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Impaired Immune Privilege Exclusive to HSCs Mediated by Tregs is associated with HSCs Exhaustion in children with Aplastic Anemia

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Abstract

A series of immune abnormalities presenting in aplastic anemia (AA) support the immune pathogenesis of AA. However, how abnormal immunity specifically damages hematopoietic stem cells (HSCs) remains ambiguous. The discovery of bone marrow immune privilege (IP) sites which are composed of CD4 $^{+}$CD25 $^{+}$FoxP3 $^{+}$ regulatory T cells (Tregs) exclusively to protect HSCs prompted us boldly assumed that it is a loss of IP protection leading to HSCs exhausted in AA. A experiment study to clinically confirm the correlation between HSCs depletion and IP abnormalities in patients with AA was conducted. The distribution of Tregs in bone marrow from children with AA, myelodysplastic syndrome (MDS) and control group was detected by immunohistochemistry. Th1, Th2, Th17 and HSCs as well as cytokines in bone marrow of these children were examined by flow cytometry. Tregs near endostea surface of bone marrow of children with AA was significantly lower than that in control and MDS. Th1 was more predominant in AA than in the control children. TNF-α, IFN-γ and IL-17 levels were also increased in AA. Compared to the control group, HSCs in bone marrow of AA, including Long-term HSCs (LT-HSCs) and Short-term HSCs (ST-HSCs), were at lower level. The results indicate that HSCs depletion is closely related to bone marrow IP abnormalities in AA, and the role of IP abnormalities in the pathogenesis of aplastic anemia deserves further research.

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Running head: HSCs Loss Protection of immune privilege

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immune privilege, Tregs, hematopoietic stem cells, aplastic anemia

Abbreviations

AA	aplastic anemia
HSCs	hematopoietic stem cells
IP	immune privilege
Tregs	$CD4^+CD25^+FoxP3^+$ regulatory T cells
MDS	myelodysplastic syndrome
LT-HSCs	Long-term HSCs
ST-HSCs	Short-term HSCs

Abstract

A series of immune abnormalities presenting in aplastic anemia (AA) support the immune pathogenesis of AA. However, how abnormal immunity specifically damages hematopoietic stem cells (HSCs) remains ambiguous. The discovery of bone marrow immune privilege (IP) sites which are composed of $CD4^+CD25^+FoxP3^+$regulatory T cells (Tregs) exclusively to protect HSCs prompted us boldly assumed that it is a loss of IP protection leading to HSCs exhausted in AA. A experiment study to clinically confirm the correlation between HSCs depletion and IP abnormalities in patients with AA was conducted. The distribution of Tregs in bone marrow from children with AA, myelodysplastic syndrome (MDS) and control group was detected by immunohistochemistry. Th1, Th2, Th17 and HSCs as well as cytokines in bone marrow of these children were examined by flow cytometry. Tregs near endostea surface of bone marrow of children with AA was significantly lower than that in control and MDS. Th1 was more predominant in AA than in the control children. TNF-α, IFN-γ and IL-17 levels were also increased in AA. Compared to the control group, HSCs in bone marrow of AA, including Long-term HSCs (LT-HSCs) and Short-term HSCs (ST-HSCs), were at lower level. The results indicate that HSCs depletion is closely related to bone marrow IP abnormalities in AA, and the role of IP abnormalities in the pathogenesis of aplastic anemia deserves further research.

1 ? INTRODUCTION

Acquired aplastic anemia (AA) is characterized by pancytopenia caused by bone marrow failure without dysplasia or fibrosis. Both clinical and laboratory evidence support that pathogenesis of bone marrow

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failure is due to injury to hematopoietic stem cells (HSCs) by abnormal immunity[1,2]. Overall, research on depletion of HSCs in AA has focused on exploring the mechanism by which abnormal immunity attacks HSCs. However, no specific antibodies or immune cells targeting HSCs have been found to date. Therefore, the "immune-mediated pathogenesis" of AA has not yet clarified how immune disorders specifically damage HSCs rather than other cells in bone marrow.

With in-depth study of bone marrow microenvironment, immune privileged (IP) sites of bone marrow specific to HSCs are discovered[3]. IP sites are mainly comprised of $CD4^+CD25^+FoxP3^+$regulatory T cells (Tregs) which surround HSCs near the endosteal surface, providing an immune tolerance microenvironment for HSCs to protect HSCs from infection, radiation and other stress damage. This urged us to shift the research on depletion mechanism of HSCs in AA from immune "attack" to "protection loss" of IP. In fact, IP sites also exist in other tissues, and their abnormal pathogenicity has been a concern in various diseases[4]. For example, IP sites in hair follicle provide protection for epithelial hair follicle stem cells, and functional impairment leads to destruction of epithelial hair follicle stem cells, which is the main pathological mechanism of persistent alopecia in alopecia areata[5]. The local immune tolerance status of IP sites can be determined by detecting $FoxP3^+/CD4^+$ cells with an immunohistochemical method[6]. But the correlation between the collapse of self-tolerance of bone marrow IP sites and AA has not been reported. This study aimed to clinically verify that HSCs failure in AA is closely related to IP abnormalities through the detection of bone marrow IP sites in AA, MDS, and countrol group, providing clinical supportive evidence of worth further study for the IP pathogenesis in AA.

2 ? METHODS

2.1 ? Patients

AA cases and myelodysplastic syndrome (MDS) cases were all from Shanghai Children's Hospital between. According to the severity, AA patients were classified into non-severe AA, severe AA and very severe AA[7]. MDS was diagnosed based on WHO classification criteria[8]. Twenty children with solid tumors without bone marrow infiltration and any hematological complications were used as normal controls. Bone marrow biopsy specimens from AA, MDS and normal controls were used for immunohistochemistry. The bone marrow fluid of the first suction and peripheral blood from AA and controls were detected by flow cytometry.

2.2 ? Immunohistochemical staining for FoxP3, CD4 and CD3

Bone marrow specimens from posterior iliac crest were obtained in all cases. Immunohistochemical staining for FoxP3, CD4 and CD3 was separately performed using Ventana Benchmark GX Immuno-AutoStainer (Roche) according to the manufacturer's instructions. The primary anti-human rabbit monoclonal antibody, incluing anti-FoxP3 (dilution 1:500; Abcam), anti-CD4 (dilution 1:100; Abcam) and anti-CD3 (dilution 1:100; Abcam), were revealed with an indirect biotin streptavidin system (HRP/DAB Detection ICH kits, Abcam). Cells of $FoxP3^+$presented brown chromogen on the nucleus, while $CD4^+$and $CD3^+$ cells showed cytoplasmic brown granules.

All slides were reviewed independently by two skilled pathologists. The positive cells were counted in 5 visual fields near endosteum of trabecular bone under high-power magnification ($\times$600). The mean value of $FoxP3^+/CD4^+$ in each case represented the local immune tolerance status mediated by Tregs.

2.3 ? Flow cytometry analysis of Th1, Th2, Th17 and HSCs

T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17) and HSCs in bone marrow of children and controls were detected by flow cytometry. The anti-human antibodies (BD Pharmingen) included APC-H7-CD3, APC-CD4, FITC-CD183, PE-Cy7-CD196, FITC-CD34, PE-Cy7-CD38,PE-Cy7-CD45RA,PE-CD90, Alexa Fluor 647-CD49f,PerCP-Cy5.5-CD3,PerCP-Cy5.5-CD56, PerCP-Cy5.5-CD11 and PerCP-Cy5.5-CD19. Th1, Th2 and Th17 are the lineages of $CD3^+CD4^+CD183^+CD196^-$, $CD3^+CD4^+CD183^-CD196^-$and $CD3^+CD4^+CD183^-CD196^+$respectively. Long-term HSCs (LT-HSCs) are the lineages presenting $lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$, while short-term HSCs (ST-HSCs) presenting $lin^-CD34^+CD38^-CD45RA^-CD90^-CD49f^-$. The monocyte was collected from bone marrow aspirates

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or peripheral blood with a Ficoll density gradient. Cells were analyzed by a FACSCalibur (BD Biosciences). Data analysis was performed using FlowJo software.

2.4 ? Cytokine assays

Cytokines in bone marrow and peripheral blood were detected by multiplex bead-based flow cytometric immunoassays. Multiplex assay kits for IL-5, TNF-α, IL-2, IL-6, IL-1β, IL-10, IFN-γ, IL-8, IL-17 and IL-12 were purchased from RAISECARE in China. Above cytokines were tested in plasma samples obtained by centrifugation of heparinized bone marrow or peripheral blood from AA and control children.

2.5 ? Statistical analysis

All graphs and statistical analysis were generated by using GraphPad Prism 5 (GraphPad Software). Continuous variables were presented as mean±SD. As the data were not distributed normally, nonparametric Mann Whitney and Kruskal-Wallis tests were used for comparison of data.P <0.05 was considered as statistically significant.

2.6 ? Ethical approval

The Children's Hospital of Shanghai Local Research Ethics Committee approved this study, and informed written consent was obtained from the patients' parents.

3 ? Results

3.1 ? Characteristics of the patients

Of 30 AA children, 56.70% (n=17) were boys, and 43.60% (n=13) were girls. The age ranged from 2 to 14 years, with a median age of 8 years. The percentages of non-severe AA, severe AA and very severe AA were 50.00%, 36.70% and 13.30%, respectively, in the 30 AA patients. There were no statistically significant differences in age or sex among the AA group, MDS group and control group.

3.2 ? Lower Tregs in IP sites of AA

HSCs have been mainly detected in close proximity to vasculature and the endosteum, which is the osteoblast niche[9]. Tregs accumulate in bone marrow and provide protection for HSCs via IP mechanisms. Therefore, FoxP3/CD4 should be able to indicate the immune tolerance status provided by IP to HSCs, especially in the area near endosteum, which has been identified as an IP site, overlapping with the osteoblast niche. We observed FoxP3$^+$, CD4$^+$ and CD3$^+$ cells near the endosteum(Figure 1). FoxP3$^+$/CD4$^+$were significantly lower in AA than in normal controls (18.09%+-5.38%*vs* . 21.72%+-4.21%, P <0.05) and MDS (18.09%+-5.38% *vs* . 22.63%+-5.98%, P <0.05) (Figure 2A). Taking the low number of Tregs in bone marrow into account, we tried to use the absolute count of FoxP3$^+$ cells to represent bone marrow immune tolerance status. The average number of Tregs in AA was also significantly lower than that in normal control (4.07+-1.41 *vs* . 5.25+-1.86 cells/HP, P <0.05) and MDS (4.07+-1.41 *vs* . 5.30+-1.49 cells/HP, P <0.05) (Figure 2B). No matter FoxP3$^+$/CD4$^+$or FoxP3$^+$ absolute count, there was no statistically significant difference between MDS patients and normal controls (P >0.05).

3.3 ? Th1/Th2 imbalance in AA

The normal IP effect also involves homeostasis between Th1 and Th2 with a predominance action of Th2 cells and a relatively low activity of Th1 and Th17[10], so Th1/Th2 can indirectly reflect IP function[11]. Th17 cells are also considered to be similar to Th1 cells in the regulation of immune tolerance. Compared with the normal control, AA group showed a significantly higher proportion of Th1 and a lower Th2 in bone marrow (for Th1 39.80%+-5.20%*vs* . 22.1%+-2.9%, P <0.0001; Th2 59.20%+-5.30%*vs* . 77.20%+-2.60%, P <0.0001), with a Th1/Th2 shift. Meanwhile Th17 was also lower in AA group than that in control group (1.00%+-0.60% *vs* . 0.50%+-0.70%, P =0.0047) (Figure 3).

For Th1/Th2 in peripheral blood of AA, it was also higher than that in the control group, but there was no statistically significant difference (P >0.05) (data not shown). The predominance Th1 of bone marrow also

supported the weakening of the local immune tolerance environment.

3.4 ? Cytokines in bone marrow

Significant increases of TNF-α, IFN-γ and IL-17 were observed in the bone marrow of AA patients comparing with that in contral group with 11.20±35.20 *vs* . 2.80±0.70 pg/mL (P <0.0001), 35.00±7.80 *vs* . 13.00±5.80 pg/mL (P <0.0001) and 28.90±11.20 *vs* 2.60±0.30 pg/mL (P <0.0001), respectively (Figure 4A). The levels of TNF-α, IFN-γ and IL-17 in peripheral blood of AA group were also higher than that in the control group, but there was no statistically significant difference (Figure 4B).

3.5 ? Decrease of LT-HSCs

Hematopoietic stem cell pools include LT-HSCs and ST-HSCs. In order to understand the status of subpopulations of HSCs in AA, we tested LT-HSCs and ST-HSCs. The results showed that both of them were significantly lower in bone marrow of children with AA than that in the control group(for LT-HSCs 1.13±0.82/100000 *vs* . 12.09±5.29/100000,P <0.0001; for ST-HSCs 2.79±1.75/100000 *vs* . 22.98±8.76/100000, P <0.0001)(Figure 5).

4 ? DISCUSSION

The initial understanding of IP was limited to eyes, testes and brain, which are organs with blood tissue barriers. Later, research expanded the concept of IP, which actually exists in many tissues, including hair follicles, intestinal mucosa, lung and so on. IP actively guides and controls the immune response through various mechanisms to maintain the integrity of immune tolerance microenvironment. Fujisaki[3] first revealed bone marrow IP sites by using high-resolution *in vivo* imaging in animal experiments. The main component cells of IP are Tregs, which are characterized by the expression of FoxP3 in the nucleus. Through the binding of membrane CXCR4 receptors with a large amount of chemokine CXCL12 in the HSCs niche, Tregs aggregate to bone marrow near the endosteum surface of trabecular bone to surround HSCs[12, 13], where the transplanted allogeneic HSCs could survive for a long time (30 days) without myeloablative conditioning regimen before transplantation, which is the gold standard experiment for IP identification. Once the Tregs were removed from bone marrow by deleting the CXCR4 receptor in mice, TNF-α and IFN-γ were enhanced, while IL-10 was decreased in the bone marrow[3]. Then, the transplanted allogeneic HSCs described in the above experiment could not survive in the osteoblast niche. HSCs located in IP immune tolerance microenvironment are LT-HSCs[3]. Subsequent studies showed that LT-HSCs were over mobilized with stress induction after Tregs were depleted[12, 14]. Therefore, in bone marrow, IP not only provides an immune tolerance microenvironment to protect LT-HSCs from various immune factors but also reduces the overmobilization of LT-HSCs during stress and maintains the static state of LT-HSCs, which are the stem cells maintaining lifelong hematopoiesis of bone marrow.

It has been proven in AA patients that the ability of Tregs to migrate to bone marrow was impaired, and the proportion of Tregs in the bone marrow detected by flow cytometry was also decreased[15,16]. However, there has been no research linking the immune abnormalities of AA with local immune tolerance status of IP. Interestingly, a series of immune disorders of AA reported in the literature and in our previous studies are strikingly similar to the excessive immune status due to the abnormal IP. The increased expression of TNF-α and IFN-γ in $CD4^+$ and $CD8^+$ T cells was similar to the changes of bone marrow cytokines in the IP damage assay (Treg deletion)[3, 17,18]. The Th1/Th2 polarization detected in a previous study of an AA animal model was highly consistent with the immune disorder caused by impaired IP function[19,20].

Drawing on other experimental methods studying the function of IP in local tissues [4,5,21], we tested the distribution of Tregs in the bone marrow osteoblast niche by immunohistochemistry. There were significantly fewer Tregs on the endosteal surface of bone marrow than in normal controls and MDS patients, accompanied by an increase in Th1/Th2, TNF-α, IFN-γ and IL-17 in bone marrow, while in another bone marrow failure disease, MDS, they were not. All these results suggested that IP structure and function were impaired in bone marrow of children with AA.

The LT-HSCs protected by IP are usually in a static state to maintain the hematopoietic stem cell pool[3].

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Generally, LT-HSCs are appropriately transition into activated ST-HSCs which in turn differentiate into various blood cells[22]. This transition increases during stress. Recent research has shown that Tregs regulate the level of reactive oxygen species in LT-HSCs to prevent LT-HSCs from being over mobilized and exhausted during oxidative stress[11,13]. Our experiment showed significantly reduced LT-HSCs in AA, which can be attributed to the loss of the protective effect of IP on LT-HSCs. Various clinical evidences also support that the ability to maintain HSCs against stress in AA is reduced. Patients usually develop AA after infection, exposure to radioactive substances or certain drugs, and the severity of the disease is often aggravated during infection[1, 23]. In clinical practice, we found that 60%-70% of children with NSAA naturally evolved into SAA during the course of continuous exposure to various environmental stresses[24, 25]. After the depletion of LT-HSCs, there are not enough reserve HSCs to be mobilized, resulting in the corresponding reduction of ST-HSCs. Therefore, we also detected a decrease of ST-HSCs in the bone marrow of children with AA.

5 ? CONCLUSION

Our study clinically confirmed the correlation between AA HSCs depletion and abnormal IP, so the hypothesis of the onset of AA caused by abnormal IP deserves further research. If this assumption is confirmed, the specific depletion of HSCs mediated by immune disorder of AA is able to be reasonably explained as the loss of HSCs under stress owing to the impaired immune tolerance microenvironment provided by IP to HSCs (Figure 6), being expected to break the bottleneck in the study of the pathogenesis of AA.

AUTHOR CONTRIBUTIONS

C.H and S.L analyzed data and write the manuscript. J.Y, X.L, Y.J, T.Z, S.L and Y.L contributed to data collection and sample preparation; S.J, F.Z and H.J contributed to designed research, performed research, analyzed and interpreted data, wrote the manuscript as well as to the approval of the final manuscript.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of the study are available on request from the corresponding author Shayi Jiang.

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Figure legends

FIGURE 1. CD3$^+$, CD4$^+$ and FoxP3$^+$ cells near endosteum detected by immunohistochemistry. The numbers of CD3$^+$, CD4$^+$ and FoxP3$^+$ cells all decreased in AA patients.

FIGURE 2. Tregs decreased in IP sites . FoxP3$^+$/CD4$^+$(A) and FoxP3$^+$(B) were analyzed in normal control, MDS and AA. Both FoxP3$^+$/CD4$^+$ and FoxP3$^+$ cells in AA were lower than that in normal control and MDS.

FIGURE 3. Proportion of Th1, Th2 and Th17 cells in bone marrow.A. The percentages of Th1, Th2 and Th17 in normal control and AA measured by flow cytometry. B. Compared with the normal control group, the percentage of Th1 and Th17 in AA group increased significantly.

ΦΙΓΥΡΕ 4. Εξπρεσσιον οφ ΤΝΦ-α, ΙΦΝ-γ ανδ ΙΛ-17 αμονγ βονε μαρροω ασπιρατες μονονυςλεαρ ςελλς. A. Compared with the normal control group, TNF, IFN- γ and IL-17 significantly increased in bone marrow of aplastic anemia. B. Determination of TNF-α, IFN-γ and IL-17 in peripheral blood by Flow cytometry, there were no differences between normal control and AA.

FIGURE 5. Bone marrow hematopoietic stem cell subsets: LT-HSCs and ST-HSCs. A. The content of LT-HSCs and ST-HSCs in normal and AA detected by flow cytometry. B. LT-HSCs and ST-HSCs in bone marrow of aplastic anemia, especially the LT-HSCs, were significantly lower than those in the control group.

FIGURE 6. A schematic diagram illustrating impared IP leading to HSCs depletion in AA.

FIGURES

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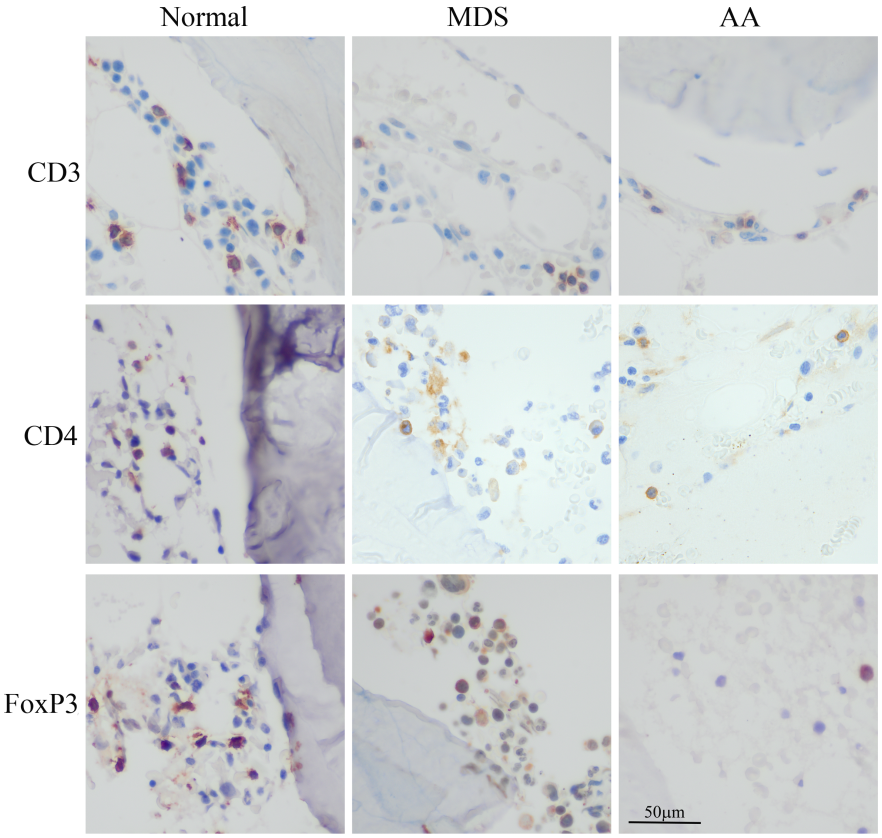

FIGURE 1

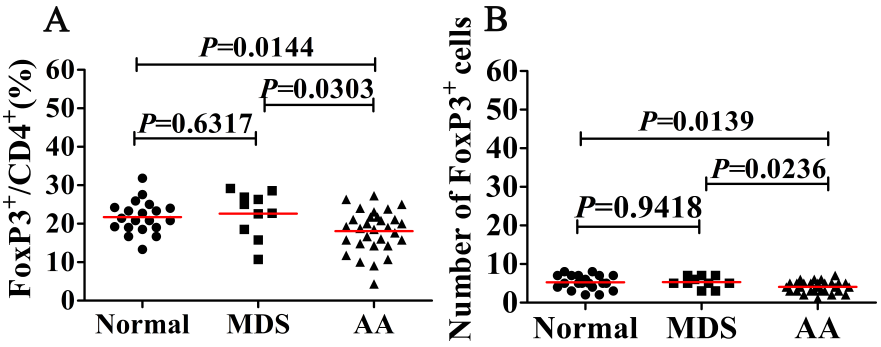

FIGURE 2

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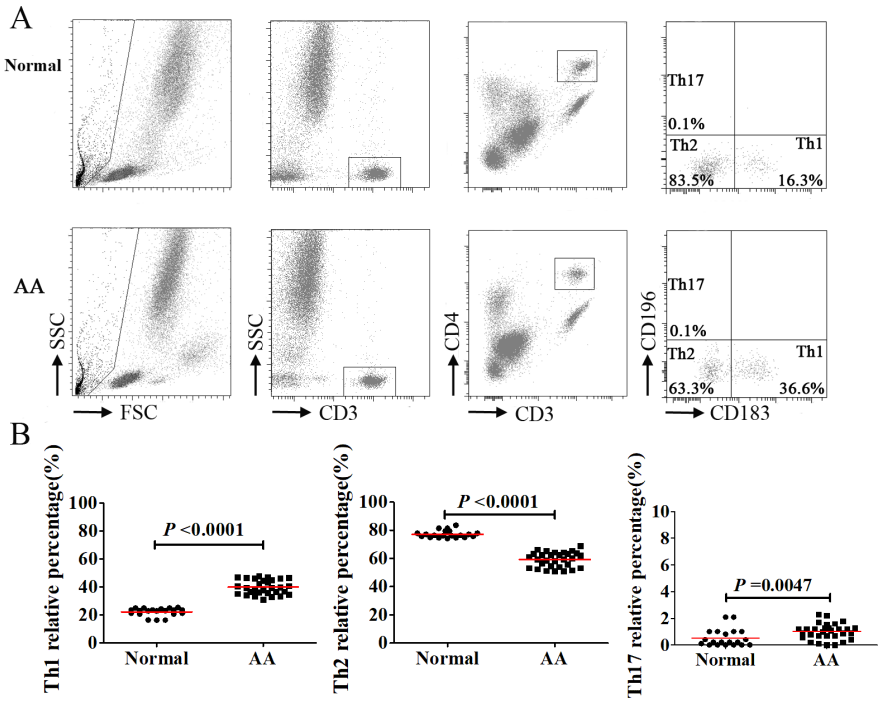

FIGURE 3

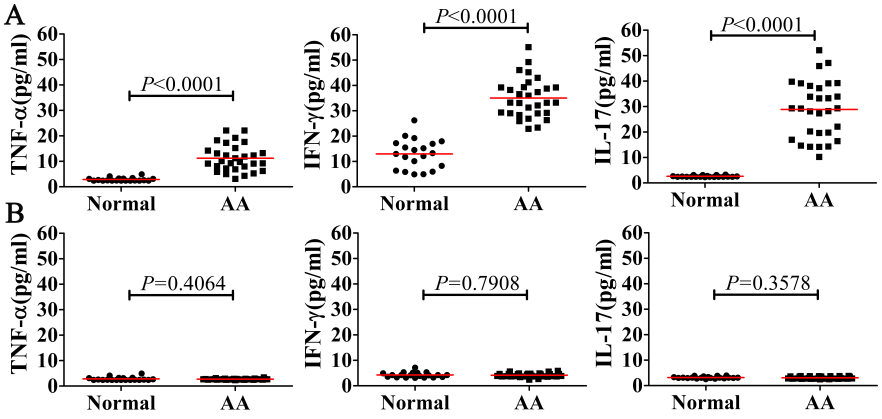

FIGURE 4

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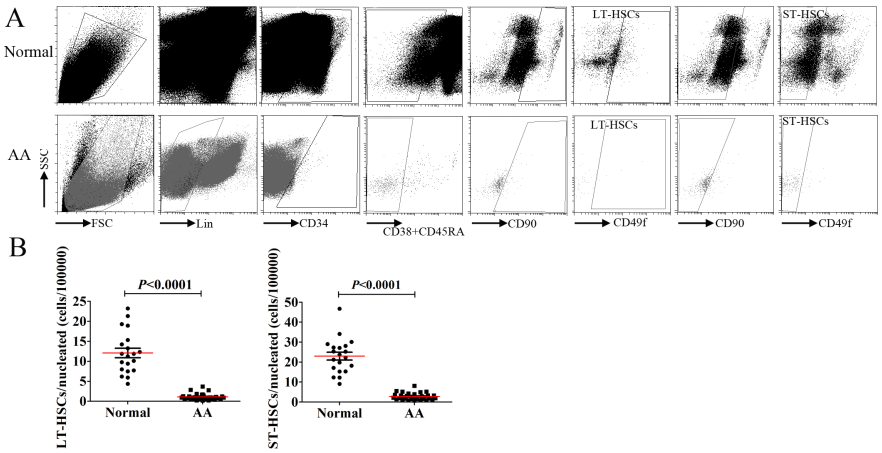

FIGURE 5

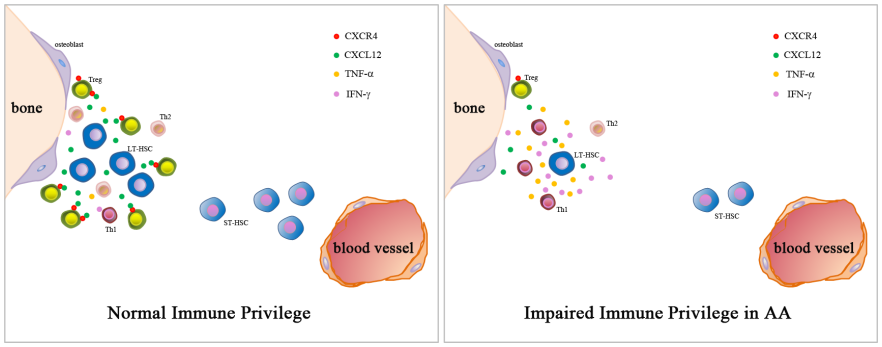

FIGURE 6