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Human spleen contains different subsets of dendritic cells and regulatory T lymphocytes

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Summary

Most knowledge about dendritic cells (DCs) and regulatory T cells in humans has been gathered from circulating cells but little is known about their frequency and distribution in lymphoid organs. This report shows the frequency, phenotype and location of DCs and regulatory T cells in deceased organ donors' spleens. As determined by flow cytometry, conventional/myeloid DCs (cDCs) CD11c^{high}HLA-DR^{+}CD123$^{-/low}$ were 2·3 ± 0·9% and LIN^{-} HLA-DR^{+}CD11c^{high} 2·1 ± 0·3% of total spleen cells. Mature CD11c^{high}HLA-DR^{+}CD83^{+} were 1·5 ± 0·8% and 1·0 ± 1·6% immature CD11c^{high}HLA-DR^{+}CD83^{-} cDC. There were 0·3 ± 0·3% plasmacytoid DCs (pDC) CD11c$^{-/low}$HLA-DR^{+}CD123^{high} and 0·3 ± 0·1% LIN^{-}HLA-DR^{+}CD123^{high}. Cells expressing cDCs markers, BDCA-1 and BDCA-3, and pDCs markers BDCA-2 and BDCA-4 were observed in higher frequencies than DCs with other phenotypes evaluated. CD11c^{+}, CD123^{+} and CD83^{+} cells were located in subcapsular zone, T cells areas and B-cell follicles. CD4^{+}CD25^{high} Tregs were 0·2 ± 0·2% and CD8^{+}CD28^{-} comprised 11·5 ± 8·1% of spleen lymphocytes. FOXP3^{+} cells were found in T- and B-cell areas. The improvement in cell separation, manipulation and expansion techniques, will facilitate the manipulation of donor spleen cells as a part of protocols for induction and maintenance of allograft tolerance or treatment of autoimmune diseases.

Keywords: dendritic cells, FOXP3, human spleen, Tregs, T suppressor cells

Introduction

The spleen is the largest lymphoid organ and it plays an important role in both innate and adaptive immune responses. The spleen is organized as a tree of branching arterial vessels, in which the smaller arterioles end in a sinusoidal system. The white pulp is formed by lymphoid sheaths with specialized T cells, B cells and marginal zones. T cells are located in the periarteriolar lymphoid sheath. The marginal zone is composed of fibroblasts, marginal-zone macrophages, B cells and dendritic cells (DCs), and it is the transit area for cells leaving the bloodstream and entering into the white pulp. In contrast to mice, the human marginal zone is divided into inner and outer zones, surrounded by a large perifollicular zone [1].

Spleen function requires the presence of all types of cells participating in the immune response; however, the frequency and distribution of cell populations able to modulate the immune response, such as DCs subsets and regulatory T lymphocytes, are not well established in human spleen.

DCs are important antigen presenting cells (APCs), functioning as a link between innate and adaptive immune responses, triggering either immunity or tolerance [2–5]. DCs include several subpopulations with different origins, anatomical distribution, phenotype and function. Two main subsets have been characterized; the conventional/myeloid DCs (cDC), derived from myeloid precursors, are characterized by the phenotype LIN^{-}CD11c^{high}HLA-DR^{+}CD123$^{-/low}$, and plasmacytoid DCs (pDCs), derived from a lymphoid precursor, exhibit the phenotype LIN^{-}CD11c$^{-/low}$HLA-DR^{+}CD123^{high} [5,6]. DCs can also be categorized by their maturation state. Immature DCs are located mainly in peripheral tissues, where they capture antigens, initiate their maturation and migrate to T zones of lymphoid organs, where they become mature to present antigen and stimulate naïve T lymphocytes [2,3,7].

Most knowledge about human cDCs has been obtained from monocyte-derived DCs, but DCs in human tissues, particularly spleen DCs, have not been extensively characterized [8,9]. Buckley *et al.* [10] showed that macrophages and

DCs are located in the same splenic anatomical compartments and share monocyte-macrophage markers, suggesting that both cell types are related and possibly derived from a common precursor [10]. Summers *et al.* reported four different interdigitating and germinal centre DCs present in tonsils [11]. Vandenabeele *et al.* demonstrated in human thymus a major population of DCs, exhibiting the phenotype $CD11b^{-}CD11^{+}CD45RO^{low}CD83^{high}CD86^{high}HLA\text{-}DR^{high}$ and a minor DC $CD11b^{+}CD11c^{high}CD45RO^{high}$ population. These authors also reported the presence of pDCs $CD123^{high}$ in the thymic cortex [12]. Although the phenotype and function of human tissue DCs are not completely dilucidated, evidence suggests that the function of DCs may change according to their anatomical location. In secondary lymphoid tissues mature DCs present antigens, captured in the periphery, to naïve T cells and induce immunity, while in the thymus DCs present self-antigens, induce negative selection of autoreactive T cells and promote the positive selection of regulatory T cells [12,13].

T-cell subsets with modulatory capabilities also are not well documented in human spleen. Regulatory T cells $CD4^{+}CD25^{high}FOXP3^{+}$ (Tregs) constitute 5–10% of mature $CD4^{+}CD8^{-}$ thymocytes and about 10% of circulating $CD4^{+}$ T cells. Tregs are essential for immunological homeostasis and self-tolerance [14–18]. Tregs function by cell–cell contact or cytokines-, IL-10 and TGF-β, dependent mechanisms [14–17]. Another important T-cell subset with modulatory proprieties is the $CD8^{+}CD28^{-}$ that exert their suppressor effect by cell–cell contact dependent mechanisms, inhibiting the expression of CD40L and IL-2 production by effector cells and inducing the expression of tolerogenic molecules ILT3 and ILT4 on APCs [19,20].

Given the importance of spleen in the immune response, this organ must contain the different cell populations able to modulate the immune effector cells. The identification of these cells in human spleen would facilitate the use of this organ for possible cell therapies in different clinical situations such as transplantation, autoimmune diseases, and immunodeficiencies. The aim of this study was to determine the frequency and location of DCs and immunomodulatory T-cell subsets in human spleen.

Materials and methods

Human spleens

Human spleen fragments (2 cm^3) were obtained from deceased organ transplant donors, according to the donation protocol of the Grupo de Trasplantes, Universidad de Antioquia-Hospital San Vicente de Paúl, Medellín, Colombia. Twenty-two donors were included, 18 men and 4 women, with mean age 30·5 years (range 8–55). The causes of death were encephalic trauma and cranial haemorrhage. Donors with active infections were excluded. Spleen fragments were surgically removed and placed in RPMI-1640 (Biowittaker, Walkersville, MD) supplemented with 10% FCS (Biowittaker) and Pen/Strep (Biowittaker) at 4°C. Samples were processed within the first 8 h after extraction. Tissue fragments were mechanically disrupted, large debris were allowed to settle for 1 min and supernatants used to obtain spleen mononuclear cells (SpMC) by Ficoll-Hypaque (Biowittaker) centrifugation. Cell viability, as assessed by trypan blue exclusion, was always higher than 85%.

Flow cytometry

cDCs and pDCs phenotypes were analysed by one, two and three colour staining using FITC, PE and PECy5-conjugated monoclonal antibodies. The phenotypes evaluated were:

Immature cDC: $CD11c^{high}CD83^{-}HLA\text{-}DR^{+}$
cDC: $CD11c^{high}HLA\text{-}DR^{+}CD123^{-/low}$, $LIN^{-}CD11c^{+}HLA\text{-}DR^{+}$
pDC: $CD11c^{-/low}HLA\text{-}DR^{+}CD123^{high}$, $LIN^{-}HLA\text{-}DR^{+}CD123^{high}$

cDCs markers BDCA-1 and BDCA-3, and pDC markers BDCA-2 and BDCA-4 [21,22] were also evaluated. To differentiate macrophages and cDC, CD11c/CD68 double stain was performed.

Spleen T cells with regulatory phenotypes $CD4^{+}CD25^{high}$, $CD4^{+}FOXP3^{+}$, $CD8^{+}FOXP3^{+}$ and $CD8^{+}CD28^{-}$ were also determined.

Previous Fc blockade with PBS containing 2% PHS during 10 min, $0{\cdot}5 \times 10^{6}$ SpMC were washed and resuspended in 50 µl of staining buffer (PBS with 2% PHS, 0·1% NaN_3), and the respective antibodies. Isotype antibodies and non-stained cells were used as controls. Cells were incubated at room temperature for 20 min, washed and fixed with 2% paraformaldehyde.

To evaluate CD68 expression on cDC, CD11c surface staining was performed with anti-CD11c-FITC. The cells were fixed with 2% paraformaldehyde for 30 min and permeabilized with PBS containing 0·1% saponin and 1% BSA followed by anti-CD68-PE staining.

$FOXP3^{+}$ cells were determined in 1×10^{6} SpMC; after surface staining with anti-CD4 or anti-CD8, the cells were permeabilized and stained with 40 µl/tube of FOXP3 (Clone PCH101, eBioscience, San Diego, CA), following the manufacturer's instructions.

One hundred thousand events were acquired in an Epics XL flow cytometer (Coulter-Beckman, Hialeah, FL). The percentage of positive cells and the mean fluorescence intensity (MFI) were determined using the Windows Multiple Document Interfase 2·8 software (WinMDI, La Jolla, CA).

Immunohistochemical evaluation of human spleens

Two spleen fragments from 10 donors were preserved for immunohistochemical evaluation. The first fragment was embedded in tissue freezing medium (JUNG, Leica Instruments, Nussloch, Germany) and stored at –70°C; the second

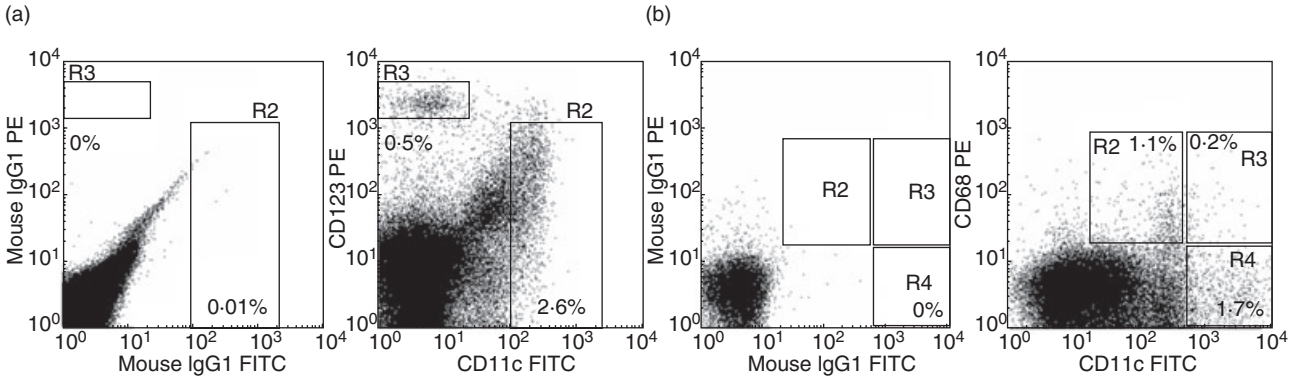


Fig. 1. Flow cytometry evaluation of cDC and pDC in human spleens. The frequency of cDC and pDC was determined according to the expression of CD11c and CD123. cDC were located in the gate $CD11c^{high}CD123^{-/low}$ (R2), while pDC were in the gate $CD11c^{-}CD123^{high}$ (R3) ($n = 17$) (a). $CD11c^{high}$ cells were mostly $CD68^{-}$ (R4) ($n = 3$) (b). 100·000 events were acquired.

fragment was preserved in 10% buffered formalin for paraffin sections. Frozen tissues were cut in 4 μm sections, fixed in acetone and endogenous peroxidase blocked with 0·5% methanol plus 50% H_2O_2. Non-specific proteins were blocked (Zymed/Invitrogen, Carlsbad, CA). Anti-CD11c at 10 μg/ml (clone 3·9, BD Biosciences), anti-CD123 at 10 μg/ml (clone 6H6, eBioscience), anti-CD83 at 5 μg/ml (clone HB15e, BD Biosciences) and anti-CD68 at 5 μg/ml (clone Y1/82A, BD Biosciences) monoclonal antibodies, were added and slides incubated for 1h at room temperature. After washing with Tris saline buffer (TSB, Sigma), secondary biotinylated antibodies (goat and rabbit anti-mouse IgG, Zymed/Invitrogen) were added for 10 min. Sections were washed with TSB and streptavidin-avidin horseradish peroxidase (Zymed/Invitrogen) was added for 10 min. The reaction was revealed with DAB (3′3′diaminbenzidine, Zymed/Invitrogen) and counter stained with Harri's haematoxilin. The slides were dehydrated with alcohol, cleared with xylol and mounted with consul-mount (Zymed/Invitrogen).

Direct immunofluorescence of blood DC-related markers was performed in frozen sections using FITC-labelled anti-BDCA-1 and anti-BDCA-3 for cDC (clones AD5-8E7 and AD514H12, Miltenyi), PE-labelled anti-BDCA-2 and anti-BDCA-4 for pDC (clones AC144 and AD517F6, Miltenyi), following the manufacturer's instructions.

The paraffin blocks, used for FOXP3 evaluation, were cut into 4 μm sections, deparaffinated overnight at 56°C and immersed three times in xylol and methanol for 5 min. Endogenous peroxidase was blocked with 6% H_2O_2 and antigen was recovered with pH 8·0 EDTA (Sigma). The next steps were the same as for frozen sections, except that Fast-Green was used as counter stain. Additionally, $FOXP3^{+}$ cells were stained in frozen sections. In both frozen and paraffin sections, anti-FOXP3 (Clone PCH101, eBioscience) was used at 5 μg/ml.

The slides were analysed by a pathologist (LAC) to determine the location of positive cells for each marker in the spleen areas at 10×, 40× and 100× magnifications.

Statistical analysis

The mean and standard deviation (SD) of the percentage of positive cells were calculated and Student's *t*-test used to compare the frequency of DCs and regulatory T lymphocyte populations. The α set was defined as $P < 0{\cdot}05$ (GraphPad Prism 4·0, San Diego, CA).

Results

Conventional/myeloid dendritic cells are more frequent than plasmacytoid dendritic cells in human spleens

DCs phenotypes according CD11c and CD123 expression were defined as follows: cDCs in the gate of $CD11c^{high}$ $CD123^{-/low}$ (R2) and pDCs in the gate $CD123^{high}CD11c^{-}$ (R3) (Fig. 1a). cDC phenotypes $CD11c^{high}$HLA-$DR^{+}CD123^{-/low}$, LIN^{-}HLA-$DR^{+}CD11c^{high}$ were observed in 2·3 ± 0·9% and 2·1 ± 0·3% of SpMC, respectively (Table 1). pDC phenotypes $CD11c^{-/low}$HLA-$DR^{+}CD123^{high}$ and LIN^{-}HLA-$DR^{+}CD123^{high}$ were observed in 0·3 ± 0·3% and 0·3 ± 0·1% of cells, respectively. The expression of the CD83 was found in 1·5 ± 0·8% of $CD11c^{high}$HLA-$DR^{+}CD83^{+}$ and 1·0 ± 1·6% of $CD11c^{high}$HLA-$DR^{+}CD83^{-}$ cells (Table 1). To further establish whether $CD11c^{high}$ cells are cDC and not macrophages, the percentage of cells expressing CD68 was determined. $CD11c^{high}CD68^{-}$ cells were 2·7 ± 0·6% of total SpMC, corresponding to cDCs. CD68 was observed in 1·6 ± 0·5% $CD11c^{low}$ cells (Fig. 1b).

Blood dendritic cells related markers are expressed in human spleens

Blood cDC-related markers, BDCA-1 and BDCA-3 were found in 10·7 ± 9·5% and 9·0 ± 7·4% of total SpMC, respectively. The expression of CD83 was found in 4·3 ± 5·2% of spleen BDCA-1^{+} and 4·3 ± 3·8% of BDCA-3^{+}

Fig. 2. Immunohistochemical evaluation of CD11c$^+$, CD68$^+$, CD123$^+$, CD83$^+$, BDCA-3$^+$ and BDCA-2$^+$ and FOXP3$^+$ cells in human spleens. The distribution and location of CD11c$^+$, CD68$^+$, CD123$^+$ and CD83$^+$ cells were evaluated in frozen sections of spleen from deceased organ donors. CD11c$^+$ cells were observed abundantly in the subcapsular compartment (a), in the T cell area (b) and surrounding and penetrating the B-cell follicles until the centre zone. Dendritic morphology of CD11c$^+$ cells inside B-cell follicles is noted in the upper right square (c). In contrast, few CD68$^+$ cells, with round morphology, penetrate B-cell follicles until the mantle cell zone. The round morphology of CD68$^+$ cells is shown in the upper right square (d). Scarce CD83$^+$ cells were observed mainly inside of the T-cell areas located in the sheaths of arteriolar sinusoids. Upper right square shows CD83$^+$ cells around the sinusoids (e). CD123$^+$ cells with dendritic morphology were observed in low frequency and located in different areas, especially inside of the B-cell follicles. Cells with dendritic morphology are shown in the upper right square (f). BDCA-3$^+$ cells were evaluated by direct immunofluorescence with anti-BDCA-3 FITC. Upper right square shows the granular fluorescence pattern (g). BDCA-2$^+$ cells were evaluated with direct immunofluorescence with anti-BDCA-2-PE. Details of granular immunofluorescence are shown in the upper right square (h). Both cDC BDCA-3$^+$ and pDC BDCA-2$^+$ cells were located in T- and B-cell zones. The distribution of FOXP3$^+$ cells was evaluated in paraffin sections. Similar results were obtained in frozen sections (data not shown). FOXP3$^+$ cells were observed in low frequency and located inside of the T-cell areas in the sheaths of arteriolar sinusoids (i) and penetrating the white pulp (j). 40× and 100× magnifications, respectively. Similar results were observed in all spleens evaluated ($n = 10$).

cells. For blood pDC-related markers, 6·3 ± 4·4% and 7·3 ± 5·3% of total SpMC were BDCA-2$^+$ and BDCA-4$^+$, respectively (Table 1).

Immunohistochemical evaluation of conventional/myeloid and plasmacytoid dendritic cells in human spleens

Immunohistochemical evaluation showed abundant CD11c$^+$ cells with round or dendritic morphology in the subcapsular spleen compartment and distributed in the T-cell areas (Fig. 2a and b). CD11c$^+$ cells were also abundant around lymphoid follicles, permeating them through the mantle, marginal and centre follicular zone (Fig. 2c). As CD11c is a myeloid marker that has a low expression on other cells, such as macrophages [23], CD68 staining was performed to determine the distribution of splenic macrophages (Fig. 2d). Although CD68$^+$ cells and CD11c$^+$ cells were distributed in similar spleen zones, few CD68$^+$ cells permeated the B-cell follicles until the mantle cell zone with very few cells present in the centre follicle zone.

Scarce CD83$^+$ and CD123$^+$ cells were observed in the same areas as CD11c$^+$ cells, in the subcapsular compartment, in T-cell areas around arteriolar sheaths and B-cell follicles (Fig. 2e and f).

The presence of cells expressing cDC-markers BDCA-1 and BDCA-3, and pDCs-markers BDCA-2 and BDCA-4, was determined by direct immunofluorescence. Scarce cells cDC BDCA-3$^+$ and pDC BDCA-2$^+$ with granular fluorescence were observed around the arteriolar sheaths and B-cell follicles (Fig. 2g and h).

Table 1. Dendritic cells phenotypes in human spleens.

DC phenotype	Mean (%) ± SD	*n*
cDC		
LIN$^-$HLA-DR$^+$CD11c^{high}	2·1 ± 0·3	5
CD11c^{high}HLA-DR$^+$CD123$^{-/low}$	2·3 ± 0·9	17
CD11c^{high}HLA-DR$^+$CD83$^+$	1·5 ± 0·8	15
CD11c^{high}HLA-DR$^+$CD83$^-$	1·0 ± 1·6	15
Blood cDC related markers		
BDCA-1$^+$	10·7 ± 9·5	17
BDCA-1$^+$CD83$^+$	4·3 ± 5·2	17
BDCA-1$^+$CD83$^-$	6·8 ± 6·9	17
BDCA-3$^+$	9·0 ± 7·4	17
BDCA-3$^+$CD83$^+$	4·3 ± 3·8	17
BDCA-3$^+$CD83$^-$	4·0 ± 2·9	17
pDC		
LIN$^-$HLA-DR$^+$CD123^{high}	0·3 ± 0·1	5
CD11c$^{-/low}$HLA-DR$^+$CD123^{high}	0·3 ± 0·3	17
Blood pDC related markers		
BDCA-2$^+$	6·3 ± 4·4	17
BDCA-4$^+$	7·3 ± 5·3	17

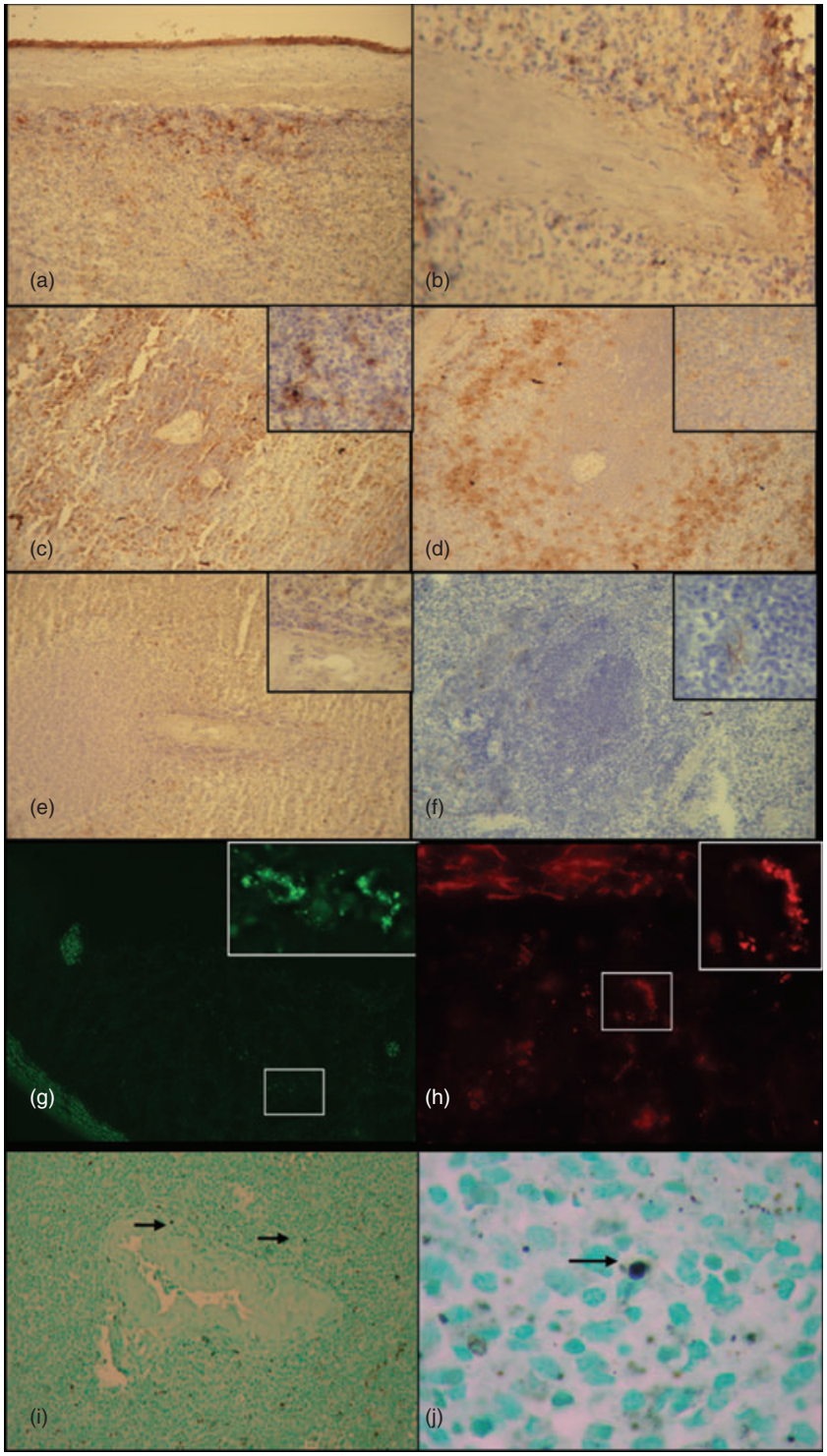
The frequency of cDC and pDC in SpMC was evaluated by flow cytometry based on the expression of CD11c, CD123 and HLA-DR ($n = 17$) or CD11c and CD123 in the gate of LIN$^-$HLA-DR$^+$ cells ($n = 5$). The frequency of blood cDC-markers BDCA-1 and BDCA-3, and blood pDC-markers BDCA-2 and BDCA-4 was also evaluated ($n = 17$). Data are presented as mean ± SD of the percentage of cells.

Regulatory T cells CD4$^+$CD25^{high} and FOXP3$^+$ cells are present in human spleens

Two colour flow cytometry evaluation of SpMC showed 8·7 ± 5·4% CD4$^+$CD25$^-$ T cells, 1·2 ± 2·2% CD4$^+$CD25^{low} and 0·2 ± 0·2% CD4$^+$CD25^{high} cells (Fig. 3a, Table 2). FOXP3 is a transcription factor considered the master control for generation and function of CD4$^+$CD25^{high} Treg cells [24–28]. The percentage of FOXP3$^+$ cells within the lymphocyte gate was 0·9 ± 0·3%, among them 0·6 ± 0·3% cells were CD4$^+$FOXP3$^+$ cells and 0·2 ± 0·1% were CD8$^+$FOXP3$^+$ (Fig. 3b).

T CD8$^+$CD28$^-$ suppressor phenotype is more predominant than CD4$^+$CD25^{high} in human spleens

The frequency of T CD8$^+$CD28$^-$ was 11·5 ± 8·1% (Fig. 3c, Table 2). Of note, CD8$^+$CD28$^-$ T cells were more frequent than CD4$^+$CD25^{high} regulatory T cells in human spleens ($P < 0·0001$).



Immunohistochemical evaluation of FOXP3 in human spleens

FOXP3$^+$ cells were present in low frequency in the T-cell areas around the arteriolar sheath and in lower proportion within the lymphoid follicles, although some FOXP3$^+$ cells were found in the centre of primary lymphoid follicles (Fig. 2i and j). The FOXP3$^+$ cells were found in frozen sections with similar frequency and location (not shown).

Discussion

The spleen is the largest lymphoid organ, with important functions in innate and adaptive immune responses [1,2].

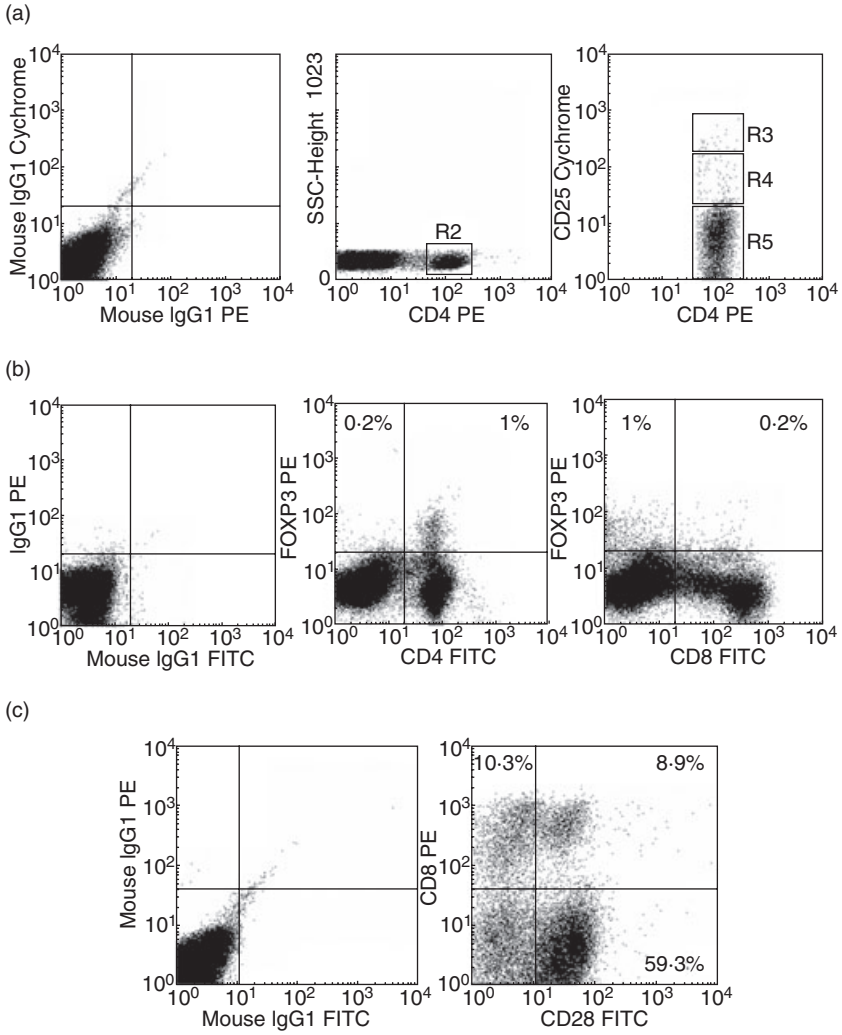


Fig. 3. Flow cytometry evaluation of $CD4^+CD25^+$, $CD4^+FOXP3^+$, $CD8^+FOXP3^+$ and $CD8^+CD28^-$ cells in human spleens. $CD4^+CD25^{high}$ (R3), $CD4^+CD25^{low}$ (R4) and $CD4^+CD25^-$ (R5) T cells in SpMC were determined by two colour flow cytometry. Dot plot of one representative experiment ($n = 20$) (a). $FOXP3^+$ cells were determined by intracellular staining with anti-FOXP3-PE after surface staining with anti-CD4-FITC or anti-CD8-FITC. Data of one representative experiment ($n = 3$) (b). The frequency of $CD8^+CD28^-$ T cells was determined with anti-CD8-PE and anti-CD28-FITC. Data of one representative experiment ($n = 21$) (c). 100·000 events were acquired. Analyses were performed in the lymphocytes gate. Isotypic antibodies were used as controls.

Most of the information about spleen cell populations has been obtained in mice, with scarce information about DCs and regulatory T cells in humans [29,30]. Deceased organ donors are a good source of tissues to study the secondary lymphoid organs, because it is possible to obtain enough tissue for immunological and histological studies and also to obtain information about the medical condition at the time of organ procurement.

Table 2. Frequency of T cell subsets in human spleens.

Spleen T cells	Mean (%) ± SD
$CD4^+$ T cells ($n = 20$)	
$CD4^+CD25^{high}$	0·2 ± 0·2
$CD4^+CD25^{low}$	1·2 ± 2·2
$CD4^+CD25^-$	8·7 ± 5·4
$CD8^+$ T cells ($n = 21$)	
$CD8^+CD28^-$	11·5 ± 8·1
$CD8^+CD28^+$	5·7 ± 4·9

The frequency of $CD4^+$ T cells: $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD4^+CD25^-$ T cells, and $CD8^+$ T cells: $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells was determined in the gate of lymphocytes, as defined by forward and side scatter, by two color flow cytometry. Data are shown as mean ± SD of the percentage of SpMC.

Our results demonstrate that $CD11c^{high}$HLA-$DR^+CD123^{-/low}$ and LIN^-HLA-DR^+CD11c^{high} cDCs are more frequent than $CD11c^{-/low}$HLA-DR^+CD123^{high}, LIN^-HLA-DR^+CD123^{high} pDCs; however, the frequencies of the two cDC and the two pDC phenotypes were similar. Interestingly, the frequencies of cDC and pDC found in spleen are higher than those reported in peripheral blood; 1% and 0·1%, respectively [31,32]. The cDC nature of the $CD11c^{high}$ cells was further demonstrated by the absence of CD68 by these cells.

Our results are in agreement with McIlroy *et al.* [30], who reported that 0·7 ± 0·5% of SpMC from organ donors are LIN^-HLA-DR^+, most of them $CD11c^+$ and exhibiting an immature $CD83^-$ phenotype. These authors also reported that $CD11c^+$ DCs are located in the periarteriolar T-cell zones, B-cell zones and marginal zone. In the T-cell zone, activated $CD11c^+CD83^+CD86^+$ DCs, were observed, suggesting *in vivo* activation [30]. In our immunohistochemical evaluation, $CD11c^+$ myeloid cells (cDC, monocytes and

macrophages) were found in the subcapsular zone, T-cell areas and surrounding and penetrating B-cell follicles until the follicle centre zone, which accords with their antigen presentation proprieties to T lymphocytes. The low frequency of the DC maturity marker, CD83, suggests that antigen presentation occurs under conditions that favour maintenance of tolerance to self antigens or differentiation and maintenance of different subtypes of regulatory cells. $CD83^+$ cells were scarce inside the T-cell area around the arteriolar sheaths and in the subcapsular zone. In the absence of infections, in organs from deceased donors the presence of $CD83^+$ cells suggests trauma-induced *in situ* maturation or migration of some mature DCs from peripheral blood into the spleen [33].

Our results demonstrate that myeloid $CD11c^+$ cells (DCs, macrophages), mature DCs $CD83^+$ and $CD123^+$ cells were located in specific spleen microanatomic locations. This observation is in agreement with Buckley *et al.* [10], who reported, in spleens obtained after surgery due to trauma or hsematological diseases, that macrophages and DCs share markers, suggesting that both cell types are related and possibly derived from common precursors; this demonstrated the phenotypic and topographical heterogeneity among spleen mononuclear cells and identified subsets of macrophages distributed in different compartments in red pulp and the marginal zone [10]. Pack *et al.* studied human spleens and reported that DEC-205, an endocytic receptor that mediates efficient antigen presentation, is expressed by DCs in the white pulp of the spleen, especially in T-cell areas [34].

The higher percentage in the spleen, compared with blood, for cDC BDCA-1 and BDCA-3, and pDC BDCA-2 and BDCA-4 markers [21,22,35,36] suggests that in the spleen there are DCs, DCs precursors, monocytes and macrophages in different stages of differentiation and activation; however, it is also possible that these markers are increased by systemic reaction to trauma.

Although DCs subsets in human lymphoid organs are not completely studied, evidence supports the heterogeneity of DCs phenotype and function on the different tissues. In tonsils, Summers *et al.* [11] identified four different interdigitating DCs based on the relative expression of HLA-DR, CD11c, CD13 and CD123; among these, $CD123^+$ pDCs were observed and DCs subsets differed in the expression of activation and costimulatory molecules. A fifth DC subset was a germinal centre DCs $CD11c^+HLA\text{-}DR^{mod}CD13^-$ [11]. In the thymus, two distinct mature DCs populations have been described [12]. The major $CD11b^-CD11^+CD45RO^{low}CD83^{high}CD86^{high}HLA\text{-}DR^{high}$ population produces IL-12. The minor DC population was $CD11b^+CD11c^{high}CD45RO^{high}$. Mature $CD83^+CD14^+$ and immature $CD83^-CD14^-$ DCs were observed within the two populations. The $CD11b^+$ DCs produced low amounts of IL-12. The same authors reported the presence of pDCs $CD123^{high}$ in the thymic cortex and medulla that did not secrete IL-12. However, upon culture, the thymic pDCs adopted a phenotype similar to $CD11b^-$ DCs [12]. The existence of several DCs phenotypes in lymphoid tissues suggests that the microenvironment and the immune function of the organ itself, dictates the differentiation of common DCs precursors to the DC subsets, located in special zones in the microanatomy of the organ to play a specialized function.

Our results indicate that in human spleen, $CD8^+CD28^-$ T cells were more frequent than $CD4^+CD25^{high}$ natural regulatory T cells. Also, the frequency of $CD4^+FOXP3^+$ cells is higher than $CD4^+CD25^{high}$ cells, suggesting that regulatory T cells down-modulate CD25 expression in the spleen, although it is also possible that some naïve T cells $CD4^+CD25^-$ are $FOXP3^+$, allowing them to acquire regulatory proprieties when stimulated under particular antigen presentation conditions [37]. Although the $CD8^+CD28^-$ cells were predominant in human spleen, our results demonstrated that the frequency of $CD8^+FOXP3^+$ cells was very low. Functional studies must be performed in the future to determine the regulatory capabilities of $CD8^+CD28^-$ cells from human spleens.

The evaluation of T cells in the spleen of deceased transplant donors was reported by Colovai *et al.* [29]. These authors compared $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes from the spleens of deceased transplant donors with reactive non-neoplasic spleens (idiopathic thrombocytopenic purpura, splenic infarct) and found that reactive spleens presented a higher percentage of $CD3^+$ T lymphocytes than normal spleens. The frequency of regulatory T cells was not evaluated in their study [29].

The immunohistochemical evaluation also demonstrated that $FOXP3^+$ cells are present in low numbers inside the T-cell area, around the arteriolar sheaths, and scarce in B-cell follicles, even in the centre zone. Although the inmunohistochemical technique employed did not allow the simultaneous evaluation of multiple cell markers, staining of separated sections of the same spleens showed the presence of $CD4^+$ and $CD8^+$ cells in T- and B-cell areas, as described for $FOXP3^+$ cells, suggesting that $FOXP3^+$ cells are functional, possibly, in the maintenance of tolerance to self antigens.

In conclusion, the results suggest that both cDC and pDC at different maturation stages and different subsets of regulatory T cells are present in human spleen. In the future, improvements in cell separation, manipulation and expansion techniques, may allow the manipulation of donor spleen cells as part of protocols for induction and maintenance of allograft tolerance or treatment of autoimmune diseases.

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