Heterogeneity of Mouse Thymic Macrophages: I. Immunohistochemical Analysis*

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Summary. As the first step toward understanding the identity and functions of thymic macrophages in situ, we examined the phenotypic heterogeneity of mouse thymic macrophages in tissue sections by the immunohistochemical double staining method with four monoclonal antibodies (F4/80, Mac-2, anti-CD32/16 and anti-I-A antibodies) as macrophage markers. Morphologically, three types of macrophages were identified: dendritic, round and flat-shaped. Dendritic macrophages were scattered throughout the thymus, and most of them were stained by all four markers. Among these macrophages, those at the cortico-medullary region (CMR) expressed a high intensity of CD32/16 antigen. Round macrophages were also distributed throughout the thymus; most of them, however, were localized in the cortico-medullary region to the medulla. These cells were F4/80-negative, Mac-2-positive, CD32/16-negative and I-A-positive. In contrast, round macrophages located at the cortex expressed F4/80. Flat-shaped macrophages were localized at the subcapsular region of the cortex where active lymphopoiesis was observed. This type was positive for F4/80 and CD32/16, but negative for Mac-2. Furthermore, most of the three types of thymic macrophages showed intense reactions of the I-A antigen within the cytoplasm in addition to the expression of I-A antigen on the cell membrane. These results indicate that morphological characteristics of thymic macrophages at different locations reflect phenotypic variations detected in immunohistochemistry, and suggest that these different type macrophages may play distinct roles at various locations in thymocyte development in the thymus.

Macrophages, one of the stromal components of the thymus, are derived from the bone marrow hematopoietic stem cells and are distributed throughout the thymus (KAISERLING et al., 1974). The major role of macrophages is the phagocytosis of dying, damaged thymocytes (KENDALL, 1991; NABARRA and ANDRIANARISON, 1991; NAKAMURA et al., 1995). In addition, several other important functions of macrophages have been proposed, including cytokine production which might affect thymocyte proliferation, maturation and differentiation, and involvement in the negative selection of potentially self-reactive T cell clones (KYEWSKI et al., 1986; SPRENT et al., 1988; LEIRA and GALLILY, 1988; DEMAN et al., 1996).

Previous investigators have reported that macrophages in each organ are generally heterogeneous in their shape and function (NAITO et al., 1996). Immunohistochemical examination in the rat, using ED1, ED2 and ED3 monoclonal antibodies, revealed distinct subpopulations of thymic macrophages in association with their characteristic distributions within the thymus (SMINIA et al., 1986). The rat thymic macrophages were also investigated ultrastructurally, revealing three different populations: cortical macrophages accounting for the majority, macrophages with dense bodies and rare phagocytosis in the cortico-medullary zone, and medullary interdigitating cells (IDCs) (MILICEVIC et al., 1987). Compared with those of the rat thymic macrophages, far fewer studies have focused on the heterogeneity of thymic macrophages in the mouse (TOUSSAINT-DEMYLLE et al., 1991). Ultrastructural analysis of the mouse thymus has revealed the shape of cortical and medullary macrophages, and of IDCs at the cortico-medullary junction and in the medulla (NABARRA and ANDRIANARISON, 1991). However, no precise immunocytochemical studies have been reported, except those in which mouse thymic macrophages were found positive for Mac-1 and Mac-2 (FLOTTE et al.,

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We undertook this study for elucidation of thymic macrophage heterogeneity in the mouse thymus in relation to their phenotypes, distributions, localization and functions. Such a study was deemed useful for comprehending the mechanisms of thymocyte differentiation within the mouse thymus. To investigate precisely the phenotypic and functional heterogeneity of thymic macrophages, we examined the mouse thymus by a double color staining method using several combinations of monoclonal antibodies specific for mouse thymic macrophages.

MATERIALS AND METHODS

Animals

Six-week-old female BALB/c mice were obtained from the Institute for Experimental Animals at the Tohoku University School of Medicine.

Antibodies

FITC-labeled anti-CD32/16 monoclonal antibody and biotin-labeled anti-I-Ad monoclonal antibody were purchased from PharMingen (San Diego, CA), and FITC-labeled F4/80 from Serotek (UK). The supernatants of the hybridoma clones, F4/80 and M3/38, were used as antibodies of F4/80 and Mac-2, respectively. FITC-conjugated goat anti-rat IgG antibody and Texas Red-labeled streptavidin were obtained from Vector (Burlingame, CA). Biotinylated goat anti-rat IgG antibody was purchased from Cappel (West Chester, PA). These monoclonal antibodies are listed in Table 1.

<table>
<thead>
<tr>
<th>Antibody/</th>
<th>Antigen</th>
<th>Molecular and cellular function</th>
<th>Cellular distribution</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>F4/80</td>
<td>160kDa glycoprotein</td>
<td>Seven-transmembrane type receptor</td>
<td>Macrophages, monocytes, promonocytes</td>
<td>Austyn and Gordon, 1981; Haidl and Jefferies, 1996; McKnight and Gordon, 1996</td>
</tr>
<tr>
<td>Mac-2</td>
<td>32kDa lectin</td>
<td>Activated macrophage (Thioglycollate-elicited peritoneal macrophage)</td>
<td>Macrophages, Langerhans cells, Kupffer cells, osteoclasts, interdigitating cells, epithelial cells</td>
<td>Ho and Springer, 1982; Flotte et al., 1983; Cherayil et al., 1989, 1990; Sato and Hughes, 1994</td>
</tr>
<tr>
<td>CD32/16</td>
<td>FcR II (CD32)</td>
<td>Receptor for the Fc region of IgG</td>
<td>Macrophages, eosinophils, neutrophils CD32 on B cells, monocytes, platelets CD16 on NK, K, T cells, Kupffer cells</td>
<td>Unkeless, 1979; Van de Winkel and Anderson, 1991; Looney, 1993; Bredius et al., 1994</td>
</tr>
<tr>
<td>I-A</td>
<td>MHC Class II</td>
<td>Antigen presentation</td>
<td>Macrophages, monocytes, B cells, Kupffer cells, Langerhans cells, thymic epithelial cells</td>
<td>Beller and Unanue, 1980; Bhattacharya et al., 1981; Van Ewijk et al., 1988</td>
</tr>
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</table>
to obtain immunofluorescence images: 1) Immunofluorescence was imaged with an epifluorescence microscope (AX-80, Olympus, Tokyo). Specimens were photographed for each specific fluorescence in combination with the Nomarski differential-interference-contrast (DIC) image. After being developed with positive films, images were digitized with an LS-1000 film scanner (Nikon, Tokyo) on a Power Macintosh 8500 personal computer (Apple Computer, Inc., Cupertino, CA). 2) Some sections were examined with Meridian/TR confocal laser scanning microscope (Meridian Instruments, Inc., Okemos, MI). Image files stored in the Meridian/TR’s computer were transferred to the Macintosh personal computer. Image data on the Macintosh personal computer were processed to enhance contrast and to combine fluorescence image with the DIC image by Photoshop image processing software for Macintosh (version 3.0, Adobe Systems, Inc., San Jose, CA). Images were reproduced on film with an LFR Mark II film recorder (Lasergraphics, Irvine, CA).

RESULTS

General

The most well-known antibodies for mouse macrophage markers, F4/80 and Mac-2, were used to take a brief look at macrophages in the thymus (Fig. 1a–c). These were distributed throughout the thymus, though much more numerously in the medulla than in the cortex. The number and distribution pattern of the F4/80 macrophages were roughly comparable to those of Mac-2 positive cells (Fig. 1a, c); this was confirmed in double staining of the thymus with these two markers, showing that the majority of macrophages were doubly stained. Mac-2 single positive macrophages, however, were detected in a substantial number in the medulla.

Cortex

Among the numerous macrophages detected throughout the cortex, two morphologically distinct types could be discriminated (Fig. 2). The major type had a dendritic shape with well developed cell processes extending into the narrow spaces around thymocytes. The other type of macrophage was rather round and obviously lacked cell processes. Although these macrophages were distributed throughout the cortex, the number of the dendritic macrophages was much greater than that of the round macrophages (Fig. 2).

Double staining of sections with F4/80 and Mac-2 demonstrated that the dendritic macrophages were intensely stained with both of these two markers. On the other hand, the round macrophages were strongly stained by Mac-2, but only weakly by F4/80 (Fig. 2).

In sections doubly stained with CD32/16 and Mac-2, the dendritic macrophages were double positive for these two markers; the round macrophages were positive for Mac-2 and negative for CD32/16 (Fig. 3). I-A antigen was detected in a reticular pattern, most of which was expressed on the thymic epithelial cells (Fig. 4). Two-color staining of sections with anti I-A and F4/80 antibodies, however, revealed that most of the dendritic macrophages were I-A-positive (Fig. 4). In addition to the detection of I-A antigen along the cell membrane, the intense reaction of I-A antigen was also observed within the cytoplasm of the dendritic macrophages (Fig. 4). Since the number of round macrophages was very small, it could not be clearly determined whether they expressed I-A antigens.

Subcapsular region

In addition to the two types of cortical macrophages described above, flat shaped macrophages were localized at the subcapsular region of the cortex (Fig. 5). These cells were F4/80-positive but Mac-2-negative (Fig. 5). Double staining by Mac-2 and anti-CD32/16 indicated that flat shaped macrophages at the subcapsular region expressed CD32/16 but not Mac-2 (Fig. 3).

Medulla

A large population of macrophages were present also in the medulla (Figs. 1a–c, 6). These were composed of both dendritic macrophages and round macrophages (Fig. 6). The number of round macrophages was greater in the medulla—especially at the periphery of this area—than in the cortex (Fig. 6).

Double staining with F4/80 and Mac-2 demonstrated that the dendritic macrophages were strongly stained by these two markers (Fig. 6), similar to cortical dendritic macrophages. The round macrophages were Mac-2-positive but F4/80-negative (Fig. 6), contrary to cortical round macrophages (Mac-2 positive and F4/80 weakly positive). Furthermore, the F4/80-positive and Mac-2-positive dendritic macrophages expressed CD32/16 while the round macrophages did not express this antigen (Fig. 7).

The medulla could be recognized as a discrete area in the immunostaining by the anti-I-A antibody (Fig. 8). This region showed a rather confluent staining
Fig. 1. Distribution of thymic macrophages double-stained with F4/80 and Mac-2. a. A F4/80-FITC (green) image combined with a DIC image. b. A F4/80-FITC (green) image combined with a Mac-2-Texas Red (red) image. c. A Mac-2-Texas Red (red) image combined with a DIC image. Macrophages are distributed throughout the thymus, both in the cortex (C) and in the medulla (M). a, b, c: ×160

Fig. 2. Double staining of the cortex of the thymus with F4/80-FITC (green) and Mac-2-Texas Red (red). The major type of macrophages are of dendritic morphology with well developed cell processes (arrowheads). On the other hand, a very small number of the round macrophages are detected in the cortex (arrow). These are strongly stained by Mac-2, but only weakly by F4/80. ×600

Fig. 3. Double staining of the cortex of the thymus with CD32/16-FITC (green) and Mac-2-Texas Red (red) in the cortex. The dendritic macrophages are double positive for CD32/16 and Mac-2 (arrowheads). The round macrophages are Mac-2 single positive (CD32/16 negative) (arrow). A flat shaped macrophage is seen at the subcapsular region (CD32/16-positive but Mac-2-negative) (double arrow). ×600

Fig. 4. Double staining of the cortex with F4/80-FITC (green) and I-A-Texas Red (red). Most of dendritic macrophages express I-A antigens along the cell membrane and in the cytoplasm (arrowheads). Confocal laser microscopy. ×600

Fig. 5. Double staining of the cortex with F4/80-FITC (green) and Mac-2-Texas Red (red). Flat shaped macrophages localized at the subcapsular region (double arrow) are F4/80-positive but Mac-2-negative. ×600
Figs. 2-5. Legends on the opposite page.
Fig. 6. Double staining of the medulla with F4/80-FITC (green) and Mac-2-Texas Red (red). Dendritic macrophages are stained by these two monoclonal antibodies (arrowheads), similar to cortical dendritic macrophages. The round macrophages are Mac-2-positive but F4/80-negative (arrows). ×600

Fig. 7. Double staining of the medulla with CD32/16-FITC (green) and Mac-2-Texas Red (red). Dendritic macrophages are double positive for CD32/16 and Mac-2 (arrowheads). Round macrophages are Mac-2 single positive (CD32/16 negative) (arrows). ×600

Fig. 8. Double staining of the medulla with F4/80-FITC (green) and I-A-Texas Red (red). Most macrophages express I-A antigens on the cell surface and in the cytoplasm (arrowheads). Confocal laser microscopy. ×600
Fig. 9. Immunohistochemical localization of CD32/16 positive cells in the thymus. A CD32/16-FITC (green) image combined with a DIC image. CD32/16 positive cells are specifically localized at the cortico-medullary region. ×120

Fig. 10. Double staining of the thymus with CD32/16-FITC (green) and I-A-Texas Red (red). CD32/16 positive macrophages are seen at the border of the medulla (M), which is strongly stained with I-A (red). ×120

Fig. 11. The cortico-medullary region double stained with CD32/16-FITC (green) and Mac-2-Texas Red (red). Dendritic macrophages are double positive for CD32/16 and Mac-2 (arrowheads). Round macrophages are Mac-2 single positive (CD32/16 negative) (arrows). ×600

Fig. 12. The cortico-medullary region double stained with F4/80-FITC (green) and Mac-2-Texas Red (red). Dendritic macrophages are double positive for F4/80 and Mac-2 (arrowheads). Round macrophages are Mac-2 single positive (F4/80 negative) (arrows). ×600
pattern. The major number of cells expressing I-A antigen were considered to be thymic epithelial cells. Most medullary macrophages, their morphology and phenotypes notwithstanding, highly expressed I-A antigen along the cell membrane as well as in their cytoplasm (Fig. 8).

Cortico-medullary region (CMR)

Staining with anti-CD32/16 antibody clearly demonstrated that dendritic macrophages strongly expressing CD32/16 were specifically localized at the CMR of the thymus (Fig. 9). Double staining of sections with anti-CD32/16 and anti-I-A antibodies confirmed that these CD32/16 positive macrophages aggregated along but slightly outside the border of the strongly I-A positive medulla (Fig. 10). Only a minor fraction of these macrophages expressed I-A antigen. These macrophages were also stained by both Mac-2 and F4/80 (Figs. 11, 12).

Round macrophages were also detected at this region. These cells were F4/80-negative, Mac-2-positive and CD32-negative (Figs. 11, 12).

The immunohistochemical heterogeneity of thymic macrophages is summarized in Table 2.

DISCUSSION

In the present study, we have performed morphological and immunohistochemical analysis of macrophages in the mouse thymus. We adopted the two-color analysis for immunohistochemistry throughout the course of the experiments. Single color staining alone could not depict the exact phenotypical identity of any cellular components of the thymus; an accurate interrelation between expression patterns of different antigens could not be elucidated by single color analyses no matter how many antigens were used in combination. Dual color analyses are thus critically important to obtain correct information about the accurate identity and distinction of, and lineage relationship between subsets, if any, of mouse thymic macrophages, when several surface markers have to be complementarily used with each other.

Although other monoclonal antibodies such as Mac-1 and Moma-1 are also available, we chose the four monoclonal antibodies listed in Table 1. Mac-1 was considered inappropriate for the study because it detected neutrophils in the thymus more strongly than thymic macrophages. Moreover, Moma-1 has so far not been used to detect thymic macrophages.

Three distinct subpopulations were phenotypically categorized as follows:

1) Dendritic macrophages

The predominant subpopulation of thymic macrophages comprises a large and dendritic type with many processes, scattered throughout the thymic parenchyme, both in the cortex and in the medulla, expressing Mac-2, F4/80, and CD32/16, as well as I-A antigens. Since these macrophages have been considered to be phagocytic in nature (NIBBERING et al., 1987; BREDIUS et al., 1993), it is reasonable to assume that they are responsible for phagocytosing dying thymocytes (KENDALL, 1991; NABARRA and ANDRIANARISON, 1991; SURH and SPRENT, 1994; NAKAMURA et al., 1995). In fact, electron microscopy revealed, in thymic macrophages with well-developed cell processes often containing a number of phagosomes with ingested thymocytes at various stages of degradation (NAKAMURA et al., 1995). In addition, a subtype of dendritic macrophages was noted in the CMR in this study. The extent of CD32/16 expression in dendritic type CMR macrophages was much greater compared with those in macrophages in other sites. As stated,

<table>
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<tr>
<th>Regions of the thymus</th>
<th>Shape</th>
<th>F4/80</th>
<th>Mac-2</th>
<th>CD32/16</th>
<th>I-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcapsular</td>
<td>flat</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cortex</td>
<td>dendritic</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>round</td>
<td>+/−</td>
<td>+</td>
<td>−</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cortico-medullary</td>
<td>dendritic</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>region</td>
<td>round</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Medulla</td>
<td>dendritic</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>round</td>
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</table>

N.D.: not determined
since CD32/16 antigens are receptors for the Fc portion of IgG (CD32, FcγRII; CD16, FcγRIII; van de Winkel and Anderson, 1991; Bredius et al., 1994), CMR macrophages strongly expressing these antigens would be a more specified population for ingesting dying thymocytes than those in other locations.

2) Round macrophages

The second major type was round small macrophages that obviously lacked cell processes, and were mainly detected in the medulla and the CMR. Phenotypically, they were positive for Mac-2 but negative for F4/80 and CD32/16; I-A antigens were detected in these cells. On the basis of their characteristic distribution pattern, small cell size, and their unique phenotype: 1) they could be involved in the final stage of thymocyte differentiation, 2) they may represent an immature thymic macrophage population which may have just entered the thymus via vessels running through the septa to the medulla and/or the CMR (Kaiserling et al., 1974), or 3) their origin may differ from that of dendritic type macrophages. Our preliminary observations by electron microscopy showed that these Mac-2 positive, small round-shaped cells were large lymphoid cells displaying little, if any, phagocytotic activities (Nakamura et al., unpublished observations). Detailed examination will be required to characterize this subset of thymic macrophages.

Small round-shaped cells present in the cortex appeared to weakly express F4/80 in addition to Mac-2, producing a slight contrast with those in the medulla and in the CMR. I-A expression in these cortical cells could not be sufficiently assessed due to their low number. Whether these populations belong to the same lineage or to separate populations remains unclear.

3) Subcapsular flat macrophages

The last type, slender and flat macrophages, extending their processes along with and underneath the capsule, is present in the subcapsular region. This type of macrophage was small in quantity. They were positive for F4/80 and CD32/16, but negative for Mac-2. We concluded that subcapsular flat macrophages constitute an independent subset of thymic macrophages from the dendritic and the medullary round types described in this study. Thymocytes divide extensively at the subcapsular region; lymphoblasts are aggregated to form the prominent zone, which always gives a positive reaction for proliferative cell nuclear antigen (PCNA) (von Gaudecker, 1991); a number of cells at various phases of mitoses are present in this region. It is, therefore, highly probable that flat macrophages may play some important roles in thymocyte proliferation and selective differentiation. Ultrastructural studies followed by functional analyses of these flat macrophages are essential to delineate their biological significance.

Interaction between major histocompatibility complex (MHC) antigen class II, I-A and the T cell antigen receptors (TCR) is considered to be the critical step for the positive and/or negative selection of thymocytes (Blackman et al., 1990; Owen and Moore, 1995). Thymic epithelial cells are the most abundant source of I-A in the thymus, and are thought to play a major role in the positive selection (Van Ewijk et al., 1988). I-A antigens expressed by bone marrow-derived cells including macrophages are a candidate molecule responsible for the negative selection (Kyeski et al., 1986; Kendall, 1991). In this context, it is noteworthy that nearly all thymic macrophage subsets, if not all, have I-A antigens. However, previous studies by flow cytometry and immunohistochemistry have demonstrated that only about half of thymic macrophages bear the I-A antigens (flow cytometry: Nabarra and Papiernik, 1988, and our unpublished observations; immunohistochemistry: Van Ewijk et al., 1980). The inconsistency may be derived from: 1) the different methods used for the detection of I-A (either immunohistochemistry or flow cytometry); 2) the different fixatives used for immunohistochemical procedures—we used the PLP method, which is considered the most suitable fixative to preserve proteins and peptides (Yoshimura et al., 1980; Murakoshi et al., 1981), as compared with the mixture of paraformaldehyde and glutaraldehyde (Van Ewijk et al., 1980); or 3) the different subcellular localization of I-A antigens. Whether or not I-A was expressed on the surface could be confirmed by flow cytometry, but not accurately by light microscopy of tissue sections; in contrast, whether or not the antigen was present in the cytoplasm could be demonstrated by immunohistochemistry, but not by flow cytometry. Farr and Nakane (1983) indicated the intense immunolabeling of I-A antigens within the cytoplasm of thymic reticular cells. Detailed immuno-electron microscopy combined with flow cytometry will have to address this controversial issue.

Investigators have stated that the IDCs, another component of the bone marrow-derived cells, are localized at the CMR and the medulla (Nabarra and Papiernik, 1988; Nabarra and Andrianarison, 1991). Previous immunohistochemical studies also demonstrated that IDCs were I-A-positive, Mac-2-positive.
and F4/80-negative (Nabarra and Papiernik, 1988; Ardavin and Shortman, 1992). Although round macrophages in this study seemed to share similar or common features in phenotype and distribution pattern with the IDCs, their size and shape were in sharp contrast with those of previously reported IDCs. This discrepancy will have to be resolved by immunoelectron microscopy, since the identity of IDCs is originally defined ultrastructurally.

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