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PHENOTYPIC SUBPOPULATIONS OF MACROPHAGES AND DENDRITIC CELLS IN HUMAN SPLEEN

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Abstract

Using immunohistochemical techniques and a large number of monoclonal antibodies, the presence and distribution of phenotypic subpopulations of macrophages (MØs) and dendritic cells in human spleen were assessed. The results of this study show that different subsets of MØs and dendritic cells are present in the spleen and that some of these occupy discrete microanatomic locations. In the red pulp (RP) certain antigens are expressed by different proportions of uniformly distributed MØs in the cords. On the other hand, some antigens are present on MØs that form clusters of variable size within the red pulp. These include CD11c, CD15 and α-1-anti-chymotrypsin. Another type of cell in the RP that is phagocytic under certain conditions is the splenic sinusoidal lining cell (SLC). These cells exhibit a phenotype that is unique: nonspecific esterase (NSE)+, lysozyme+, and HLA-DR+, CD36+, factor VIII-related antigen+, CD8+ and CD71+. MØs in the splenic marginal zone (MZ) share some antigens with red pulp MØs, but in addition express CD11b, CD14 (Mo2;63D3) and 61D3. These antigens are found on only a few RP MØs. MZ cells expressing one antigen shared with RP MØs (CD4) and one present largely on the MZ cells (CD14;63D3) form clusters around small vessels; these structures resemble the so-called splenic ellipsoids that may play a role in the trapping of circulating antigens. Phagocytic MØs (tingible body MØs) of the white pulp follicular germinal centers were also shown to differ from RP and MZ cells with respect to the expression of the antigens CD11b, CD14 (Leu M3;Mo2), CD16 and the antigen detected by antibody 25F9. The unique topographical and surface antigenic features of dendritic cells were confirmed by this study. Furthermore, these cells were found to share a number of antigens with RP, MZ, and white pulp MØs, which suggests that they may be derived from a common progenitor. The presence of phenotypic subpopulations and variation in distribution among human splenic phagocytic cells and dendritic cells may be indicative of functional specialization.

Key Words: Human macrophages, macrophage subsets, human spleen, cell surface antigens, monoclonal antibodies, dendritic cells.

Introduction

The long range goal of our studies is to define phenotypic subpopulations of human tissue macrophages (MØs) in order to correlate specific functions with identifiable MØ subsets. To accomplish this, the issue of human MØ heterogeneity must be addressed, i.e., can tissue MØs, like lymphocytes, be subdivided into discrete populations?

The human spleen was chosen as one model for these studies for several reasons. First, the spleen has a high content of MØs (about 16%), as determined by morphometric analysis [3,6]. Secondly, this organ is divided into relatively distinct "compartments" (Fig 1). The compartmentalization facilitates the identification of phenotypic subpopulations of MØs. The splenic red pulp occupies about 80% of the splenic parenchyma [3, 6]. The major portion of the splenic mononuclear phagocytes is located in the red pulp "cords". Other components of this anatomic subdivision are the splenic sinusoids and their lining cells and circulating erythrocytes and leukocytes [3,6]. The white pulp of the spleen makes up the remaining 20% of the tissue and is composed of a majority of small lymphocytes that are segregated into B-cell and T-cell regions, and a much smaller number of MØs and dendritic cells [3, 6, 39; Fig 1]. The marginal zone (MZ) of the spleen is a third compartment that is defined as the interface between the red pulp and the white pulp lymphoid tissue [19, 25]. These microanatomic subdivisions greatly aid in the identification of different MØ types *in situ* because they can be recognized more easily in this setting, especially in the red pulp and MZ, than in other tissues where they are associated with a larger number of other cell types. Using a large panel of antibodies directed against monocyte and MØ antigens, we show that phenotypic subsets of MØs do exist in the spleen and that some of these subpopulations occupy discrete locations within the splenic tissue.

Materials and Methods

Spleens

Tissue was obtained fresh from spleens that had

been removed from accident victims (5), incidental to gastric surgery (1) and from patients with idiopathic thrombocytopenic purpura (3), splenic vein thrombosis (1), pyruvate kinase deficiency (2), hereditary spherocytosis (1), β-thalassemia (2) and Hodgkin's disease (9). The latter were stage I or II and none of these spleens contained Hodgkin's disease. All antibodies were tested on a minimum of 5 different spleens, including spleens from accident victims, and the results were similar for all the spleens tested.

The antibodies used in these studies and their published reactivity with mononuclear phagocytes and related cells are shown in Table 1.

In addition to those described above, sections of routinely fixed and processed spleen from 10 patients with an extensive history of blood transfusions for β-thalassemia were examined. The sections were stained with hematoxylin and eosin and also with a stain for iron (Prussian blue). Specifically, the sections were examined for the presence and locations of cells containing hemosiderin pigment and iron from phagocytosed red blood cells.

Detection of antigens

Cellular antigens were detected in sections of frozen spleen as described before [18, 44]. Briefly, fresh spleen was embedded in OCT compound in plastic tissue molds (both from Ames, Elkhart, IN), quickly frozen in a bath of isopentane submerged in liquid nitrogen and stored in air-tight plastic bags at -70°C. Sections (4-6 μM thick) of the frozen spleen were fixed immediately for 10 seconds in cold (4°C) acetone, dried and stored in a vacuum desiccator no longer than one day. Just before incubation with antibodies, the sections were fixed for an additional 5 minutes in acetone (4°C), rinsed for 10 minutes in phosphate-buffered saline (PBS; 0.02M phosphate, 0.15M NaCl, pH 7.4) and incubated 15 minutes with diluted normal serum. Antigens were detected by the avidin-biotinylated horseradish peroxidase method as described by Hsu et al [18] and modified by Wood and Warnke [44]. The results were confirmed in most cases by using biotinylated glucose oxidase (Vector Laboratories, Burlingame, CA) as the enzyme marker and tetranitroblue tetrazolium as the developing reagent. The latter method was especially useful when endogenous peroxidase obscured antibody reactivity. Normal serum or irrelevant antibodies of the same isotype were substituted for the primary antibody in controls. Most of the antibodies were used at final dilutions of 1:20-1:200 and the biotinylated second antibodies at 1:400. All antibodies were centrifuged at 100,000xG for 30 minutes before use.

Histochemical analyses for specific and nonspecific esterases (NSE)

The nonspecific esterases, α-naphthyl acetate esterase and α-naphthyl butyrate esterase (in the presence and absence of sodium fluoride), and the granulocytic specific enzyme, chloroacetate esterase, were assayed in sections from frozen spleen according to the method of Li et al [24]. Only cells with strong, diffuse cytoplasmic reaction product were scored as positive.

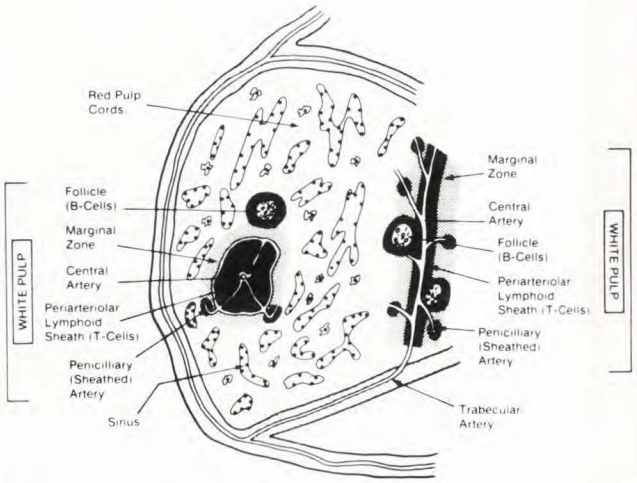


Figure 1: Diagram of splenic "microanatomy" (with permission, American Journal of Pathology).

Quantitative estimates of antigen-positive cells

Precise quantitation in frozen sections of the proportions of cells reacting with the antibodies used in this study could not be performed using classical morphometric analysis. The inability to clearly delineate cytoplasmic borders in areas containing numerous positive cells and varying numbers of negative cells was a major reason. Another was the difficulty in determining the total number of cells in the various splenic "compartments" in frozen sections, in particular in the white pulp that contains numerous, closely packed small lymphocytes. However, in order to compare the relative densities of antigen positive cells, sections from at least 5 different spleens stained with each antibody were scored microscopically. The proportions of positive cells were averaged and divided into the following broad categories: < 10%, 10-25%, 25-50%, 50-75%, and > 75% positive.

Results

Distribution of antigens on human splenic MØs and sinusoidal lining cells

The distribution of MØ-associated antigens was assayed in sections from frozen human spleen using a panel of monoclonal antibodies (Table 1). To simplify the data, they will be presented for each of the microanatomic "compartments" of the human spleen (Fig. 1).

Red Pulp Cords.

The results for the mononuclear cells of the red pulp cords are shown in Table 2. Several patterns were observed. A relatively uniform pattern of staining by more than 50% of the cord cells was obtained with NSE (most inhibited by fluoride), HLA-DR, CD4, CD16 and CD45. A similar uniform pattern, but involving fewer positive cells (25-50%), was seen with anti-lysozyme, anti-α-1-AT and anti-α-1-ACT. On the other hand, a uniform but different pattern of staining was exhibited

TABLE 1 - Antibodies Used in This Study

Antibody	CD Designation	Major Specificities	Source[q]	Refs.
HLA-DR(3)		Mo;MØs; IRC[a]; DRC[b]; LH[c] cells; B lymphocytes	BD[h], NEN[o], S. Melvin[p]	15, 22
HLA-DQ		Mo;MØs; IRC; DRC; LH cells; B lymphocytes	BD	15, 8
HLA-DP		Mo;MØs; IRC; DRC; LH cells; B lymphocytes	BD	15, 11
3G8	CD16	FcR (IgG):MØs; neutrophils; NK[d] cells	Fliet	15, 12
Leu 11b	CD16	FcR (IgG):MØs; neutrophils; NK cells	BD	15, 32
anti-CR1[e]	CD35	CR1 (C3b):B lymphocytes; Mo; neutrophils; DRC	D	15, 14
Mol	CD11b	CR3[f](C3bi):Mo; MØs; neutrophils	C[k]	15, 9
OKM1	CD11b	CR3 (C3bi):Mo; MØs; neutrophils	O[l]	15, 45
Mac-1	CD11b	CR3 (C3bi):Mo; MØs; neutrophils	Clone M1/70	15, 1
Leu M1	CD15	Mo; some MØs; IRC; neutrophils; Reed-Sternberg cells	BD	15, 27
Leu M3	CD14	Mo; MØs; IRC; DRC; LH cells	BD	15, 43
Mo-2	CD14	Mo; MØs; IRC; DRC	C	15, 40
61D3		Mo; MØs; DRC	BRL[m]	15, 33
63D3	CD14	Mo; MØs; DRC	BRL	15, 33
Leu M5	CD11c	Mo; MØs; IRC; DRC; large granular lymphocytes	BD	15, 19
OKM5	CD36	Mo; endothelial cells; splenic SLC[g]	O	15, 35
EBM 11	CD68	MØs; IRC; LH cells	D	15, 40
25F9		Cultured Mo; MØs; IRC	A[n]	15, 46
G16/1		MØs in granulomas; placental MØs	A	15
PHM-2		Mo; alveolar, peritoneal, and some tissue MØs	A	15, 16
PHM-3		Mo; placental MØs	A	15, 16
R4/23		DRC	D	15, 30
Leu 3a, b	CD4	T helper lymphocytes; Mo; MØs; IRC; LH cells	BD	15, 30
Ber-H2 (Ki-1)	CD30	Reed-Sternberg cells; some lymphomas; some MØs	D	15
Leu6	CD1a	Cortical thymocytes; LH cells; some MØs		15
CR2	CD21	B lymphocytes; DRC	BD	15, 42
Anti-transferrin receptor	CD71	Transferrin receptor:replicating cells; splenic SLC	BD	15
Anti-leukocyte common antigen	CD45	All leukocytes	BD	2
Leu 1	CD5	T lymphocytes; chronic lymphocytic leukemia; some B-cell lymphomas	BD	4
Leu 2	CD8	T lymphocyte subset; SLC	BD	4
Leu 4	CD3	T lymphocytes	BD	4
Leu 5	CD2	T lymphocytes (sheep erythrocyte receptor)	BD	4
Leu 8		T lymphocyte (CD4) subset; some B lymphocytes	BD	4
Leu 9	CD7	T lymphocytes;some acute non-lymphoblastic leukemias	BD	4
anti-lysozyme		Myeloid cells; Mo; MØs	D	28
anti-α-1-antitrypsin		MØs; hepatocytes; Mo:myeloid cells	D	20
anti-α-1-antichymotrypsin		MØs;small intestinal epithelium	D	10
Factor VIII		Endothelial cells; megakaryocytes	D	34

[a] Interdigitating reticulum cells; [b] Dendritic reticulum cells; [c] Langerhans cells [d] Natural killer cells
[e] Complement receptor 1 (C3b) [f] Complement receptor 3 (C3bi) [g] Sinusoidal lining cells [h] Becton Dickinson
[i] Dr. Howard Fliet, Dept. of Pathology, SUNY Stonybrook, NY [j] Dako [k] Coulter
[l] Ortho [m] Bethesda Research Laboratories [n] Accurate
[o] New England Nuclear [p] Dr. Sue Melvin, St. Jude Children's Research Hospital, Memphis, TN
[q] All monoclonal antibodies were purified immunoglobulin except for HLA-DR (Melvin), 3G8, Mac-1, EBM-11, R4/23, and Ber-H2, which were tissue culture supernates

TABLE 2 Antigenic Phenotypes and Distributions of Splenic MØs, SLC and Dendritic Cells

RED PULP		MARGINAL ZONE	WHITE PULP		
Cords	SLC		T-zone (interdigitating reticulum cells)	B-zone (dendritic reticulum cells)	B-zone (tingible body MØs)
>75%	>75%	>75%			
[1]NSE	[1]NSE	[1]NSE	HLA-DR	[10](HLA-DR?)	[10](HLA-DR?)
CD16	Lysozyme	CD16	HLA-DQ	[10](HLA-DQ?)	[10](HLA-DQ?)
[2]CD45	HLA-DR	CD45	[11]25F9	61D3	[1]NSE
50-75%	CD36	50-75%	[12]CD4	R4/23	Lysozyme
HLA-DR	FVIII	HLA-DR	[3,11]CD11c	CD11c	α-1-AT
CD4	61D3	α-1-ACT	[11]CD14(LeuM3)	[14]CD14	α-1-ACT
[3]CD15	CD8	CD68	CD15	[13]CD21	25F9
[4]CD68	CD71	25-50%	CD45	[13]CD35	CD11c
25-50%		CD11b	[11]CD68	[10](CD45?)	CD14(LeuM3)
Lysozyme		Lysozyme			[10](CD21?)
α-1-AT		α-1-AT			[10](CD35?)
[3]α-1-ACT		61D3			[10](CD45?)
10-25%		10-25%			CD68
[3]HLA-DQ		HLA-DQ			
[3]CD11c		[9]CD4			
<10%		[3]CD11c			
[5]61D3		[9]CD14(Mo-2,63D3)			
25F9		[3]CD15			
[6]CD11b		<10%			
[6,7]CD14		25F9			
[8]PHM3		CD14 (LeuM3)			

1. Most inhibited by fluoride; 2. Most weakly positive, some clusters of strongly positive cells;
3. Clusters of positive cells; 4. Strongly positive cells evenly distributed in the cords;
5. Sinusoidal lining cells weakly positive;
6. Randomly scattered positive cells in cords; some positive granulocytes (Leu M3)?;
7. Sinusoidal matrix positive (63D3); 8. Positive cells in sinusoids (monocytes?);
9. Clusters of positive cells around small vessels in MZ (Leu3a,b, 63D3);
10. Large number of positive small lymphocytes made evaluation difficult;
11. Positive cells with dendritic morphology concentrated around central arterioles as well as scattered diffusely within the T-zones; 12. Many positive small lymphocytes; some positive cells with dendritic morphology close to central arterioles in some spleens; 13. Some positive small lymphocytes; 14. Polarized pattern in germinal centers.

by sections stained for the CD68 antigen: sharply outlined red pulp cords (Fig. 2). An apparently random distribution of individual positive cells (less than 25%) was exhibited by HLA-DQ, 61D3, 25F9, CD11b, CD11c and the CD14 antibodies (Leu M3, Mo-2, 63D3). In addition to these patterns, the CD11c and anti-α-1-ACT antibodies stained scattered clusters of cells in the cords while the CD15 antibody (LeuM1) stained large, irregularly distributed aggregates of cells (Fig. 3). Rare PHM3+ cells were present within the sinusoids. The latter resembled circulating monocytes. Cord mononuclear cells did not react with OKM5, G16/1, PHM2, R4/23, CD21 or CD35 antibodies.

Sinusoidal Lining Cells (SLC).

The cells lining the red pulp sinusoids were strongly and uniformly positive for the following: NSE (fluoride inhibited), lysozyme, HLA-DR, CD36, factor VIII-related antigen, CD8 and CD71 (Table 2 and Figures 4-7). These cells also appeared to be weakly positive with the antibody 61D3. An interesting pattern of reactivity was observed within the SLC cytoplasm with the antibody OKM5: parallel, dark bands of staining alternating with lighter bands, perpendicular to the long axis of the SLC (Fig. 4). The CD14 antibody 63D3, while not staining the SLC themselves, appeared to react with the supporting matrix of the sinusoids, much like

the silver-based "reticulin" stains (Fig 8).

Previous studies from our laboratory reported that the SLC also reacted with CD4 antibodies [4, 5]. Further studies have shown that the SLC are probably CD4- and that the strongly CD4+ cord MØs (Table 2) had obscured this fact.

Examination of sections of spleen from patients with long histories of blood transfusions for β-thalassemia revealed a striking concentration of hemosiderin pigment in the SLC, presumably from phagocytosed erythrocytes (Fig. 9). Iron stains confirmed its presence in the SLC. This phenomenon was not identified in any of the other 24 spleens examined in this study.

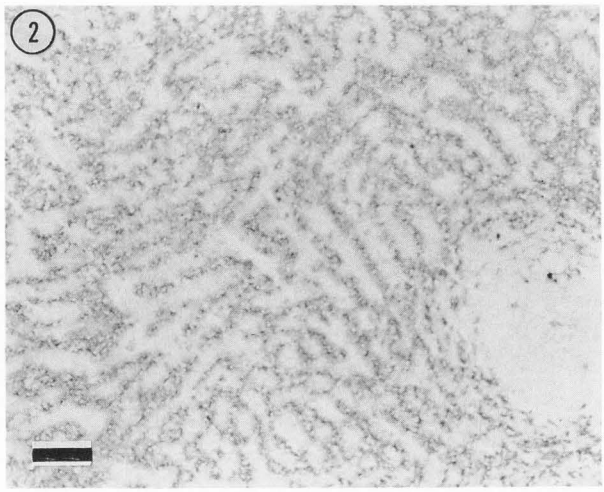
Marginal Zone (MZ)

One group of antibodies (Table 2) showed a distribution of positive cells that was qualitatively and quantitatively similar to that seen in the red pulp. These included NSE (most inhibited by fluoride), HLA-DR, HLA-DQ, 25F9, anti-lysozyme, anti-α-1-AT, CD14 (Leu M3), CD15, CD16, CD45 and CD68. A larger number of positive cells than that observed in the red pulp was detected with CD11b, CD14 (63D3, Mo-2), 61D3, and anti-α-1-ACT (Fig 10). The antibodies 63D3 (CD14) and Leu 3a,b (CD4) detected a different pattern of positive cells in the MZ in most of the spleens tested: distinct clusters of cells as well as scattered, individual positive cells (Fig 11). Many of the cell clusters appeared to be arranged around small vessels and resembled the structures called ellipsoids (see Discussion).

The splenic MZ is variably populated by endogenous-peroxidase-positive granulocytes. For this reason, antigen-positive MZ mononuclear cells are sometimes obscured by these cells when the immunoperoxidase technique is used to demonstrate them. To confirm the presence of various antigens on MZ mononuclear cells, the experiments were repeated with avidin-biotinylated glucose oxidase complexes substituted for avidin-biotinylated-horseradish peroxidase complexes in the antigen detection system. Since there is no glucose oxidase in human tissues, the problem of endogenous enzyme activity is obviated. The results confirmed the data obtained with the immunoperoxidase method. To exclude the possibility that MZ granulocytes either expressed some of the antigens being assayed or nonspecifically bound the antibodies (FcR?), spleens were analyzed for the granulocyte-specific enzyme, chloroacetate esterase, for comparison. In all cases, antigen-positive mononuclear cells exceeded the proportion of esterase positive granulocytes by at least 50-100% (data not shown).

White Pulp

The splenic white pulp is divided into predominantly T-lymphocyte and B-lymphocyte zones as defined by studies using antibodies to T- and B-cells [4, 5]. Table 2 lists the results for the distributions of cells expressing MØ-related antigens in the white pulp T-zone and B-zone, respectively. In the T-zone, strongly positive cells with the prominent cytoplasmic processes characteristic of interdigitating reticulum cells were detected



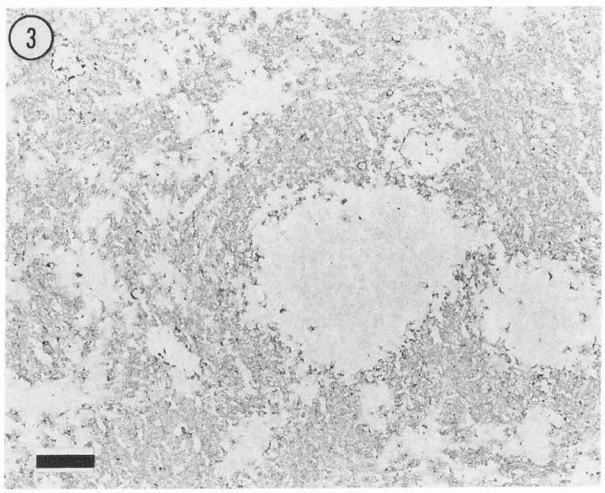


Figure 2: CD68 (EBM-11). The red pulp cords are sharply outlined by the positive MØs. White pulp is seen at lower right. Bar = 250 μm.

Figure 3: CD15 (LeuM1). Irregular, variably sized clusters of positive cells are seen in the red pulp. White pulp is at center right. Contrast the pattern shown in Fig. 2. Bar = 250 μm.

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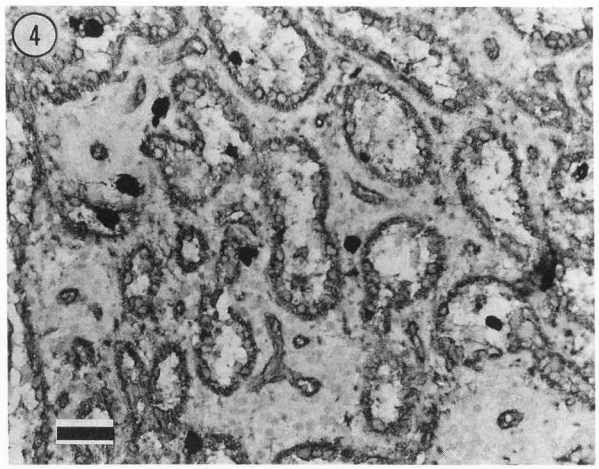
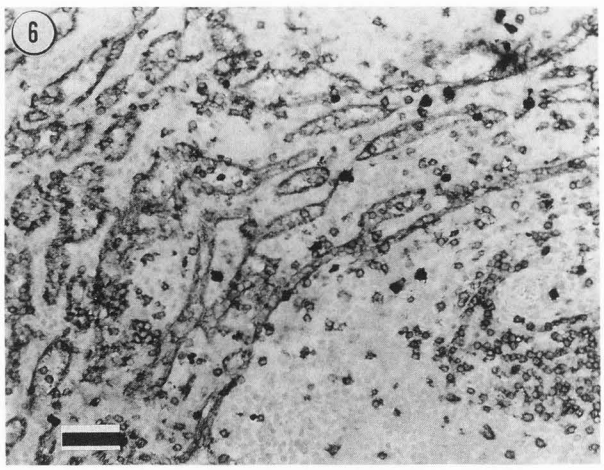
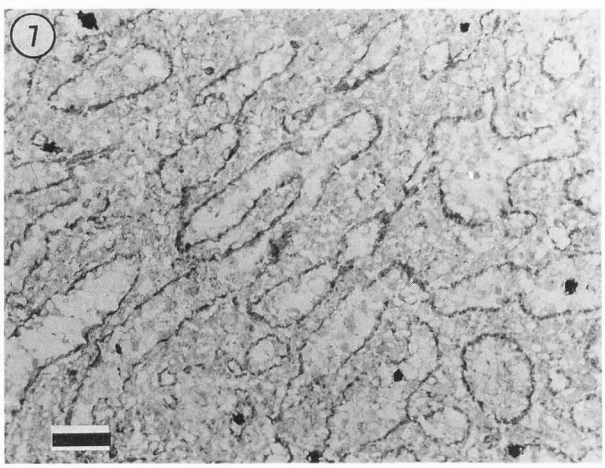
by the antibody 25F9 and those to HLA-DR, HLA-DQ, CD11c, CD14 (Leu M3), CD15 and CD68 (Fig 12). Interestingly, staining of these cells by the other two CD14 antibodies (Mo-2 and 63D3) was not observed. Some of the cells identified by Leu M3 (CD14), EBM11 (CD68) and 25F9 were very closely associated with the central arterioles (Fig 12) as opposed to the more even distribution in the T-zone of cells positive for HLA-DR, HLA-DQ and CD11c. The latter antibody also stained some clusters of cells. Staining of numerous small lymphocytes (T-cells) by Leu 3a,b (CD4) made the detection of other cell types difficult. However, in some spleens, CD4+ cells with dendritic morphology were seen close to central arterioles (Table 2).







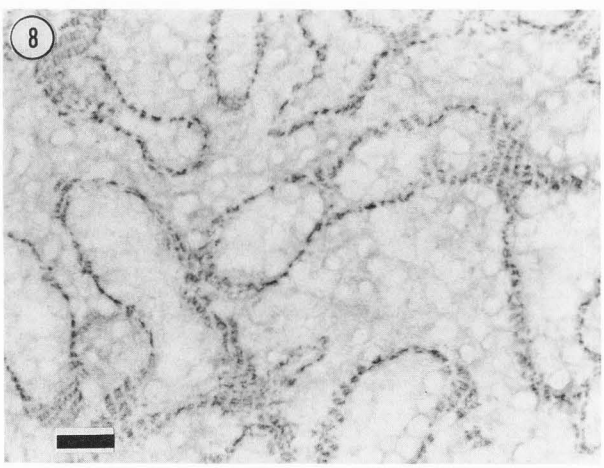


Figure 4: CD36. The SLCs are strongly positive for this monocyte-related antigen. Note dark and light bands of staining perpendicular to the long axis of the SLC's. Dark cells are endogenous peroxidase positive granulocytes. Bar = 50 μm.

Figure 5: Factor VIII-related antigen. SLCs are positive, similar to the endothelial cells of other types of vessels. White pulp is at right of photo. Bar = 50 μm.

Figure 6: CD8 (Leu 2a). This antigen is strongly expressed by the SLCs. Note the positive "suppressor/cytotoxic" lymphocytes around the central arteriole of the white pulp at lower right. Bar = 50 μm.

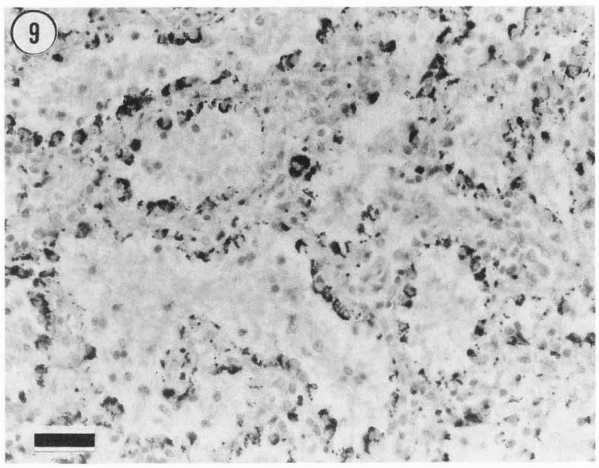
Figure 7: CD71 (transferrin receptor). The SLCs are positive for this antigen that is also seen on replicating cells and some "activated" MØs. Bar = 50 μm.

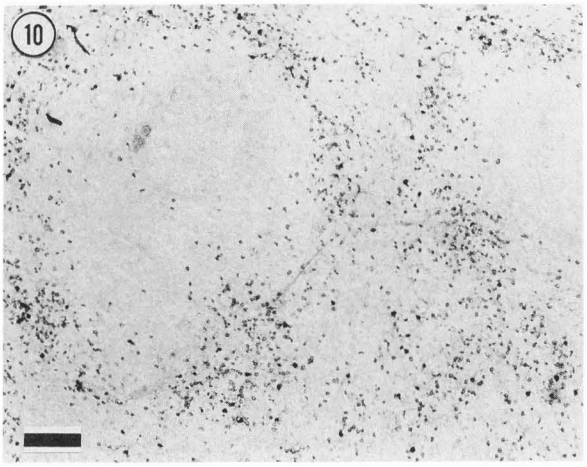
Figure 8: CD14 (63D3). The matrix surrounding the sinusoids of the red pulp is positive but the SLCs do not stain. Bar = 25 μm.

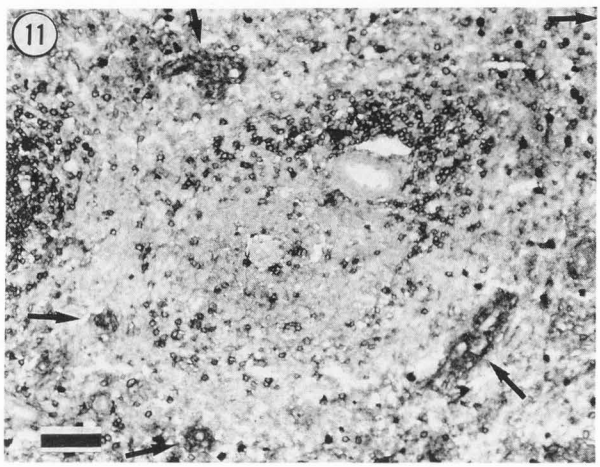
Figure 9: Iron pigment (hemosiderin) in SLCs in spleen from a patient with β-thalassemia. Bar = 25 μm.

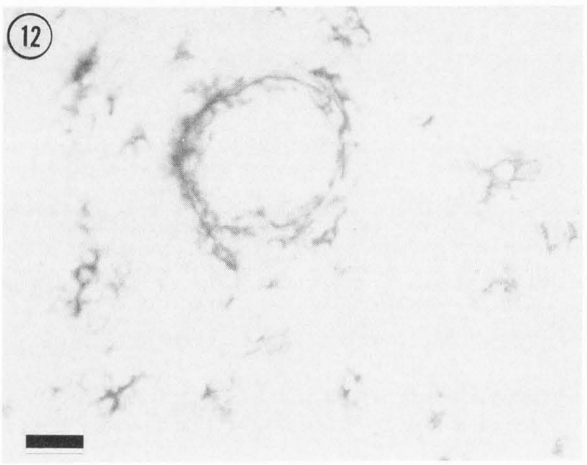
Figure 10: CD11b (CR3;OKM1). This antigen is strongly expressed by cells in the marginal zone around the white pulp. Bar = 100 μm.

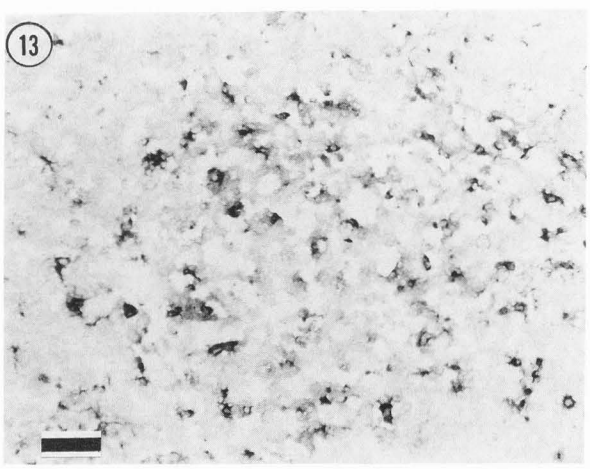
Figure 11: CD4 (Leu3a,b). The CD4 antigen is present on cells around small vessels in the MZ (arrows). Note also the positive small lymphocytes around central arterioles at left and upper center. Bar = 50 μm.











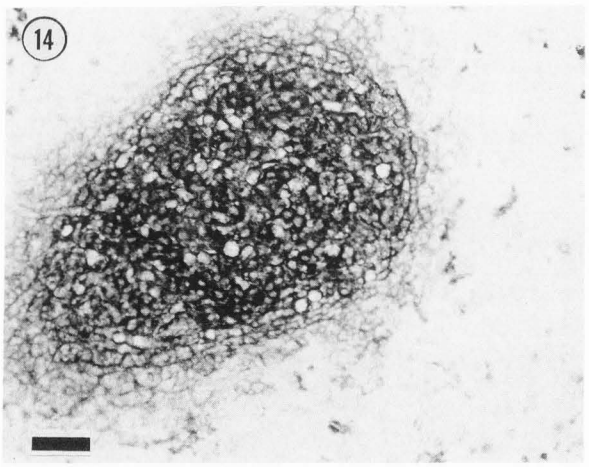


Figure 12: CD11c (LeuM5). The IRC of the white pulp show characteristic "dendritic" morphology. Note close relationship to the central arteriole (center). Bar = 25 μm.

Figure 13: CD68. The germinal center of this large white pulp follicle shows positive "tingible body" MØs. Bar = 25 μm.

Figure 14: 61D3. This white pulp germinal center shows strongly staining dendritic reticulum cells. The white circles interspersed among the labeled cell processes are unlabeled lymphocytes and DRC nuclei. Bar = 25 μm.

In the B-cell follicles (Table 2), diffuse, apparently background staining made evaluation of germinal center staining difficult with some of the antibodies. Nonetheless, three distinct patterns could be recognized. Scattered cells in the germinal centers of the follicles with abundant cytoplasm that contained cellular debris, tingible body (TB) MØs, were identified with NSE (fluoride inhibitable), CD68, 25F9, lysozyme, anti-α-1-AT and anti-α-1-ACT (Fig 13). In contrast, only cells with dendritic morphology (dendritic reticulum cells) were detected in germinal centers with the antibodies 61D3, 63D3 (CD14) and R4/23 (Fig 14). Similar cells were stained by anti-CD21 and anti-CD35 antibodies, along with some germinal center lymphocytes. The third pattern, exhibited by CD11c and CD14 antibodies, was positive staining of both TB MØs and dendritic reticulum cells (Table 2). Additionally, the CD14+ cells were concentrated at one end of the germinal center, creating a polarized pattern. No reaction of the CD4 antibody (Leu3a,b) with TB MØs or dendritic reticulum cells could be detected (Table 2).

Discussion

A major conclusion suggested by these studies is that phenotypic subpopulations of MØs are concentrated in different locations within the spleen. For example, cells that reside in the splenic MZ (Fig 1) share antigens with the red pulp MØs but, in addition, express antigens seen on few red pulp cells. These include CD11b, CD14 (63D3, Mo-2) and 61D3 (Fig. 10). Furthermore, two antigens, CD4 and CD14 (63D3), are present on cells that are distributed differently in these two splenic "compartments": CD4+ and CD14 (63D3)+ cells exhibit distinct clustering (Fig 11). These clusters were present to a variable degree in most spleens and often appeared to be centered around small vessels. This configuration resembles structures in human and animal spleens called ellipsoids or Schweigger-Seidel sheaths [7, 38;see below]. The apparent MZ location of these clusters is different from that of ellipsoids, however. The latter are located primarily in the red pulp, according to the studies of Buyssens et al [7]. Some studies have suggested that ellipsoids may be involved in the trapping of circulating antigens [31] while others have likened them to the high endothelial venules of the lymph node [7]. If the structures in the MZ are somehow analogous to ellipsoids, the presence of MØ-associated antigens on them supports the former interpretation.

The presence of subpopulations of cells within the same "compartment", i.e. red pulp, is also suggested by the results of this study. Inspection of Table 2 shows that certain groups of antigens are expressed by different proportions of red pulp MØs. Furthermore, the results show different patterns of distribution of cells expressing certain antigens. For example, CD11c+, CD15+ and α-1-ACT+ cells are clustered irregularly within the cords as opposed to the uniform distribution observed for the cells expressing many of the other antigens (Fig. 3). On the other hand, CD68+ MØs are sharply demarcated within the cords in a rather distinct pattern (Fig. 2).

The other population of resident cells in the splenic red pulp, the SLC, has been suggested by some authors to be phagocytic [17, 26, 37]. Results presented here on spleens from extensively transfused patients showed iron stores in SLC from phagocytized red cells (Fig. 9). Thus, the SLC would seem to qualify, functionally, as a type of MØ. As such, this cell exhibits a distinct phenotype that differs from red pulp cord MØs and from those in the MZ and white pulp (Table 2). Specifically, the presence of the CD8, CD71 and factor VIII-related antigens and the antigen detected by the OKM5 (CD36) antibody makes these cells unique. They express a combination of surface antigens, enzymes and cell products in common with monocytes, MØs, T-lymphocytes and endothelial cells [4, 5].

Comparison of antigens expressed by MØs in the germinal centers of follicles (B zone; tingible body MØs) with those expressed by MØs in other compartments suggests that the former is another phenotypic MØ subpopulation. TB MØs are 25F9+, CD14+, CD11b- and CD16- while MZ MØs are 25F9-, CD14 variable (Leu M3- but Mo2+), CD11b+ and CD16+.

In addition to showing phenotypic variation among splenic mononuclear cell subpopulations, the data from this study point out major antigenic differences between spleen cells, circulating monocytes and other types of MØs. Results from our laboratory and from published studies show, for example, that about 80% of blood monocytes express the antigens detected by the monoclonal antibodies PHM2, PHM3, and OKM5 [Buckley et al, unpublished results; 16, 41]. Splenic mononuclear cells either do not express these antigens (OKM5, PHM2, PHM3) or they are present on only a minority (Table 2). These results agree with the results of Hancock et al for PHM3 (seen only on circulating monocytes in the splenic sinusoids) but differ from their data for PHM2 [16]. These authors state that splenic red pulp MØs are weakly PHM2+ and that this antibody stains thymic, peritoneal, alveolar and exudative MØs and MØs in granulomas [16]. We have been unable to detect the antigen recognized by this antibody on any of the spleens we tested (Table 2). Compared with MØs in normal tonsils, lymph nodes and placental villi (Hofbauer cells), splenic MØs display other differences [Buckley et al, unpublished results; 15]. For example, only splenic red pulp and MZ MØs appear to express CD15. On the other hand, very few splenic MØs in these same two compartments are positive for the "pan-MØ" antibody, LeuM3 (CD14), while nearly all the other tissue MØs are uniformly positive. Similarly, the antibody 25F9 is not expressed by red pulp and MZ MØs (Table 2) but is present on most other tissue MØs. Finally, MØs in the red pulp and MZ compartments share the presence of CD16 (FcR for IgG) only with placental villous MØs but not with those in lymph nodes and tonsils [Buckley et al, unpublished observations; 15].

Unique topographical and antigenic features for dendritic cells (DC) are also illustrated by this study. This class of cells is identified by characteristic cell processes in tissue sections. The major subtypes are classified by their tissue locations and cell surface antigens and include T-zone interdigitating reticulum cells (IRC), follicular (B-zone) dendritic reticulum cells (DRC) and Langerhans cells (LC) of the skin [39]. DCs have been shown to be potent accessory cells but their relationship to MØs is not well understood [21, 39]. This study confirms the specific locations of two of the major DC subsets, more fully defines their surface antigenic phenotypes, and also suggests that DC and MØs may be related. Table 2 and Figs. 12 and 14 show that T-zone IRC and B-zone DRC exhibit characteristic cell surface antigenic phenotypes. Although these cells express class II major histocompatibility antigens, CD14 (Leu M3) and CD45 in common, they differ in the expression of a number of others; specifically, IRC are R4/23-, 61D3-, 25F9+, CD4+, CD14 (63D3)-, CD15+, CD21-, CD35- and CD68+. In contrast, DRC are R4/23+, 61D3+, 25F9-, CD4-, CD14 (63D3)+, CD15-, CD21+, CD35+ and CD68-. Unlike results of Wood et al. [43], we were unable to detect CD4+ DRC. These authors pointed out that DRC are often only trace positive and it is possible that we may not have detected such weakly positive cells in our preparations [43]. Our data do confirm the observation by Wood et al that IRC are CD4+ [43].

The data in Table 2 also show that IRC share the expression of EBM-11 (CD68), Leu M5 (CD11c), Leu M1 (CD15), 25F9 and Leu 3a,b (CD4) with splenic MØs and that some of the latter cells and DRC express 61D3, CD11c and CD14 (Leu M3, 63D3) in common. These findings suggest that MØs and DC may derive from a common progenitor and are the products of morphologic, phenotypic and functional differentiation. Wood et al. [43] and Franklin et al. [13] offered a similar conclusion based on their studies of MØs and dendritic cells in various lymphoid tissues. Similarly, a unique study of bone marrow transplantation patients reported by Murphy et al. [29] showed repopulation of epidermal LC apparently derived from phagocytic precursor cells MØs) in the dermis.

The presence and arrangement of subsets of mononuclear cells in the spleen may be indicative of functional specialization. The functional implications of the differences in phenotype and distribution between red pulp and MZ cells, for example, are not known but there are some intriguing possibilities. Humphrey and Grennan [19] studied mouse spleen and showed that MØs in the MZ differ from those in the red pulp in their ability to ingest labeled neutral but not acidic polysaccharides. MacLennan et al. [25] pointed out that this property is not shared by follicular dendritic cells, interdigitating cells of the T-zone or by red pulp MØs. The latter investigators [25] also showed that MZ B-lymphocytes in the rat express surface immunoglobulin isotypes different from other splenic lymphocytes and stated that the same was true of human splenic MZ B-lymphocytes. They suggested that this B-cell subset and the MZ MØs may interact in the immune response to specific kinds of antigens [25]. An important example of this could be the phenomenon of post-splenectomy sepsis, caused most often by pneumococcus [36]. The removal of the spleen may result in a deficit of MØs (MZ MØs?) that recognize the capsular polysaccharide of this organism (a neutral polysaccharide) as well as a decrease in the B-cells that respond to this antigen (MZ lymphocytes?). The results would be an inability to mount an appropriate immune responses to this organism followed by septicemia that is often fatal in these patients [36]. Functional studies to test the ability of isolated MZ MØs to recognize and ingest pneumococcal polysaccharides compared with similar studies using other types of MØs would be of great interest.

The presence of FcR for IgG (CD16) on red pulp and MZ cells, the expression of both FcR(IgG) and CR3 (CD11b) on MZ cells and the apparent lack of either on the phagocytic cells in the germinal centers and on the dendritic cells may reflect differences in the way these various cell populations recognize antigen-antibody complexes (Table 2). A major function of the spleen is the recognition and clearance of senescent cells from the circulation. This activity appears to predominate in the red pulp and is most evident in conditions such as immune cytopenias in which active phagocytosis of antibody-coated cells is observed in the splenic red pulp. The presence of FcR on red pulp MØs may facilitate recognition of antibody-sensitized, circulating cells. On the other hand, evidence from animal studies suggests that the cells of the MZ participate in the trapping of circulating particulate antigens and may recognize, as noted above, specific types of antigens [19, 31]. Although pure speculation, the presence on MZ cells of an additional recognition element, the C3bi complement receptor (CR3), could aid in this process (Fig. 10). The absence of the FcR (IgG) and of CR3 on tingible body MØs and DC may be a reflection of alternative mechanisms for antigen recognition and processing. Kapsenberg et al. [21] presented evidence in support of this interpretation, showing that FcR+ MØs and FCR- DC processed antigen differently. Our data do not address, on the other hand, the possibility that these cell surface receptors (or any of the antigens detected on splenic MØs and DC) are subject to physiologic up or down regulation in response to antigenic challenge. For example, the antibody 3G8 recognizes an FcR for IgG on granulocytes but not on circulating monocytes [12]. After 7 days in culture, however, some monocytes do express this antigen; furthermore, the majority of pulmonary alveolar MØs also express the antigen recognized by 3G8 [12]. The presence (or absence) and proportions of antigens on the various cell populations was uniform among all the spleens we tested; this suggests that major changes in receptor density is not a dominant phenomenon in the spleen or is not detectable by the techniques used here. Assays for FcR, CR and other antigens in

vitro under various conditions could help to elucidate this issue.

An interesting ancillary finding from this study is the staining of the SLC by the antibody 63D3 (Fig. 8). This antibody stains what seems to be the supporting matrix of the sinusoid rather than the lining cells themselves. Distinct bands oriented perpendicular to the sinusoids that are themselves composed of fine, parallel striations are seen. Further studies on the nature of these matrix structures are needed.

These data, unfortunately, do not shed any light on the important topic of the origin of macrophage heterogeneity. However, they do suggest potential experiments to test the hypothesis that microenvironment may be a determining factor in macrophage phenotype function, whether they originate from single or multiple cell lines. For example, the effect on phenotype of culturing macrophages from one microanatomic compartment with stromal elements from another compartment could be tested.

The information derived from the work presented here demonstrates that phenotypic and topographical heterogeneity exists among splenic MØs, both within and between microanatomic "compartments". Furthermore, some of these MØ subpopulations are phenotypically different from MØs in other tissues and from circulating monocytes. The presence and distribution of these MØ subsets suggests functional specialization. As the roles of more of the membrane antigens described here are elucidated, it is likely that MØ phenotypic heterogeneity will be matched by functional heterogeneity.

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Discussion with Reviewers

H.B. Fliet: Table 2 demonstrates that an inverse relationship exists with respect to the expression of CD14 and CD16. Could the author comment on the possible basis for this observation.

Author: My guess is that the "resident" red pulp cord macrophages are CD14- and that the few clusters of positive cells are circulating monocytes that are CD14+. The macrophages populating the cords either never were CD14+ or the local environment modulated them to become CD14-. There is ample data to show tissue influence on macrophage phenotype (see Ref. 16).

H.B. Fliet: The spleen is not a static organ. Could cells migrating into it from the circulation reflect

different degrees of maturation?
Author: This is certainly a possibility but I doubt it. For example, one would expect to see varying proportions of cells carrying monocyte restricted antigens since these are the likely cells that could contribute to the splenic macrophage pool. In fact, we see very few cells expressing these antigens (e.g., CD36, PHM3).

H.B. Fliet: Could anatomic location regulate expression of specific macrophage antigens? What role does proximity to splenic T and B cells play in macrophage antigen expression?
Author: I certainly do think that anatomic location is a potential regulator of macrophage phenotype. The fact that different tissues contain phenotypically different macrophages supports this, although not excluding a possible role for different macrophage lineages. I do not know what role T and B cells play in macrophage antigen expression but the fact that lymphokines can modulate macrophage antigens makes this a distinct possibility, e.g., in the marginal zone that is so near the lymphocytes.

H.B. Fliet: How do the observations of splenic macrophage antigen expression correlate with macrophage expression *in vitro* by monocyte-derived macrophages?
Author: As a model, I think the study of monocyte-to-macrophage maturation has some merit. On the other hand, I do not know how it relates to reality because the local environmental factors that most surely play a role in this process *in vivo* are not operative. More practically, I don't know of any study of monocyte-derived macrophages that assays more than a few antigens thus, comparisons with our data are difficult.