A field guide to human Fc-gamma receptors

Genetics, cellular expression and interaction with immunoglobulins

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Citation for published version (APA):

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Blauwe pauw
*Pavo cristatus*
Indian peafowl

Om vrouwtjes te lokken voert de blauwe pauw een dans uit, waarbij hij de staartveren omhoog zet. De staartveren lijken ogen te hebben en vormen een kleurrijk geheel van blauw, groen en paars.
Red pulp macrophages in the human spleen represent a distinct cell population with a unique expression pattern of Fc-gamma receptors

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Manuscript in preparation
ABSTRACT

Tissue-resident red pulp macrophages in the spleen are presumed to play a major role in the clearance of IgG-opsonized blood cells, as occurs in immune thrombocytopenia (ITP) and autoimmune hemolytic anemia (AIHA). Uptake of the blood cells is mediated by receptors for IgG (Fc-gamma Receptors or FcγRs). Recently, studies in rodents have shown distinct characteristics and origin of tissue-resident macrophages as opposed to monocyte-derived macrophages, and have challenged the concept of tissue-resident macrophages deriving from circulating monocytes. However, little is known about red pulp macrophages in humans, and studies on expression of different FcγR isoforms show conflicting results.

We developed a novel method to isolate red pulp macrophages from human spleens, taking advantage of the natural autofluorescence of these cells. As shown in spleens from 78 individuals, the red pulp macrophages are a distinct cell population different from splenic monocytes and monocyte-derived macrophages. Human red pulp macrophages express the low-affinity receptors FcγRIIa and FcγRIIIa, but not the inhibitory FcγRIIb. The high-affinity receptor FcγRI is not constitutively expressed, but was expressed only under inflammatory conditions. This is in stark contrast to monocyte-derived macrophages, which constitutively express FcγRI, FcγRIIa and FcγRIIb.

Concluding, we describe a novel method to isolate pure populations of bona-fide splenic tissue-resident red pulp macrophages, and confirm the distinct nature of this cell population in human subjects. Knowledge on the FcγR expression of these cells is crucial for understanding and improving treatment strategies for autoimmune diseases such as ITP and AIHA.
INTRODUCTION

Circulating blood cells that are opsonized by immunoglobulin G (IgG) auto-antibodies can be rapidly cleared from the circulation. For instance, auto-antibodies against platelets result in immune thrombocytopenia (ITP), whereas auto-antibodies against erythrocytes result in autoimmune hemolytic anemia (AIHA). These blood cells are cleared by macrophages that have direct contact with blood cells, i.e. macrophages of the reticuloendothelial system, which comprises of macrophages in the spleen and the liver\(^1,2\). In vivo studies with radioactively labeled IgG-opsonized platelets and erythrocytes have revealed that in the majority of patients, the spleen, not the liver, is the predominant site of platelet destruction\(^3,4\), a notion that is supported by the fact that splenectomy can be an effective last-resort treatment for refractory ITP\(^5\). Macrophages clear these IgG-opsonized blood cells by phagocytosis, which is mediated by the receptors for IgG (Fc-gamma Receptors, FcγRs). However, splenic macrophages have been poorly characterized thus far in humans, especially with regard to FcγR expression. Functional studies of IgG-mediated phagocytosis in humans often use monocyte-derived macrophages instead\(^6,7\), because these are readily available.

Splenic tissue can be grossly divided into red pulp, which consists of an open circulation and contains many erythrocytes, and white pulp, which consists of lymphoid tissue partly associated in follicles\(^8\). Surrounding the follicles lies a marginal zone, which contains mainly B lymphocytes\(^9\). Within these tissues, different subsets of macrophages have been identified in humans. The most abundant type of macrophage is the red pulp macrophage, characterized by expression of CD163, which is thought to be responsible for the clearance of aged red blood cells from the circulation and iron metabolism\(^8,10\). The open circulation of the red pulp functions as a filter for blood, resulting in close contact of red pulp macrophages with blood cells, facilitating uptake of senescent erythrocytes\(^8\). Another type of macrophages has been identified in a so-called perifollicular zone that is present at the border of the marginal zone and the red pulp, which contains a CD169 positive macrophage population known as perifollicular zone macrophages\(^11\). Finally, macrophages have been observed in the human marginal zone, but these have not been well-characterized and may resemble red pulp macrophages\(^9\).

In mice, the situation is different, with a well-characterized and specialized subset of marginal zone macrophages (SIGNR1 and MARCO positive), which are thought to be responsible for the uptake of apoptotic cells\(^12\). Furthermore, a CD169-positive subset is present as well in the marginal zone, known as marginal zone metallophilic macrophages\(^8\). Finally, rodent red pulp contains many red pulp macrophages, similar to the situation in humans\(^8\). In the past years, many reports have focused on the origin and functional diversity of different tissue-resident macrophages in rodents. This has led to a paradigm shift, as many tissue-resident macrophage populations, including the red pulp macrophages of the spleen, have been shown to consist of self-renewing populations derived from embryonic macrophages, established prior to birth\(^13,14\). Circulating monocytes, which were previously believed to be the precursors of all macrophages,
contribute nothing or at most very little to various tissue-resident macrophages including the red pulp macrophages in the spleen. These tissue-resident macrophages have distinct expression profiles enabling specific functions, and the specific expression profiles are often driven by environmental factors in the local tissue\textsuperscript{14}. For instance, rodent red pulp macrophages are dependent on the transcription factor Spi-C\textsuperscript{15}, which was later shown to be regulated by high heme concentrations in the spleen\textsuperscript{16}.

In humans, the various types of tissue-resident macrophages are much less well characterized, although the concept of stable tissue-resident macrophage populations not deriving from monocytes has been proven in humans\textsuperscript{17,18}. Altogether, human red pulp macrophages and other splenic macrophages may be very different from monocyte-derived macrophages. This raises the question whether monocyte-derived macrophages are a valid model for functional phagocytosis studies regarding IgG-opsonized blood cells.

For instance, expression of the different FcγRs may be different on splenic macrophages. Five of the six isoforms of human FcγRs, all with differences in affinities for IgG and function, can be found on monocyte-derived macrophages\textsuperscript{19}. However, there is little conclusive evidence on which isoforms of FcγR are expressed on splenic macrophages in humans, with contradicting reports. Macrophages throughout the red pulp of the spleen do indeed express receptors for IgG and bind IgG-opsonized erythrocytes\textsuperscript{20}, and immunohistochemistry has indicated that FcγRIII is present throughout the red pulp\textsuperscript{21}. Another study actually showed that both FcγRI, FcγRII and FcγRIII are present on macrophages of the spleen\textsuperscript{22}. These studies could not distinguish between the isoforms of FcγRII and FcγRIII. A recent study investigated the expression of the inhibitory FcγRIIb more specifically, and found that it was expressed on splenic macrophages in most individuals, but was decreased in patients suffering from ITP\textsuperscript{23}. Remarkably this study also suggested that in most individuals, FcγRIII was absent from splenic macrophages\textsuperscript{23}, contradicting the earlier findings. In phagocytosis assays with ex vivo obtained splenic macrophages, FcγRI was suggested as the critical FcγR, whereas blocking FcγRIII had little or no effect\textsuperscript{24,25}.

Altogether, the evidence on which FcγRs are expressed on human splenic macrophages is confusing. In the present study, we developed a protocol for specifically obtaining splenic red pulp macrophages, and used a set of isoform-specific monoclonal antibodies (MoAb) to determine the cell-type specific expression as compared to monocytes and monocyte-derived macrophages.

**METHODS**

**Human Subjects**

Spleen tissue was from organ transplant donors as described before\textsuperscript{26}. Written informed consent for organ donation was obtained according to national regulations regarding organ donation. Splenic tissue of the organ donor was obtained during transplantation surgery, as part of the standard diagnostic procedure for HLA-typing, and was transported in University of Wisconsin
Fluid at 4°C. In case there was an excess of splenic tissue for diagnostic procedures, this excess of splenic tissue was used in an anonymous fashion for research in the present study, in accordance with the Dutch law regarding the use of rest material for research purposes. Blood samples were rest material from blood samples of organ donors drawn at the time of surgery as a standard diagnostic procedure, and were not always available. In some cases, control blood from age matched healthy volunteers was used. Written informed consent was obtained from all healthy volunteers. The study was approved by the Medical Ethics Committee of the Academic Medical Center and Sanquin in Amsterdam, and was performed in accordance with the Declaration of Helsinki.

**Preparation of single cell suspension of splenocytes**

Splenocytes were isolated by injecting a piece of spleen at several sites with collagenase buffer, containing 100 U/ml collagenase CLSPA (Worthington Biochemical Corporation), 2 Kunitz Units/ml DNase (Deoxyribonuclease I, bovine recombinant) (Sigma-Aldrich), 0.5 μg/ml Aggrastat (MSD), 1 mg/ml Glucose (Sigma-Aldrich) and 1mM Calcium Chloride (Merck) in HEPES. Connective tissue was removed and the tissue was subsequently incubated in the collagenase buffer for 30 minutes at 37°C. Tissue was then filtered using a 100μm filter. Subsequently, erythrocytes were lysed with an isotonic ammoniumchloride buffer for 5 minutes at 4°C, after which lysis buffer was washed away.

**Preparation of blood leukocytes and monocyte-derived macrophages.**

Whole blood leukocytes were isolated from heparin or EDTA blood (spleen donors) or from heparin blood (healthy controls) by lysis of red blood cells with an isotonic ammonium chloride buffer. Monocyte-derived macrophages were cultured for 9 days with either M-CSF or GM-CSF from monocytes isolated from heparin blood of healthy controls, as previously described.

**Elutriation**

To enrich for large cells from the single cell suspension of splenocytes prior to sorting, the splenocytes were purified by counterflow centrifugal elutriation (JE-6B Beckman-Coulter centrifuge, Beckman Instruments Inc.; Palo Alto, CA, USA). During this purification the fractions were monitored on a Forward scatter, Side scatter plot on a FACS Canto II machine (BD).

**Cell sorting**

The elutriated fraction of splenocytes was stained with CD163-PE (Trillium diagnostics) and CD14-PE-Cy7 (BD Pharmingen) for 30 minutes in the dark, shaking, at 4°C, and washed twice. Cells were sorted on a FACS ARIA II machine (BD), and were collected at 4°C. Purity of the sorted fractions was checked by flow cytometry on a FACS Canto II machine (BD). Cells for morphological analysis were subjected to a second round of sorting, yielding a purity of > 95%. Cytospins were made after sorting and stained with May-Grünwald Giemsa for morphological analysis.
Flow cytometry

For determination of expression levels on different cell populations from the spleen, the single cell suspension of splenocytes was stained with specific MoAbs. Samples were measured on a FACS CANTO II (BD), using the following channels and wavelengths: Forward scatter, Side scatter, Pacific blue: 450/50 nm, AmCyan: 510/50 nm, FITC: 530/30 nm, PE: 585/42 nm, APC: 660/20 nm, PerCP-Cy5.5: 710/50 nm, PE-Cy7: 780/60 nm, APC-Cy7: 780/60 nm.

For a list of MoAbs see Table S1.

Genetic analysis

Genomic DNA was isolated from splenocytes with the QIAamp® Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

CNV and SNPs in the low-affinity FCGR genes FCGR2A, FCGR2B, FCGR2C, FCGR3A, and FCGR3B were routinely determined with an FCGR-specific multiplex ligation-dependent probe amplification (MLPA) assay (MRC-Holland, Amsterdam, The Netherlands) as described previously27.

Immunohistochemistry

Small pieces of human spleen were embedded in Tissue Tek, frozen in liquid nitrogen vapor and stored at -80°C. Cryostat sections of 7 µm thickness were cut, fixed in acetone for 10 minutes and rehydrated in PBS. The sections were blocked with 5% human serum in PBS for 15 minutes prior to 30 minutes staining with specific MoAbs (Table S1). After washing with PBS, the sections were embedded in vinol containing DAPI to stain the nuclei. Isotype-matched antibodies were used as negative controls. Stainings were analyzed using a DM6000 Leica immunofluorescence microscope.

RESULTS

The spleen contains an autofluorescent cell population of the monocyte/macrophage lineage

In order to characterize the resident splenic macrophages we obtained a single cell suspension by incubation of splenic tissue with a collagenase buffer. We first analyzed the single cell suspension of splenocytes by flow cytometry, which revealed a consistent population of autofluorescent cells in unstained samples (Fig.1a). Such autofluorescent cells never occur in a suspension of whole blood leukocytes (Fig.1a). Apart from the autofluorescent cells, the splenocyte suspension contained, based on canonical forward sideward scatter patterns, populations of neutrophils, monocytes, and a large population of lymphocytes, that were in part much larger than blood lymphocytes (Fig.1a). Autofluorescence is a known characteristic of macrophages, and these cells showed a similar pattern in autofluorescence as monocyte-derived macrophages (Fig.1b). To confirm that the autofluorescent cells represented the splenic macrophages, we stained for canonical lineage markers of leukocytes, corrected for appropriate isotype controls. First, these
Figure 1. Splenocytes contain an autofluorescent cell population of the monocyte/macrophage lineage
A representative flow cytometry plots of unstained single cells suspensions of splenocytes (left) and blood leukocytes (right) showing an autofluorescent cell population in splenocytes but not in blood. Another consistent feature of splenocytes is the large proportion of lymphocytes, which are in part larger than blood lymphocytes. Percentages are indicated, figure is representative of n=78 spleens. B MFIs for different fluorescent channels in unstained samples, comparing the autofluorescent cells in the spleen and spleen monocytes gated as in a, with blood monocytes, M-CSF Mφ and GM-CSF Mφ cultured monocyte-derived macrophages. C, D, E, F stainings of
cells were positive for the Leukocyte Common Antigen CD45 (Fig.1c) confirming hematopoietic origin. Second, these cells were positive for the common monocyte/macrophage markers CD14, CD33 and CD36 (Fig.1d-f), while being negative for the lymphocyte markers CD3 (T cells), CD19 (B cells) and CD56 (NK cells) (Figure S1). Thus, we can conclude that the autofluorescent cells are of the monocyte/macrophage lineage.

The autofluorescent cells in single cell suspensions of splenocytes are red pulp macrophages

Intracellular staining with the macrophage marker CD68 showed the autofluorescent cells to be macrophages, not monocytes (Fig.2a). We then set out to stain these macrophages with the known markers for splenic macrophages, CD163 and CD169. The autofluorescent cells were highly positive for the red pulp macrophage marker CD163 (Fig.2b), although a small percentage of the cells was usually negative, especially in spleens that had been removed > 24 hours before handling (data not shown). CD169 appeared to be present in very low levels, just above background staining, but we never detected a CD169\textsuperscript{high} population (Fig.2c). The monocytes in the spleen also expressed low levels of CD163 (Fig.2b), but no CD169 (Fig.2c). To further confirm that the autofluorescent CD163 positive cells in the spleen are red pulp macrophages, we set out to obtain pure fractions of these cells for morphological analysis. We first enriched for large cells by elutriation of the splenocytes, increasing the percentage of autofluorescent CD163\textsuperscript{high}CD14\textsuperscript{low} cells by approximately 4 times, while not affecting expression levels of surface antigens (data not shown). Cells were subsequently sorted for autofluorescent CD163\textsuperscript{high}CD14\textsuperscript{low} cells, and non-autofluorescent CD163\textsuperscript{int}CD14\textsuperscript{high} monocytes (Fig.2d). Morphological analysis of autofluorescent CD163\textsuperscript{high}CD14\textsuperscript{low} cells revealed a typical macrophage phenotype, with a round nucleus, multiple granular bodies and in some case whole ingested erythrocytes (Fig.2d). The monocytes were similar to normal blood monocytes (Fig.2d). The small population of CD163\textsuperscript{neg} autofluorescent cells consisted merely of cellular debris and dying cells upon morphological analysis (data not shown), revealing that this is not a bona fide cell population in the spleen.

Red pulp macrophages are distinct from monocytes and monocyte-derived macrophages.

To characterize the red pulp macrophages further for several canonical phagocyte surface receptors, we compared these cells with monocytes and monocyte-derived macrophages by flow cytometry. This revealed several marked differences, such as an absence of the integrin CD11b

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**Figure 1. Splenocytes contain an autofluorescent cell population of the monocyte/macrophage lineage (legend continued)**

CD45, CD14, CD33 and CD36 on various cell types in spleen and blood. MFI: median fluorescence intensity, ΔMFI: MFI corrected for staining with an isotype control. M-CSF Mφ: monocyte-derived macrophages cultured for 9 days with M-CSF, GM-CSF Mφ: monocyte-derived macrophages cultured for 9 days with GM-CSF. Means + s.e.m. of n≥8 are shown for each group.
on red pulp macrophages (Fig.3b), whereas the other integrins were present (Fig 3a,c,d). Various other surface markers (Fig 3e-l) in some cases show marked differences from both monocytes and monocyte-derived macrophages, most notably a very high surface expression of gp91PHOX, one of the proteins of the NADPH oxidase complex (Fig.3i). Altogether, red pulp macrophages are highly distinct from both monocytes and monocyte-derived macrophages. On the other hand, monocytes of spleen and blood were similar in their expression of all tested surface markers.

**Red pulp macrophages have a distinct FcγR expression pattern.**

FcγR isof orm expression on spleens from 74 individuals revealed a distinct expression pattern (Fig.4a). When compared to monocytes and monocyte-derived macrophages, levels of the high-affinity FcγRI (CD64) were very low (Fig.4c,d). In fact, red pulp macrophages from many individuals had no expression at all, while others showed low expression of this receptor. Because FcγRI is known to be induced in inflammatory states, we investigated whether this could be the cause in our spleen donors. Indeed, there was a strong correlation of FcγRI expression on red pulp macrophages with FcγRI levels on neutrophils in the same spleen (Fig.4b). FcγRIIa (CD32a) was expressed in both monocytes and red pulp macrophages with similar expression levels, although lower than the levels detected in monocyte-derived macrophages (Fig.4c,d). The inhibitory FcγRIIb (CD32b) appeared not to be present on red pulp macrophages at all (Fig. 4c,d). On monocytes, only a small subset expresses FcγRIIb19, whereas FcγRIIb levels on monocyte-derived macrophages were high (Fig.4d). FcγRIIC (CD32c), an activating Fc receptor which has an extracellular domain identical to FcγRIIb, was not present either; although FcγRIIC cannot be differentiated from FcγRIIb by MoAbs directly, its presence can be deducted by comparing different FCGR2C genotypes19. However, we could not confirm presence of FcγRIIC on red pulp macrophages when comparing individuals of different genotypes (Figure S2). Finally, FcγRIII (CD16) was always present in the whole population of red pulp macrophages(Fig. 4c,d), strikingly different from both monocytes and monocyte-derived macrophages, which have only a small subset of CD16 positive cells, and in which CD16 is low or absent on the majority of cells7,19. We then investigated which isoform of FcγRIII was present on the red pulp macrophages by staining with the MoAb 5D7 (CLBgran11), which specifically recognizes FcγRIIIb. This revealed that the red pulp macrophages express exclusively FcγRIIIa (Figure S3).

**Immunohistochemistry confirms the expression pattern of FcγRs on red pulp macrophages, and reveals FcγRIIa as the major FcγR on perifollicular zone macrophages**

To confirm the FcγR expression pattern of splenic macrophages, we performed immunofluorescent stainings on section of human spleen tissue. This confirmed expression of FcγRII and FcγRIII, and the absence of FcγRI and FcγRIIb (Fig.5a,c,e). In tissue sections, we could also detect the CD169 positive macrophages in the perifollicular zone, which could not be found in the single cell suspension of splenocytes. These perifollicular zone macrophages only had clear expression of FcγRII, but no or at most very low expression of FcγRI, FcγRIIb and FcγRIII (Fig.5b,d,e).
Figure 2. Spleen autofluorescent cells are macrophages and represent the red pulp macrophages of the spleen

A-C Flow cytometry stainings on monocytes in the spleen, blood monocytes, spleen autofluorescent cells, M-CSF and GM-CSF cultured monocyte-derived macrophages. Top panel: representative histogram on spleen autofluorescent cells (black line: specific staining, gray shading: isotype control). Bottom panel: summary of Δ MFIs of multiple stainings. A intracellular staining for CD68 B CD163 staining C CD169 staining. D Sorting strategy for splenocytes sorted into autofluorescent CD163<sup>high</sup>CD14<sup>low</sup> red pulp macrophages and non-autofluorescent CD163<sup>int</sup>CD14<sup>high</sup> spleen monocytes, and May-Grünwald-Giemsa stainings of the sorted cell populations, representative of n=8 experiments.

MFI: median fluorescence intensity, Δ MFI: MFI corrected for staining with an isotype control. M-CSF Mφ: monocyte-derived macrophages cultured for 9 days with M-CSF, GM-CSF Mφ: monocyte-derived macrophages cultured for 9 days with GM-CSF.

Means + s.e.m. of n≥7 are shown for each group.
Figure 3. Red pulp macrophages exhibit an expression pattern distinct from monocytes and monocyte-derived macrophages

A-K Flow cytometry stainings on non-autofluorescent CD163\textsuperscript{int}CD14\textsuperscript{high} spleen monocytes, CD14\textsuperscript{high} blood monocytes gated on FSC/SSC pattern, autofluorescent CD163\textsuperscript{high}CD14\textsuperscript{low} red pulp macrophages from spleen, and M-CSF and GM-CSF cultured monocyte-derived macrophages, A CD11a, B CD11b, C CD11c, D CD18, E CD89, F CD200R, G HLA-DR, H gp91PHOX (NADPH oxidase complex), I TLR4.

MFI: median fluorescence intensity, Δ MFI: MFI corrected for staining with an isotype control. M-CSF Mφ: monocyte-derived macrophages cultured for 9 days with M-CSF, GM-CSF Mφ: monocyte-derived macrophages cultured for 9 days with GM-CSF.

Means + s.e.m. of n≥8 are shown for each group.
Figure 4. FcγR expression on red pulp macrophages is distinct from the expression on monocytes and monocyte-derived macrophages

A overview of expression of the FcγR isoforms on autofluorescent CD163<sup>hi</sup>CD14<sup>lo</sup> red pulp macrophages from spleen, showing individual values. FcγRI n=74, FcγRIIa n=49, FcγRIIb n=39, FcγRIIla n=74, FcγRIIIa n=74, FcγRIIIb n=7. B Correlation of FcγRI expression on Red Pulp Mφ with FcγRI levels on neutrophils in the same spleen (n=74). C representative histograms on autofluorescent CD163<sup>hi</sup>CD14<sup>lo</sup> red pulp macrophages from spleen for FcγRI, FcγRIIa, FcγRIIb and FcγRIII (black line: specific staining, gray shading: isotype control). D comparison of flow cytometry stainings on non-autofluorescent CD163<sup>int</sup>CD14<sup>hi</sup> spleen monocytes, CD14<sup>hi</sup> blood monocytes gated on FSC/SSC pattern, autofluorescent CD163<sup>hi</sup>CD14<sup>lo</sup> red pulp macrophages from spleen, and M-CSF and GM-CSF cultured monocyte-derived macrophages. FcγRI: n≥24 for each group. FcγRIIa: n≥10 for each group. FcγRIIb: n≥15 for each group. To ensure specificity for FcγRIIb, only samples from individuals that cannot express FcγRIIc and with wild-type FCGR2B promoters are presented here. FcγRIII: n≥23 for each group. MFI: median fluorescence intensity; Δ MFI: MFI corrected for staining with an isotype control. M-CSF Mφ: monocyte-derived macrophages cultured for 9 days with M-CSF, GM-CSF Mφ: monocyte-derived macrophages cultured for 9 days with GM-CSF. Means + s.e.m. are shown for each group.
FCγR expression on human splenic macrophages

Figure 5. Immunohistochemistry of spleen tissue confirms the FCγR expression pattern of Red Pulp Macrophages

Immunofluorescent co-stainings of 
A: CD163, FCγRIII and CD19, 
B: CD169, FCγRIII and CD19, 
C: CD163, FCγRI and FCγRIIb, 
D: CD169, FCγRI and FCγRIIb, 
E: CD163 and FCγRIIa+b and 
F: CD169 and FCγRIIa+b. CD163 marks the red pulp macrophages, CD169 marks the perifollicular zone macrophages, CD19 marks B cells in the follicles. B cells in the follicle are also positive for FCγRIIb. Figures are representative of n=3 spleens from different donors.
DISCUSSION

We have characterized the major resident macrophages in the human spleen, the red pulp macrophages, as a unique population with major differences in expression for surface receptors such as the FcγRs, as opposed to monocytes and monocyte-derived macrophages. Our newly developed method takes advantage of the autofluorescence of these cells and allows for immediate specific isolation of red pulp macrophages from the spleen, by a combination of autofluorescence, CD163 and CD14 (Fig.2). No surface markers that absolutely distinguish red pulp macrophages from monocytes could be found. We show that human red pulp macrophages express the low-affinity FcγRIIa and FcγRIIIa, but not the inhibitory FcγRIIb, and appear to have an inducible (but not constitutive) expression of the high-affinity FcγRI, as we detected FcγRI in these cells only under inflammatory conditions. This is in stark contrast with monocytes (either from spleen or blood) and monocyte-derived macrophages, which constitutively express FcγRI, and express FcγRIIIa only on a small subset of the population. We could not characterize the perifollicular zone macrophages in the human spleen in detail, but found these cells to express FcγRIIa but not FcγRI, FcγRIIb or FcγRIII by immunohistochemistry stainings (Fig.5).

Although it is not exactly known which macrophage population in the spleen is responsible for the uptake of IgG-opsonized blood cells from the circulation, red pulp macrophages are a likely candidate. First, they are the most abundant macrophages in the spleen. Second, they are in direct contact with the cells in the circulation (as opposed to the perifollicular zone macrophages which surround endothelial cells in capillary sheaths\textsuperscript{11}). Another finding that supports the importance of red pulp macrophages is the notion that in patients with hereditary spherocytosis, who develop a hemolytic anemia as a result of an increased splenic uptake of red cells by red pulp macrophages, failed to develop thrombopenia when injected with platelet-specific antibodies\textsuperscript{28}, suggesting saturation of the red pulp macrophages.

Our finding of FcγRIIIa being constitutively expressed on all red pulp macrophages is in line with several older publications showing FcγRIII in the red pulp of the spleen, but is in contrast with a more recent publication by Wu et al., proposing that FcγRIII is only expressed on splenic macrophages in a minority of individuals\textsuperscript{23}. However, we found FcγRIII consistently in red pulp macrophages of 74 individuals, as tested by flow cytometry of single cell suspensions, and confirmed by immunohistochemistry. A possible explanation for this discordance is that Wu et al. may have described macrophages in the marginal or perifollicular zone of the spleen\textsuperscript{23}, which we show to express no FcγRIII, as opposed to the more numerous red pulp macrophages. Furthermore, circumstantial evidence for the presence of FcγRIII on macrophages of the reticuloendothelial system comes from studies in which in vivo blocking of specific FcγRs in individual cases of patients with ITP suggests that FcγRIII is important for the in vivo clearing of opsonized platelets\textsuperscript{29,30}, whereas the high-affinity FcγRI is not\textsuperscript{31}. It must be said that the patients in these studies had been previously splenectomized, so these findings should reflect
the situation in macrophages in the liver. More supporting evidence for an important role of the low-affinity FcγRII and FcγRIII, comes from the finding that genetic variation in these receptors is associated with ITP, and that the removal of transfused IgG-opsonized erythrocytes in human volunteers correlates with SNPs in FcγRIIa and FcγRIIIa.

The inhibitory FcγRIIb is absent or at most very lowly expressed on red pulp macrophages, both in normal state and inflammatory circumstances. Given the fact that FcγRIIb is often attributed an important role in balancing the immune response and preventing uncontrolled immune activation, this absence is remarkable. Recently, it was proposed that FcγRIIb was highly expressed on (CD33⁺) monocytes in a limited series of 3 human spleens, but we did not find a significantly higher expression in splenic monocytes than in blood monocytes in 24 donors, even after correction for genotypes that could express also the homologous FcγRIIC (excluding 9 donors). In fact, the splenic monocytes showed expression of FcγRIIb only on a subset of the cells, similar to the situation in blood (data not shown).

Previous publications have found expression of FcγRI on splenic phagocytes. Our data now show that red pulp macrophages do not constitutively express FcγRI, although its expression can be induced under inflammatory conditions. The most likely explanation for this difference is that these previous studies have investigated spleen monocytes or spleen monocyte-derived macrophages, which do not reflect bona fide tissue-resident red pulp macrophages. These studies enriched for phagocytes of the spleen either by selecting for adherent cells or by CD14 bead isolation. The spleen contains a high amount of monocytes, which are present because the spleen is a blood filled organ which receives approximately 180 ml/min of blood in healthy adults, and functions as a reservoir for monocytes. Therefore, spleen monocytes actually outnumber red pulp macrophages in single cell suspensions of splenocytes (by approximately 3-5 times, (Fig.1,2)). They appear to be much more similar to blood monocytes than to red pulp macrophages (Fig.1-4). Indeed, selecting for adherent cells in fact resulted in preparations that mainly consist of monocyte-derived macrophages, with a similar FcγR expression pattern (Figure S4). These findings underline the importance of specifically isolating splenic macrophages using both autofluorescence, CD14 and CD163.

Considering the abundance of red pulp macrophages in tissue sections of the spleen (Fig.5), the low prevalence of these cells (especially when compared to monocytes) in single cell suspensions is remarkable. Presumably, the difficulty of getting these tissue-resident cells out of the tissue matrix (despite a 30-minute incubation with collagenase) plays a role here, whereas all blood cells – always present in the spleen in relatively high amounts as a consequence of the blood-filled nature of this organ – will easily be dissociated from other cells. This may also explain the total absence of CD169 positive perifollicular macrophages in the single cell suspensions, as these cells may be even more difficult to release, being closely associated with endothelial cells.

Working with human spleen tissue, several aspects are important to consider. For instance, knowledge of the high autofluorescence of macrophages in the spleen is crucial to any researcher...
who performs flow cytometry with spleen cells, because the autofluorescence may appear as false-positive stainings of any antigen stained for, if not properly corrected by isotype controls. The best way to circumvent this would be to gate out all autofluorescent cells by using other channels, and subsequently look for specific staining in the non-autofluorescent cells. Furthermore, many techniques suitable for blood cell isolation do not automatically work for suspensions of splenocytes; for instance an percoll or ficoll isolation step did not separate lymphocytes and monocytes/macrophages from polymorphonuclear cells as it does in blood (data not shown).

Concluding, we describe a novel method for the specific isolation of splenic red pulp macrophages, a macrophage population that has a distinct expression profile from monocytes and monocyte-derived macrophages. Possibly, this reflects a different origin of these cells, similar to the situation in rodents, where red pulp macrophages consist of a self-renewing and highly specialized population established prior to birth13. However, since there are many differences between rodent and human spleen, caution must be taken in the translation of these findings to the human situation.

Regarding the expression of FcγRs on red pulp macrophages, especially the fact that mainly low-affinity FcγRs are expressed, and the absence of the inhibitory FcγRIIb is striking. The functional consequence of this different expression pattern for the phagocytosis of IgG-opsonized blood cells is currently being investigated. In the end, this knowledge will help to better understand the process of clearance of IgG-opsonized blood cells from the human circulation, and may guide more targeted therapies for ITP and AIHA.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Landsteiner Foundation for Bloodtransfusion Research (LSBR #0916). The authors would like to thank mrs. Swi Fan Cheung (Sanquin, Amsterdam, the Netherlands) for help in providing tissue samples, mrs. Ellen Borg (VU University Medical Center, Amsterdam, The Netherlands) for assistance with the experiments, and Prof. dr. Georg Kraal (VU University Medical Center, Amsterdam, The Netherlands) for intellectual input.

AUTHOR CONTRIBUTIONS

S.Q.N. designed the study, performed experiments, analyzed data and wrote the manuscript, C.W.B performed experiments and analyzed data, E.M. performed and designed experiments, J.H. and T.K.B. discussed data, R.B. designed experiments and discussed data, T.W.K. discussed data, designed the study and wrote the manuscript.
REFERENCE LIST


## SUPPLEMENTARY DATA

Table S1. List of MoAbs.

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Figure S1. spleen autofluorescent cells are negative for canonical lymphocyte markers

A CD3 staining on monocytes in the spleen (n=10), spleen autofluorescent cells (n=10), and circulating blood T cells (n=23). B CD19 staining on monocytes in the spleen (n=8), spleen autofluorescent cells (n=8), and circulating blood B cells (n=23). C CD56 staining on monocytes in the spleen (n=8), spleen autofluorescent cells (n=8), and circulating blood NK cells (n=23). MFI: median fluorescence intensity, Δ MFI: MFI corrected for staining with an isotype control. Means + s.e.m. are shown for each group.
Figure S2. Expression of FcγRllb and/or FcγRllc on Red Pulp Macrophages per genotype

Comparison of staining of MoAb 2B6, which detects the identical extracellular domain of both FcγRllb and FcγRllc, on red pulp macrophages. Individuals with different combinations of haplotypes for FCGR2C (FCGR2C-Stop vs FCGR2C-ORF), promoter haplotypes for FCGR2B (2B.4 promoter vs no 2B.4) and deletion of Copy Number Region 1 (CNR1, resulting in ectopic expression of FcγRllb on specific cell populations, i.e. NK Cells) were compared for 2B6 expression. FcγRllc-Stop n=36, FcγRllc-Stop with 2B.4 promoter (1x) n=3, FcγRllc-ORF without 2B.4 promoter n=10, FcγRllc-ORF with 2B.4 promoter (1x) n=6, FcγRllc-ORF (2x) with 2B.4 promoter (1x) n=1, FcγRllc-ORF (2x) with 2B.4 promoter (2x) n=1, FcγRllc-Stop with deletion of CNR1 n=4, FcγRllc-ORF with deletion of CNR1 n=1.

MFI: median fluorescence intensity, Δ MFI: MFI corrected for staining with an isotype control. Statistics by unpaired t test. ns: non-significant; ** P <0.01. Means + s.e.m. are shown for each group.
Figure S3. Red Pulp Macrophages express FcγRIIIa, not FγRIIIb

Staining with different MoAbs for FcγRIII. FcγRIIIb staining by MoAb 5D7 (also known as CLBgran11), which specifically binds FcγRIIIb (white bars), is compared to staining by MoAb 3G8 (black bars), which binds both FcγRIIIa and FcγRIIIb. Stainings are on monocytes in the spleen, Red Pulp Mφ and neutrophils in the same spleen. This shows that FcγRIIIb is not expressed on Red Pulp Mφ, and therefore FcγRIII expression on these cells must be FcγRIIIa, for which no specific MoAbs exist. MFI: median fluorescence intensity, Δ MFI: MFI corrected for staining with an isotype control. Means + s.e.m. of n=7 are shown for each group.
Figure S4. Culturing splenocytes and selecting for adherent cells yields a cell type similar to monocyte-derived macrophages
Figure S4. Culturing splenocytes and selecting for adherent cells yields a cell type similar to monocyte-derived macrophages (legend)

Comparison of FcγR staining, comparing spleen adherent cells cultured for 2-3 days in medium (enriched for phagocytes by washing all non-adherent cells away after 1 day) with red pulp macrophages and monocyte-derived macrophages cultured with M-CSF or GM-CSF for 3 days.

A Staining for FcγRI (MoAb 10.1). Red Pulp Mφ n=69, spleen adherent cells n=9, M-CSF Mφ n=16, GM-CSF Mφ n=17.

B Staining for FcγRII (MoAb AT10). Red Pulp Mφ n=69, spleen adherent cells n=9, M-CSF Mφ n=16, GM-CSF Mφ n=16.

C Staining for FcγRIIb (MoAb 2B6). Red Pulp Mφ n=39, spleen adherent cells n=6, M-CSF Mφ n=9, GM-CSF Mφ n=9. To ensure specificity for FcγRIIb, only samples from individuals that cannot express FcγRIIc and with wild-type FCGR2B promoters are presented here.

D Staining for FcγRIII (MoAb 3G8). Red Pulp Mφ n=69, spleen adherent cells n=9, M-CSF Mφ n=15, GM-CSF Mφ n=17.

E Staining for CD14 (MoAb M5E2). Red Pulp Mφ n=22, spleen adherent cells n=6, M-CSF Mφ n=17, GM-CSF Mφ n=17.

MFI: median fluorescence intensity, Δ MFI: MFI corrected for staining with an isotype control. M-CSF Mφ: monocyte-derived macrophages cultured for 9 days with M-CSF, GM-CSF Mφ: monocyte-derived macrophages cultured for 9 days with GM-CSF. Means + s.e.m. are shown for each group.