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

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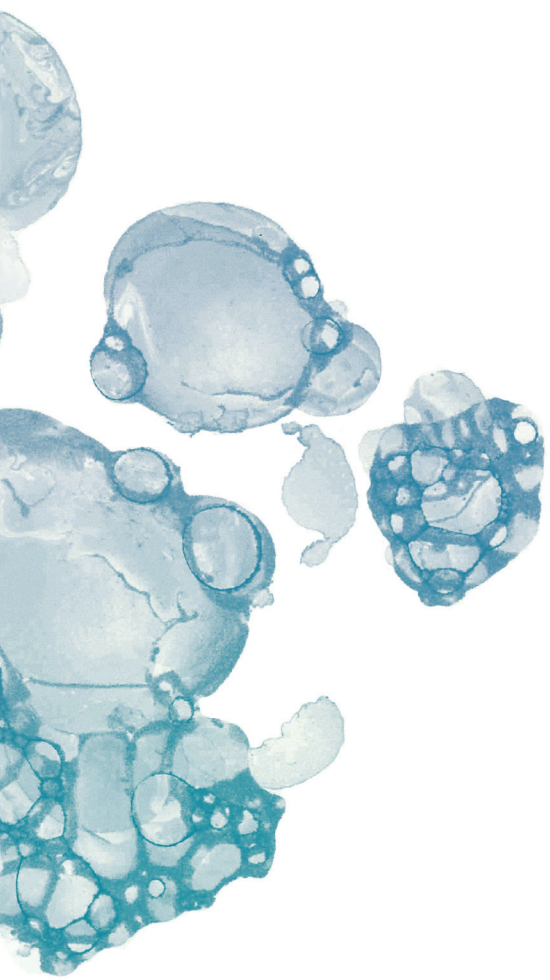

Introduction

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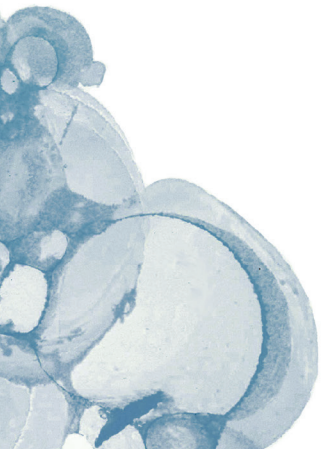

Hematopoiesis

The bone marrow is the primary site for blood cell production, hematopoiesis[1]. The bone marrow produces erythroid cells (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets), responsible for oxygen transport, protection against infections and blood clotting, respectively.

The hematopoietic stem cell is the most primitive cell in the bone marrow and maintains normal hematopoiesis. It has the ability to develop new 'daughter' hematopoietic stem cells through self-renewal, or to differentiate into committed precursor cells that can develop into one of two hematopoietic cell lineages: myeloid or lymphoid[1] (Figure 1).

Commitment to the myeloid lineage gives rise to the common myeloid progenitor, which can differentiate into three major groups of myeloid cells, depending on its development into the megakaryocyte-erythrocyte precursor or the granulocyte-monocyte precursor[1] (Figure 1). The first group of myeloid cells consists of erythroid cells and megakaryocytes, the latter of which are responsible for the production of thrombocytes. The second group of myeloid cells consists of monocytes and dendritic cells of the innate immune system. These cells can activate immune responses by antigen presentation in peripheral blood or tissues. Monocytes are also the precursor cells for macrophages, which can clear pathogens through phagocytosis, or regulate immune responses through antigen presentation and cytokine production. The last group of myeloid cells are the granulocytes, which include the neutrophils, the main effector cells of the innate immune system. These cells provide a first line of defense in case of infections by their ability to capture, engulf and kill pathogens[1].

Commitment to the lymphoid lineage gives rise to the common lymphoid progenitor, which can differentiate into the innate lymphoid cells (ILCs) of the innate immune system, or the T- and B-cells of the adaptive immune system[1]. The ILCs include natural killer (NK)-cells, which have the ability to kill infected and stressed cells without any prior antigenic exposure through the secretion of cytotoxic cytokines and granules. This is how NK-cells differ from T- or B-cells, which depend on the recognition of a specific antigen via the T-cell receptor (TCR) or B-cell receptor (BCR) on their cell surface for their activation. The development of T- and B-cells occurs through two distinct pathways that both involve a highly controlled screening process. Progenitor T-cells migrate from the bone marrow to the thymus for maturation. There, T-cells undergo positive selection to select those T-cells with TCRs that moderately bind antigens, and negative selection to eliminate all T-cells with self-reactive TCRs to prevent autoimmunity. After maturation and selection, naïve T-cells enter the circulation and acquire an effector function upon activation through interactions with antigen-presenting cells. In contrast, B-cell development occurs completely in the bone marrow. Similar to T-cells, B-cell

progenitors undergo a selection process to remove any B-cells with self-reactive BCRs. After selection, mature B-cells circulate in the blood, and following their activation by the binding of an antigen to the BCR, they can differentiate into either memory B-cells, which help prevent future infections, or effector B-cells called plasma cells, which secrete antibodies that can bind and protect against pathogens[1].

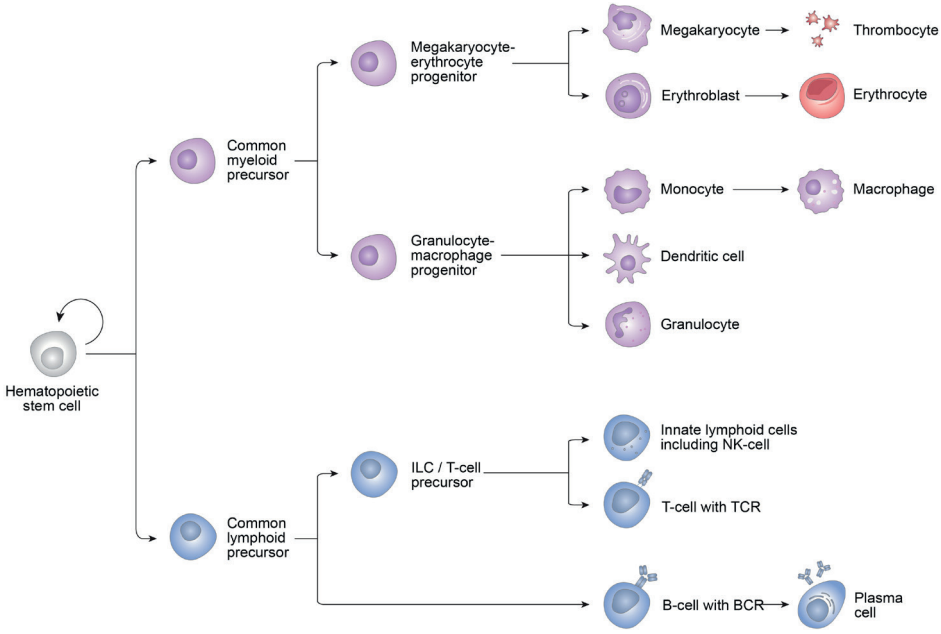

Figure 1. Overview of normal hematopoiesis in the human bone marrow. Figure adapted from The Immune System by Peter Parham[1].

The lifespan of myeloid and lymphoid cells differs considerably: while lymphoid cells can survive for weeks to a lifetime, most myeloid cells have a short half-life of only a few hours or months[1]. Therefore, hematopoiesis needs to be a continuous process to replenish these cells. The bone marrow microenvironment provides signals that support and regulate normal hematopoiesis[2,3]. This microenvironment consists of a complex network of mostly non-hematopoietic cells that can regulate the hematopoietic stem cells through the production of growth factors and chemokines. This includes the adipocytes, which cover most of the bone marrow tissue, are specialized in storing fat, and give the bone marrow its characteristic sponge-like structure (white round 'holes' in Figure 2, left). Other cells of the bone marrow microenvironment that can control stem cell function include the osteoblasts lining the bone, endothelial cells, mesenchymal stromal cells and sympathetic nerve fibers[2].

Bone marrow failure syndromes and aplastic anemia

If the bone marrow fails to produce a sufficient number of leukocytes, erythrocytes and/or thrombocytes, it may be termed bone marrow failure. The complications of bone marrow failure are the result of this decreased hematopoiesis and can involve potentially life-threatening bleeding, bruising, infections and/or fatigue, depending on which branches of the hematopoietic tree are affected.

Bone marrow failure syndromes are non-malignant conditions that result from a profound decrease in the hematopoietic stem cells in the bone marrow. This depletion leads to a characteristic hypocellular bone marrow, where the hematopoietic tissue (purple area in Figure 2, left) is replaced by adipocytes (white areas in Figure 2, right). This 'empty' bone marrow results in pancytopenia with decreased numbers of leukocytes, erythrocytes and/or thrombocytes in peripheral blood. Bone marrow failure can be either inherited or acquired[4]. Immune-mediated aplastic anemia (hereafter referred to as AA) is an acquired bone marrow failure syndrome caused by an autoimmune response targeting the hematopoietic stem cells in the bone marrow[4], and will be the focus of this thesis.

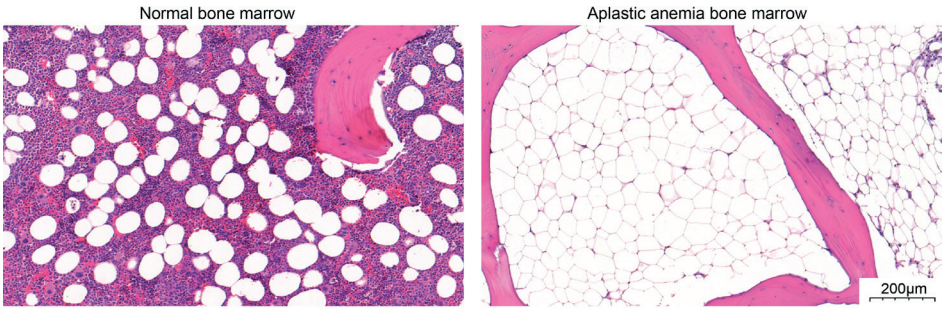

Figure 2. Histology of normal (left) and 'empty' aplastic anemia (right) bone marrow. White areas are adipocytes, long pink structures are bone, and dark purple areas represent hematopoietic tissue.

Immune-mediated aplastic anemia

AA is a rare hematological disorder with an estimated incidence of two per million[5]. Approximately 30 adults in the Netherlands are diagnosed with AA each year based on bone marrow hypocellularity without dysplasia, and pancytopenia (<100 g/L hemoglobin, $<50{\cdot}10^9$ thrombocytes/L and/or $<1.5{\cdot}10^9$ neutrophils/L)[6,7]. Diagnosing AA can be challenging since there are no biomarkers available to definitively confirm the disease. Therefore, the diagnosis of AA primarily relies on the exclusion of other causes of pancytopenia[6]. Clinically, AA is classified as non-severe (NSAA), severe (SAA) and very severe (VSAA) depending on peripheral blood counts[6]. AA can develop at any age but is most often diagnosed in young adults and

older individuals over the age of 60 years[5]. This thesis will focus on adult AA (patients ≥18 years of age). The strongest evidence for an immune-mediated cause is the recovery of the bone marrow and peripheral blood counts following immunosuppressive therapy. The widely accepted view is that the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow are targeted by an autoreactive T-cell response[4]. However, the precise mechanisms leading to AA development remain poorly understood.

Treatment of AA

There are two potentially curative treatment options for AA: allogeneic hematopoietic stem cell transplantation (allo-HSCT) or immunosuppressive therapy (IST). The international treatment guidelines recommend allo-HSCT as the first-line approach in AA patients aged ≤40 years for whom a HLA-matched sibling donor is available[6]. In recent years, allo-HSCT with a HLA-matched unrelated donor has also been applied in younger AA patients with severe cytopenias that require rapid recovery of peripheral blood counts to reduce the risk of infections[6]. However, the advantages of allo-HSCT decline with age, since older age associates with a higher risk of toxicity, graft-versus-host disease and mortality[8]. Therefore, IST is the recommended first-line treatment option in AA patients aged ≥40 years, or for patients under the age of 40 years who are ineligible for allo-HSCT due the lack of a matched donor[6]. This applies to the majority of AA patients.

Immunosuppressive therapy

The aim of IST is to deplete the autoreactive immune cells that target the hematopoietic stem cells in the bone marrow in order to enable the recovery of hematopoiesis and peripheral blood counts[4]. The discovery of IST as a possible treatment for AA occurred unintentionally in the 1970s, when it was found that patients who underwent allo-HSCT and did not engraft their donor still experienced bone marrow recovery[9]. This suggested a therapeutic effect of the immunosuppression that was used with allo-HSCT. Subsequent studies confirmed the efficacy of immunosuppression as a treatment for AA by demonstrating recovery of peripheral blood counts in 40-60% of patients[10-13].

Based on these findings, IST with four consecutive days of 40 mg/kg/day anti-thymocyte globulin (ATG) and long-term ciclosporin is still the golden standard in the treatment of AA[6]. ATG is a polyclonal preparation of primarily IgG antibodies that can be produced by immunizing horses or rabbits with cells from the human thymus (also referred to as thymocytes). In the treatment of AA, both horse-derived ATG (hATG; ATGAM®, Pfizer) and rabbit-derived ATG (rATG; Thymoglobulin®, Sanofi) have been applied. In a study comparing

hATG to rATG, it was believed that rATG would be superior to hATG in the treatment of AA since it 1) induces more profound lymphodepletion; 2) induces the expansion of regulatory T-cells that can dampen pro-inflammatory immune responses; and 3) is effective in patients who relapse after first-line hATG-based IST[14,15]. Unexpectedly, however, response rates were higher with hATG-based IST compared to rATG-based IST[14,16,17]. Therefore, hATG has been the preferred source since.

Horse-derived anti-thymocyte globulin (hATG)

Although IST is the golden standard the treatment of most AA patients, its precise effects are poorly characterized. The short course of hATG is widely believed to induce rapid lymphodepletion, mainly T-cell depletion. However, data on the immunological effects of hATG in AA patients is limited, and experience from other settings is scarce since hATG is almost exclusively used to treat AA. Furthermore, although hATG and other ATG preparations share some characteristics due to similarities in the way they are produced, data from other preparations presumably do not apply to hATG since they are not the same drug[14,18].

hATG is a purified antibody preparation that is made by immunizing horses against cells from the human thymus, the primary site of T-cell development[1]. Therefore, it can be assumed that hATG contains antibodies against surface molecules presented on immature and mature T-cells. However, it is likely that hATG also targets other immune and stromal cells since 1) many surface markers are not exclusive to T-cells (e.g. HLA and CD45), and 2) the thymus is made up of a spectrum of (supportive) cells beyond T-cells[19]. The clinical study that demonstrated the efficacy of hATG has confirmed that hATG binds T-cells and induces T-cell depletion in AA patients treated with first-line hATG-based IST[14,15]. This study further showed that hATG affects other immune lineages, although the relevance of these findings remained unclear[15]. Thus, while these findings suggest hATG exerts a broad impact on the immune landscape, the immunomodulatory mechanisms associated with its therapeutic effects and the mechanisms by which hATG induces immune cell depletion remain to be determined.

Ciclosporin

While hATG is considered the main element of IST, it is not the only component. Long-term ciclosporin is added to standard IST since higher response rates are observed with hATG + ciclosporin-based IST compared to hATG alone[6,7,20]. This implies that ciclosporin could be important by suppressing residual immune cells that remain after hATG and/or by preventing the activation of reconstituted immune cells post-hATG. Ciclosporin is a calcineurin inhibitor that largely exerts its immunosuppressive effects by inhibiting T-cell activation by blocking IL-2 release, a cytokine required for T-cell proliferation[21]. Through this mechanism, ciclosporin

may also indirectly reduce other cytokines such as IFN-γ and TNF-α and therefore influence other immune cells, including antibody-secreting B-cells, macrophages and dendritic cells[22-25]. Therefore, it can have a broad immunosuppressive effect. Ciclosporin is a well-characterized drug commonly used in patients requiring immunosuppression. However, how it modulates the immune landscape in AA has not been studied in detail.

The need for improved treatment strategies for AA

While hATG-based IST can be successful in the treatment of AA, it comes with two major issues. First, bone marrow recovery after IST is delayed, with recovery of peripheral blood counts typically occurring three to six months after start of hATG[6,7]. Therefore, response to IST is determined six months after start of IST. Second, IST is not universally effective. Most patients responding to IST only achieve partial hematological recovery, with few reaching complete normalization of peripheral blood counts[6,7]. In addition, one-third of patients do not respond to IST and face prolonged pancytopenia with the accompanied risk of severe bleeding and infectious complications[6,7].

Since IST is not successful in all AA patients, there is a need to improve the treatment of AA. For this reason, alternative treatment strategies have been investigated. First, the optimal dose of hATG has been studied since it remains poorly defined[26]. Studies have compared the recommended dose of four consecutive days of 40 mg/kg/day hATG[6] to lower or higher dosing regimens[12,27,28], but no improvements in response rates have been observed so far. Second, the efficacy of alternative immunosuppressive drugs has been explored. However, no benefits of other commonly used immunosuppressive drugs such as alemtuzumab[29] or cyclophosphamide[6,30,31] have been reported. Finally, the use of combination therapies have been investigated. A recent approach that combines hATG-based IST with eltrombopag (Revolade®, Novartis) has shown some success. Eltrombopag is a thrombopoietin receptor agonist that was studied due to its ability to stimulate hematopoiesis by inducing the differentiation of the hematopoietic stem and progenitor cells in the bone marrow[32]. In 2022, a multicenter clinical trial showed improved response rates and faster hematologic response with hATG-based IST plus eltrombopag[32]. As a result, treatment guidelines have since recommended adding eltrombopag to standard first-line hATG-based IST, or as a second-line treatment starting six months after the start of IST[6].

Despite these efforts, IST, even in combination with eltrombopag, comes with numerous issues beyond the challenges regarding the suboptimal response rates described above. IST is a nonspecific therapy and therefore is associated with toxicities and adverse events, both in the short- and long-term[32]. Another concerning complication is clonal evolution to

secondary hematological malignancies including acute myeloid leukemia, which occurs in approximately 10-15% of AA patients treated with IST[33,34]. Despite efforts to identify high-risk patients and to delineate the underlying cause[35-38], the precise mechanisms driving clonal evolution are not clear and predicting its occurrence remains challenging.

Towards targeted treatment in AA

Targeted therapy would be the golden ticket to improve the treatment of AA. Such therapies could specifically deplete the immune cells that are believed to target the hematopoietic stem cells in the bone marrow without causing off-target effects. However, the development of targeted therapies is currently impossible because it remains unclear which immune cells are precisely involved in the pathogenesis of AA and which antigen(s) on the surface of hematopoietic stem and progenitor cells is targeted. Unraveling the immune-mediated pathogenesis of AA is essential to identify pathogenic immune cell populations that could serve as therapeutic targets for targeted therapy. Furthermore, it could help improve patient classification to individualize treatment. The overarching aim of this thesis is to decipher the immune-mediated pathogenesis of AA.

The immune system in AA: what do we know?

To better understand the immune mechanisms involved, several studies already have aimed to delineate the immune cells mediating bone marrow destruction in AA. Historically, AA has been regarded as a T-cell mediated autoimmune disorder, mainly due to the efficacy of hATG, which is generated against T-cells and T-cell precursors (thymocytes)[4,18]. As a result, there has been a focus on T-cells in research investigating the immune response in AA. The earliest studies demonstrating T-cell abnormalities date from the 1980s, when altered $CD4^+/CD8^+$ T-cell ratios and increased HLA-DR expression by T-cells hinted towards activation of the T-cell compartment[39]. Around the same time, activated lymphocytes from AA patients were found to produce the pro-inflammatory cytokine interferon and to suppress hematopoiesis when cultured with normal bone marrow *in vitro*[40,41]. Additional evidence supporting a T cell-mediated immune response was provided when studies identified a significant expansion of activated cytotoxic $CD8^+$ T-cells that was more prominent in bone marrow compared to peripheral blood[42].

By making use of the advancements in immune profiling techniques, more recent studies have taken a deeper dive into the T-cell compartment to identify the precise effector cells driving bone marrow destruction. These studies can be divided into two groups based on their two distinct approaches. The first group has used immunophenotyping to explore the frequencies

of CD8+ T-cell subpopulations, based on the hypothesis that effector T-cells mediating stem cell destruction would be more abundant in AA patients compared to healthy controls. Early immunophenotyping studies pointed towards a relative increase of effector memory and terminally differentiated memory T-cells in both peripheral blood and bone marrow in AA[43]. Further analysis demonstrated that these terminally differentiated memory CD8+ T-cells often co-expressed CD57 and contained cytotoxic granules loaded with granzyme B and perforin, emphasizing their strong cytotoxic potential[44].

The second group of studies has investigated the clonality of the TCR, and reasoned that pathogenic T-cells mediating bone marrow destruction could be detected by their clonal expansion following antigen recognition and activation. Despite the technical difference, this approach has complemented the findings from the immunophenotyping studies by demonstrating that clonally expanded CD8+ T-cells are present in AA[45,46], can kill patient-derived hematopoietic progenitor cells, co-express CD57[47,48], and are primarily found within the terminally differentiated memory T-cell population[45]. However, the cellular interactions and antigenic specificity, and therefore the precise relevance, of these T-cells remain largely unknown. There is recent evidence that suggests that the pathogenic T-cells in AA are virus-specific T-cells cross-reactive for an antigen presented by hematopoietic stem and progenitor cells[47]. However, data from a large cohort of AA patients are missing. Furthermore, while CD57+ CD8+ T-cells may appear as the key effector cells, the possibility that other CD8+ T-cell populations play a role in the pathogenesis of AA cannot be excluded.

CD4+ T-cells

Although CD8+ T-cells have been the primary focus in studies on AA, there has been a growing interest in the CD4+ T-cell compartment, mainly due to the importance of CD4+ T-cells in other autoimmune diseases. CD4+ T-cells include various helper T-cell populations that provide support for other cells of the immune system including cytotoxic T-cells and B-cells through cytokine release[1]. This includes T-helper 1 (Th1) cells that secrete interferon, Th2 cells that produce IL-4, and Th17 cells that release IL-17[1]. In contrast, regulatory T-cells of the CD4+ T-cell compartment can dampen immune responses by producing immunosuppressive cytokines such as IL-10 and TGF-β[1]. In AA, total CD4+ T-cell counts do not significantly differ from controls[49,50]. However, early research on CD4+ T-cells demonstrated a shift in the Th1/Th2 balance[51], and a profound decrease of regulatory T-cells[52], consistent with a state of inflammation. Subsequent work on the CD4+ T-cell compartment confirmed these findings and further showed that regulatory T-cells tend to correlate with disease severity[49] and IST-response[53]. Intriguingly, Th1 cells are clonally expanded in AA patients[49], but like CD8+ T-cells, their antigenic specificity and precise role remain unknown in AA. Data suggest that these

cells may be recruited during the early stages of bone marrow failure by pro-inflammatory Th17 cells[49,54], although the precise relevance of these phenotypes as well as the full complexity of the $CD4^+$ T-cell compartment remain poorly defined.

B-cells

Given the focus on T-cells in the pathogenesis of AA, there is little data supporting a role for other immune cell populations. Autoantibodies have been detected in AA[4,55,56], indicating potential B-cell involvement in AA, even though the functional relevance of these antibodies is debated. Direct evidence supporting a role for B-cells in AA is limited. The most comprehensive analysis of the B-cell compartment to date showed no significant differences in total $CD19^+$ B-cell counts nor frequencies in peripheral blood between AA patients and healthy controls[50]. Analyses at the cell subpopulation level did reveal significantly lower frequencies of $CD24^{high}CD38^{high}$ regulatory B-cells, which have since been confirmed in multiple analyses[57,58]. The biological meaning of this finding is yet to be determined. Some studies suggest that this decrease in regulatory B-cells could contribute to the inflammatory environment in AA through a decline in the anti-inflammatory cytokine IL-10, which suppresses cytotoxic T-cells and T-helper 1 responses under normal conditions[50].

Beyond these regulatory B-cells, no significant differences have been reported in mature naïve B-cell, memory B-cell or plasma cell frequencies between AA patients and controls[50]. However, functional markers were not studied and therefore the state of the B-cell compartment remains unclear. Notably, there is a recent study that found an increase of a B-cell subpopulation with pro-inflammatory properties[59]. This could suggest an inflammation-associated shift in the B-cell compartment in AA.

Innate immune cells

Adaptive immune cells including T- and B-cells depend on innate immune cells for activation[1]. Therefore, it is unsurprising that there has been an interest in the role of innate immune cells in AA. Among these, NK-cells have been of particular interest due to their ability to kill aberrant cells without needing prior exposure, and to secrete high quantities of pro-inflammatory cytokines[1,57,60-63]. Dendritic cells and macrophages have also gained interest due to their capacity to present antigens and to control inflammation, respectively[64-68]. However, interpreting the presence of innate immune cells in AA is complex. Increased frequencies of these cells may not necessarily be pathogenic but may instead result from reduced hematopoiesis, given their short half-life, typically of a few days. Additionally, if pathogenic, it remains unclear whether innate immune cells are primary players contributing to stem cell destruction or rather have a supportive function.

Immunological questions in AA

There is substantial data suggesting that the development of AA results from an autoimmune response against the hematopoietic stem cells in the bone marrow. However, after 50 years of research, the precise identity of the effector cells driving bone marrow destruction remains unknown. The growing body of research described above strongly suggests that AA results from an autoimmune response involving a spectrum of immune cells, rather than just a T-cell mediated attack. However, four critical limitations in prior research have obstructed a comprehensive understanding of the immune response underlying the disease.

First, most studies have focused on specific immune cell populations, rather than characterizing the full immune landscape in AA patients. This is mostly due to technical limitations in the number of immune cell subpopulations that could be analyzed simultaneously. As a result, potentially pathogenic immune cell subpopulations most likely have been overlooked.

Second, most studies have analyzed peripheral blood instead of bone marrow, mostly due to the scarce availability of bone marrow samples. Consequently, the presence and function of potentially pathogenic immune cell populations at the site of hematopoietic stem cell destruction remain unclear. Since immune cell composition and function can differ considerably between blood and bone marrow[69-71], it is plausible that critical immune cell populations that only occur at the site of stem cell destruction have been missed.

Third, even in studies that have studied bone marrow samples, analyses have mostly been performed on bone marrow aspirates instead of bone marrow biopsies. The few studies that have investigated bone marrow biopsies have relied on conventional imaging techniques without the possibility to characterize neighboring cells[54,68]. As a result, the interactions between potentially pathogenic immune cell populations and their cellular target remain within the bone marrow microenvironment remain unclear.

Fourth, the precise effects of hATG-based IST have not been delineated. Therefore, it remains unclear how successful IST reshapes the immune landscape and which immune cells must be eliminated in order to enable bone marrow recovery.

The need for high-dimensional analyses of the immune response in AA

The limitations of prior research illustrate the need for comprehensive approaches to analyze the immune landscape in AA to 1) delineate which immune cell subpopulations precisely are involved in the pathogenesis of AA, and 2) elucidate the cellular interactions and processes leading to stem cell destruction within the bone marrow microenvironment.

Recent developments of high-dimensional single-cell analysis techniques have enabled such comprehensive analyses of the immune landscape in both liquid and tissue biopsies. This includes mass cytometry (cytometry by time of flight; CyTOF), which allows for the simultaneous detection of over 40 cell markers and therefore facilitates in-depth phenotyping of immune cell subpopulations within one sample[72]. Suspension mass cytometry is an advanced version of flow cytometry that uses metal isotope-tagged antibodies instead of fluorochrome-conjugated antibodies to study liquid biopsies. By detecting these metal tags, mass cytometry is not limited by autofluorescence. This enables the detection of large antibody panels and the identification of both common and rarer immune cell subpopulations that may be difficult to distinguish with flow cytometry. Unlike single-cell RNA sequencing, which quantifies gene expression, mass cytometry measures protein expression, which offers a more detailed view of the immune landscape and its functional state. As a result, the use of mass cytometry has led to the discovery of new immune cell subpopulations and has provided unique insight into disease mechanisms, including cancer and autoimmunity.

A recent adaptation of mass cytometry is the development of imaging mass cytometry to study in-depth phenotyped cells within their native tissue microenvironment in tissue biopsies[73]. Imaging mass cytometry differs from more conventional imaging techniques such as immunohistochemistry or immunofluorescence by enabling the detection of a larger number of cellular markers at a single-cell resolution. Therefore, imaging mass cytometry facilitates comprehensive analyses of both the spatial localization and cellular interactions of in-depth phenotyped cells within the tissue. Recently, imaging mass cytometry has improved our understanding of the spatial organization of immune response in both health and disease, including autoimmunity[74-79]. However, its application on bone marrow tissue samples remains limited and comes with significant challenges due to the need for tissue decalcification.

Aims of this thesis

AA is a rare immune-mediated bone marrow failure syndrome that results from a profound decrease of hematopoietic stem cells in the bone marrow. The exact immunological mechanisms underlying the development of the disease remain poorly understood. This impedes the development of targeted therapies that could improve clinical outcome. The research described in this thesis aims to unveil the immunological mechanisms leading to bone marrow destruction.

In **Chapter 2**, we construct a comprehensive map of the immune landscape in bone marrow aspirates from AA patients to investigate which immune cell subpopulations may contribute

to the pathogenesis of AA. Using mass cytometry, we discover that AA is characterized by a disease-specific immune cell network that is significantly enriched in AA patients compared to healthy donors. We find that this network is composed of a spectrum of cells from multiple immune lineages, consistent with a state of chronic inflammation. Specifically, it includes $CD16^{+}$ myeloid cells, $CCR6^{high}$ B-cells, Th17-like $CCR6^{+}$ memory $CD4^{+}$ T-cells, $CD45RA^{+}CCR7^{+}CD38^{+}$ $CD8^{+}$ T-cells and $KLRG1^{+}$ terminally differentiated $CD8^{+}$ T-cells. Importantly, this network shows a trend toward normalization after successful IST, suggesting a pathogenic role. These observations provide the foundation for new research questions that are studied in **Chapter 3** and **Chapter 4**.

In **Chapter 3**, we investigate the immune response in AA within its native microenvironment in the bone marrow. This analysis aims to elucidate the chronological order of events occurring during disease development and to define the interactions between potential pathogenic immune cells and their cellular target. Using imaging mass cytometry on bone marrow biopsies, we generate a spatially-resolved single-cell atlas of the bone marrow in AA both before and six months after hATG-based IST. We confirm the presence of the identified immune cell network from **Chapter 2** in the bone marrow pre-treatment. Additionally, we identify immune 'hotspots' 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 progenitor cells, these immune hotspots are nearly absent and more differentiated cells remain. Combined, these observations provide a unique visualization of the immune response during bone marrow destruction and reveal which immune cell interactions are critical in AA.

In **Chapter 4**, we perform an in-depth analysis of hATG-based IST to elucidate its precise effects *in vivo* and to evaluate how successful IST reshapes the immune landscape to enable bone marrow recovery. Since ATGAM (commercially available hATG produced by Pfizer) is generated in horses, it is a polyclonal antibody preparation with both human and non-human specificities. Therefore, we apply flow cytometry to focus on the 'active' ATGAM fraction capable of binding to human lymphocytes and inducing its therapeutic lymphodepleting effect. We provide evidence there is significant between-patient variability in active ATGAM exposure that may influence hematologic recovery. By interrogating the immunomodulatory effects of ATGAM exposure, we find that ATGAM exerts differential effects across T-, NK- and B-cells. Intriguingly, we also discover that successful treatment with ATGAM-based IST does not require a full immune 'reset' but rather depends on the selective depletion of the potentially pathogenic immune cell populations identified in **Chapter 2**. This depletion occurs either immediately upon ATGAM infusion, or through sustained immunosuppression with long-term ciclosporin use. Overall, these findings provide unique insight into the impact

of ATGAM-based IST and confirm the relevance of populations described in **Chapters 2 and 3**.

In **Chapter 5**, we explore how AA treated with first-line hATG-based IST impacts the biology of the bone marrow in the long term. We use a case study to investigate the biological mechanisms underlying clonal evolution to acute myeloid leukemia, a common and concerning long-term complication occurring in approximately 10-15% of patients who survive AA[6]. We propose a model in which the development of secondary acute myeloid leukemia is the result of the uncontrolled expansion of a preleukemic clone that may thrive in the bone marrow microenvironment created by the initial bone marrow failure. These findings underscore the added value of studying bone marrow biopsies as shown in **Chapter 3** and propose a potential role of the bone marrow microenvironment in determining long-term outcome.

In **Chapter 6**, we summarize the main findings of this thesis, discuss the autoimmune response in AA in a broader context, and explore future perspectives.

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