity in suspension.

Recommendations for the analysis of spatial bone marrow datasets

Despite the importance of analyzing bone marrow biopsies, there are few reports that have provided an in-depth analysis of the bone marrow tissue through spatial proteomics (analysis of protein expression) or transcriptomics (analysis of gene expression). This is largely attributable to the challenges introduced by the bone marrow biopsies themselves. On the one hand, the processing of bone marrow tissue samples involves a relatively harsh procedure that can result in protein, DNA and RNA degradation. For example, tissue decalcification is required to enable bone marrow tissue sectioning, but can alter proteins and therefore affect tissue staining[72]. Furthermore, decalcified bone marrow biopsies require embedding

in formalin-fixed paraffin-embedded (FFPE) blocks, which can further dissociate proteins, DNA and RNA. On the other hand, the bone marrow microenvironment consists of a broad spectrum of both hematopoietic and non-hematopoietic cells that is far more heterogeneous than most other tissues[73]. Accordingly, proper profiling of bone marrow biopsies can only be performed when applying the most advanced techniques to distinguish all cell types based on their distinct phenotypes and morphologies.

This thesis provides a technically relevant contribution through the optimization of imaging mass cytometry that overcomes both issues. **Chapter 3** presents a 37-marker imaging mass cytometry panel that was designed by carefully screening more than 100 antibodies and confirming their performance on decalcified bone marrow tissue. This panel can visualize the full spectrum of bone marrow cell types in their true morphological shapes and is not limited by issues of autofluorescence and the need for cyclic staining[74]. Therefore, imaging mass cytometry is a useful alternative to fluorescence-based imaging (e.g. CODEX or CyCIF imaging platforms) and spatial transcriptomics (e.g. Xenium or CosMx techniques), which can be affected by spectral overlap and the lack of a direct visualization of cell morphology, respectively[75,76]. It is worth noting that our imaging mass cytometry panel can be adapted to study other hematological disorders by replacing AA-specific markers including CCR6, while keeping its backbone. Like with other antibody-based approaches, however, several challenges warrant consideration. This includes the limited availability of suitable antibody clones for decalcified FFPE bone marrow tissue, and the fact that the analyses are not fully unbiased. Yet, with the increasing number of antibody-based studies on bone marrow biopsies, imaging mass cytometry is a promising technique for research driven by a clear question on specific cell subpopulations.

A prominent, but less recognized, hurdle in spatial profiling besides data acquisition are the downstream data analyses that require tailoring to each tissue type. To appropriately extract single-cell data from the acquired images, we established a data analysis pipeline that provides a detailed guideline on how to handle spatially-resolved datasets of the bone marrow, from cell segmentation to spatial analysis. We showed that cell segmentation should always be based on multiple cell markers, beyond the nuclear DNA stain. This is particularly important for segmenting megakaryocytes, which are large in size and lack the markers typically used (e.g. CD45, Vimentin)[77]. In addition, unlike previous reports[73], we found that stromal cells can be segmented appropriately, but only when a suitable marker that captures their complex shape (e.g. CD271) is included. We do recommend that the number of markers should be selected carefully based on the research question, since every additional marker increases the risk of data loss due to overlapping features in the cell segmentation mask. These segmentation errors can be expected to wane as imaging resolution continues to improve.

For the downstream subpopulation and spatial analyses several approaches were evaluated. The major difficulty here was to overcome the obstacles introduced by AA itself, which were 1) depletion of the myeloid compartment and 2) bone marrow hypocellularity. We found that both challenges could best be addressed by analyzing cell abundance as an absolute cell count, normalized to the tissue area of the hematopoietic compartment. This helped avoid analyzing skewed data of cell frequencies, and thereby facilitated a fair comparison between AA and control bone marrow tissue. As a result, our approach differed from previous spatial analyses of tissue microenvironments[71,73,78]. We envision that this approach will be of interest to future studies of normal and diseased bone marrow, as the cellular composition of the bone marrow shifts with age[73,79] and displacement of healthy hematopoietic cells is a hallmark of hematological malignancies[34].

For the spatial analyses, we recommend another customized approach. We discovered that the 'traditional' pairwise interaction analysis between all cell types as proposed by Schapiro *et al*[80] was inadequate when comparing tissues with heterogeneous cellularities. The Schapiro approach calculates the average number of cell-cell interactions an returns an estimate of the interaction or avoidance of cell subpopulations, corrected for randomness. In the context of AA, however, the reduced number of cellular interactions hampered comparison to a normal, cellular bone marrow architecture. Therefore, we applied the spatial cellular neighborhood analysis proposed by Schürch *et al*[81], which clusters cells based on the information contained in their direct microenvironment. The main advantages of this approach were that it 1) enables analysis of all segmented cells simultaneously, 2) provides a visualization of the identified neighborhoods, and 3) considers a maximum distance in its calculations to control for differences in tissue cellularity. Spatial cellular neighborhood analyses have recently become an increasingly applied approach in studies of the tissue microenvironment, and can be extended to detect spatial niches or trajectories[82,83]. We confirm their value and advocate that cellular neighborhood analyses should be used in future studies of the bone marrow microenvironment as well. These analyses could be particularly useful in unraveling the distinct pathophysiologies of bone marrow failure syndromes, or the heterogeneity in the immune landscape in acute myeloid leukemia subtypes that is reported to influence clinical outcome[84].

Given that our analyses set the stage for further exploration of bone marrow biopsies through spatially-resolved profiling techniques, it is important to consider when such techniques may be applied. The work performed in this thesis revealed several bottlenecks that limit their widespread use. The first concerns the large quantities of data that these techniques provide, which are time-consuming to analyze and require knowledge on both basic biology and bioinformatics. It can be expected that the complexity of these datasets will only increase as the number proteins or genes that can be measured increases, or as methods

are combined[76,85]. The second relates to the low throughput of most spatially-resolved profiling techniques, as data acquisition can take several hours or even days[86]. Therefore, these techniques are primarily useful in exploratory research, where to provide a platform to both test existing hypothesis and generate new ones in health and disease. This limits their clinical applicability. In patient care, however, such detailed information is hardly needed. There, it would be more relevant to detect key findings from exploratory research with more accessible techniques such as immunohistochemistry or immunofluorescence.

Future perspectives: towards identifying the antigenic target in AA

Collectively, this thesis illustrates the feasibility and power of applying high-dimensional single-cell analysis techniques on bone marrow samples to investigate the pathogenesis of AA. Using (imaging) mass cytometry, we successfully identified potentially pathogenic immune cell populations, and thereby address a major gap in the current knowledge on AA. We recognize that our findings, although significant, require further functional studies to confirm the relevance of the immune cell populations identified in **Chapters 2 to 4**. Therefore, continued research is essential. This thesis highlights that identifying the antigenic specificities of these cells should be the next step forward.

Despite the increasing number of TCR sequencing studies[21,25,26], the targeted antigen in AA remains unknown. Recent evidence suggests that AA may result from cross-reactivity, where virus-specific T-cells mistakenly attack the hematopoietic stem cells in the bone marrow[25]. However, it is probable that the origin of the autoimmune response is not so 'simple'. First, the lack of strong HLA-associations[62,87] could suggest that the antigenic target differs between AA patients. Second, it is conceivable that a committed progenitor population(s) instead of the hematopoietic stem cell is the primary target of the immune response. In this scenario, hematopoietic stem cells, which are susceptible to environmental stress[88], could be destructed as a consequence of the development of the inflammatory environment.

The data from this thesis support a more targeted approach than previously applied. Specifically, we propose selecting the $CCR6^{++}$ B-cells, $CCR6^{+}$ $CD4^{+}$ T-cells and $KLRG1^{+}$ terminally differentiated memory $CD8^{+}$ T-cells identified in **Chapters 2 and 3** and screening these cells for reactivity towards autologous stem cells. While this idea appears straightforward, its execution is complicated by the limited availability of materials collected from AA patients at diagnosis. To overcome this issue, a model could be developed to mimic AA *in vitro*. This would involve isolating the $CCR6^{++}$ B-cells, Th17-like $CCR6^{+}$ $CD4^{+}$ T-cells and $KLRG1^{+}$ terminally differentiated memory $CD8^{+}$ T-cells in order to analyze their T- and B-cell receptor repertoire to identify clonally expanded populations that may predominate the T- and B-cell compartments following antigen recognition in AA (Figure 3, top row). These T- and B-cell

receptors could then be transduced into cell lines to generate large numbers of potentially autoreactive T- and B-cells. In parallel, induced pluripotent stem cell (iPSC) technology could be applied to reprogram peripheral blood cells from the same patient, in order to produce sufficient numbers of target cells for coculture experiments (Figure 3, middle row). This model would allow functional testing of potentially autoreactive T- and B-cell receptors and would set the stage for further identification of their targeted antigen.

Another interesting approach is to identify the targeted antigen from the side of the target cell (Figure 3, bottom row). In AA, the autoimmune attack could cause a shift in the hematopoietic stem and progenitor cell compartment, in which stem cells expressing the targeted antigen are depleted but progenitor cells lacking the antigen survive. This phenomenon would explain clonal hematopoiesis, often observed in AA at diagnosis[16]. Detecting this possible shift in the hematopoietic stem and progenitor cell compartment by single-cell RNA sequencing could provide important insight into the identity of the targeted antigen(s). This approach does not need to stand on its own but can complement T- and B-cell receptor sequencing data to provide strong evidence of the antigen(s) involved in AA[25,26].

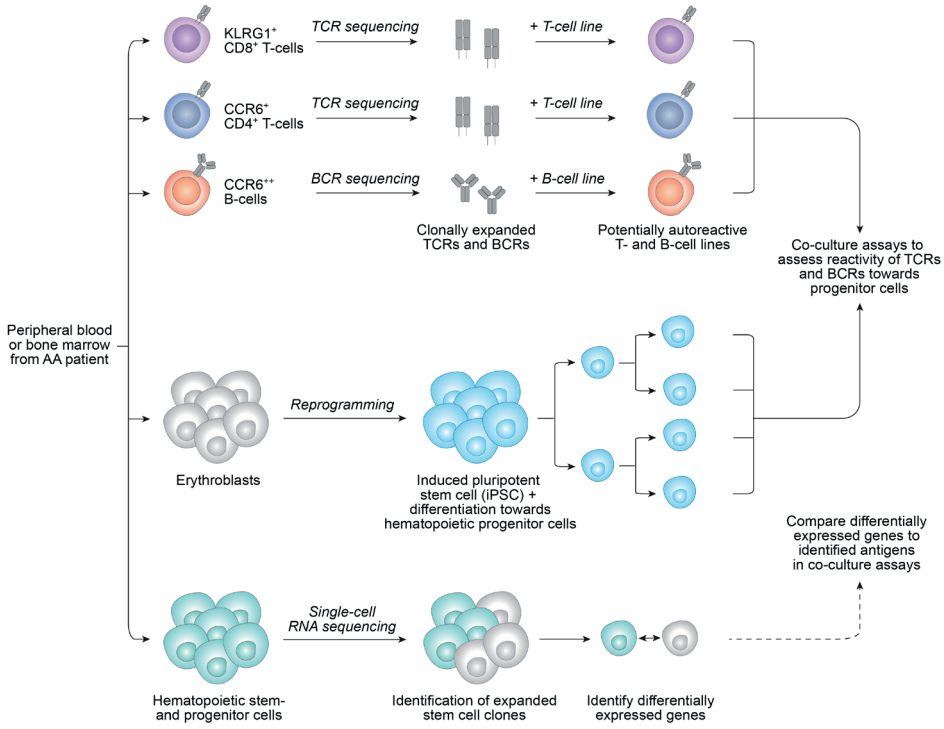


Figure 3. Proposed strategy and experimental workflow towards identifying the targeted autoantigen(s) in AA. BCR: B-cell receptor; TCR: T-cell receptor.

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