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Analysis of the *in vivo* dendritic cell response to the bacterial superantigen staphylococcal enterotoxin B in the mouse spleen

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Summary. To investigate the *in vivo* effects of Staphylococcal enterotoxin B (SEB) on dendritic cells (DCs) in the spleen, a single dose of SEB (50 μg/kg) was administered to BALB/c mice by intraperitoneal injection. Afterwards, the mice were sacrificed at 2, 6 and 24 hr, 2, 4, 7 and 15 days, and the spleens were removed. The immunocytochemical characterization of the cells was carried out using various monoclonal antibodies in cryostat-cut sections.

The distribution patterns of DCs and their major costimulatory molecules, CD80, CD86 and CD40 in the spleen were identified, and the evidence for maturation of DCs *in vivo* in response to SEB was obtained. It was found that systemic administration of SEB induced the migration of most of the immature, splenic DCs from the marginal zone to the periarterial lymphatic sheath within 6 hr. This movement paralleled a maturation process, as assessed by upregulation of CD40, CD80 and CD86 expression in the interdigitating dendritic cells (IDCs). The upregulation of costimulatory molecule expression was conspicuous only in DCs in contrast to other antigen-presenting cells (APCs) such as macrophages and B cells which did not show any significant alterations in their costimulatory molecule expression. We also demonstrated the temporal expression pattern of these costimulatory molecules on the activated DCs. The upregulation of costimulatory molecules on DCs reached a peak level 6 hr after SEB injection, while the increase in number of T cells expressing T cell receptor Vß8 reached a peak level on day 2 after SEB treatment.

In conclusion, we demonstrated the *in vivo* DC response to SEB in the mouse spleen, especially a potent stimulative effect of SEB on DCs *in vivo*, a temporal distribution pattern of DCs as well as T cells including TCR Vß8$^+$ T cells, and a differential expression pattern of costimulatory molecules on the activated DCs. The results of the present study indicate that DCs are the principal type of APCs which mediate T cell activation by SAg *in vivo*, and that each costimulatory molecule may have different role in the activation of DCs by SAg. Thus, it is plausible to speculate that DCs play a critical role in the T cell clonal expansion by SAgs and other SAg-induced immune responses *in vivo*.

Key words: Dendritic cells, Staphylococcal enterotoxin B, Superantigen, Costimulatory molecules, Mouse spleen

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Introduction

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) with extraordinary capacity for initiating primary immune responses (Steinman, 1991). Activation of naive T cells by APCs requires costimulatory signals in addition to the primary signal provided by the engagement of the T cell receptor (TCR) (Schwartz, 1990; Janeway and Bottomly, 1994). The most potent membrane-associated costimulatory molecules are CD80 (B7-1), CD86 (B7-2) and CD40 (Freeman et al., 1991; Grewal and Flavell, 1996; Van Gool et al., 1996; Van Kooten and Banchereau, 1997; Slavik et al., 1999). Furthermore, these costimulatory molecules play a role in the pathogenesis of various pathological conditions including infection, autoimmune diseases, atherosclerosis, ischemia/reperfusion injury and transplant rejection (Bluestone, 1996; Reiser and Stadecker, 1996; Takada et al., 1997; Mach et al., 1998).

Superantigens (SAgs), a class of immunostimulatory molecules, are produced by some bacteria and viruses, and are powerful T cell mitogens that activate T cells in a TCR Vß8-specific manner, and are responsible for various human diseases including food poisoning, septic

shock, toxic shock syndrome and skin diseases (Marrack and Kappler, 1990; Scherer et al., 1993; Leung et al., 1995; Johnson et al., 1996). SAgs do not require antigen processing, but efficient presentation requires binding of the SAg to a major histocompatibility complex (MHC) class II molecule at the surface of an APC (Fraser, 1989; Scherer et al., 1993). SAgs bind to class II at sites distinct from the conventional peptide-binding groove on the MHC class II protein (Marrack and Kappler, 1990; Scherer et al., 1993). The critical role of invariant chain and HLA-DM was found in the modulation of SAg-binding specificities of MHC class II-expressing cells (Lavoie et al., 1997).

Recent evidence suggests that SAgs may be relevant to the pathogenesis of autoimmune diseases (Brocke et al., 1993, 1998; Youinou et al., 1995; Schiffenbauer et al., 1998). Especially, the activation of APCs by SAgs is considered as a possible mechanism for SAg involvement in autoimmunity (Schiffenbauer et al., 1998). It was found that SAgs can stimulate APCs such as macrophages, leading to the release of cytokines, superoxides or other mediators of inflammation (Rink et al., 1994). In particular, it was reported that small amounts of SAg, when presented on DCs, are sufficient to initiate T cell responses *in vitro* (Bhardwaj et al., 1993). Bhardwaj et al. (1992) also showed that DCs are 10- to 50-fold more potent than monocytes or B cells in inducing T cell responses to a panel of SAgs, and that DCs can present femtomolar concentrations of SAg to T cells even at numbers where other APCs are inactive.

However, little information is available so far on the *in vivo* effects of SAgs on DCs and their costimulatory molecule expression. Thus, in the present study, the *in vivo* APC response to *Staphylococcus aureus* enterotoxin B (SEB), one of the most important bacterial SAgs, with special emphasis on DCs in the spleen of mice was analyzed.

Materials and methods

Experimental animals

Six- to eight-week-old male BALB/c (H-2^d) mice were housed in a specific pathogen-free animal care facility. They were maintained at room temperature on a 14 hr light to 10 hr dark cycle and were provided with food and water *ad libitum*.

In vivo SEB treatment

Purified exotoxin staphylococcal enterotoxin B (SEB) was purchased from Toxin Technology, Inc. (Sarasota, FL, USA) and Sigma Immunochemicals (St. Louis, MO, USA). Mice were injected intraperitoneally with 50 μg SEB solubilized in pyrogen-free normal saline. Control animals were injected with the same volume of normal saline. The animals were sacrificed in groups of five at 2, 6 and 24 hr, 2, 4, 7 and 15 days after injection.

Tissue preparation

The mice were anesthetized with diethylether. The spleens were removed and rapidly frozen in isopentane cooled with liquid nitrogen. Frozen sections (3 and 5 μm thick) were cut on a Reichert cryostat and placed on 3-aminopropyltriethoxysilane-coated slides. After being dried, the cryosections were fixed in cold acetone for 10 min at -20 °C .

Immunohistochemistry

Immunostaining was performed by using the streptavidin-biotin complex (ABC) method. In brief, the sections and the adjacent serial sections were incubated for 10 min in a solution of phosphate-buffered saline (PBS) containing 0.3% H_2O_2. After a wash in PBS, the sections were incubated with a bovine serum albumin (BSA) solution (Sigma, 10 mg/ml in PBS). The sections were incubated for 16-18 hr at 4 °C with the primary monoclonal antibodies (mAbs) specified in Table 1. Following incubation with the primary mAbs, the sections were incubated with biotinylated anti-rat and anti-hamster antibodies (Jackson Immunoresearch Labs., West Grove, PA, USA; Vector Labs., Burlingame, CA, USA). Then the sections were incubated for 60 min with an ABC reagent (Vectastain Elite kit, Vector Labs.) according to the manufacturer's instructions. After a PBS rinse, the sections were developed with a 0.05% 3-3'-diaminobenzidine-H_2O_2-medium under microscopical control. Afterwards, the sections were either counterstained with Harris' hematoxylin or not counterstained, and mounted in a xylene-based mounting medium (Permount).

Two-color immunohistochemical analysis was performed in order for the precise identification of the types of cell which express CD80, CD86 and CD40 costimulatory molecules according to the procedure of Matsuno et al. (1996) with a slight modification. In brief, the cryosections were fixed in cold acetone for 10 min, and then in formol calcium solution for 2 min after rehydration in PBS. After washing in PBS and incubation with 2% BSA solution for 20 min, the sections were incubated with the first primary mAbs for 16-18 hr at 4 °C. Following incubation with the primary mAbs, the sections were washed with PBS and incubated with biotinylated secondary antibodies (Jackson Immunoresearch Labs. and Vector Labs.). Then the sections were further fixed with 1% glutaraldehyde in PBS for 30 seconds. After the sections were incubated with an ABC reagent (Vectastain Elite kit), they were developed with a 0.05% 3-3'-diaminobenzidine-H_2O_2-medium. Thereafter, the sections were incubated with the second primary mAbs, and then with alkaline phosphatase-labeled secondary antibodies. Finally, the labeled cells were colored blue by the alkaline phosphatase substrate kit (Vector Blue, Vector Labs.). The sections were mounted in Vectashield (Vector Labs.).

Results

In control mice, a large number of N418$^+$ DCs was predominantly present in the marginal zone and scattered profiles of them were also found between T cells in the periarterial lymphatic sheath (PALS) of spleen (Fig. 1). There was no significant alteration in the distribution of N418$^+$ DCs in the cryosections from the spleen 2 hr after SEB injection. However, the number of N418$^+$ DCs was strikingly increased in the PALS, while there were few N418$^+$ DCs in the splenic marginal zone 6 hr after SEB administration (Fig. 2). One day after SEB injection, many N418$^+$ DCs reappeared in the marginal zone and the number of N418$^+$ DCs in the PALS became fewer than that of the PALS from the splenic sections 6 hr after SEB treatment, but still larger than that of the PALS of control mice (Fig. 3). Two days after SEB injection, the number of N418$^+$ DCs in the marginal zone was further increased and their number in the PALS was further decreased. From day 4 of SEB administration, the number and distribution pattern of N418$^+$ DCs in the PALS and marginal zone appeared to be similar to those of the splenic sections from control mice (Fig. 4).

The NLDC-145 mAb stained DCs only in the PALS of control mice (Fig. 5). Their number was notably increased in the PALS 6 hr after SEB injection (Fig. 6). From day 1 of SEB treatment, the number of NLDC-145$^+$ DCs was decreased gradually. So, from day 4 onwards, their number and distribution pattern appeared to be similar to those of control mice.

The MIDC-8 mAb, similarly to the NLDC-145 mAb, stained DCs only in the PALS of control mice (Fig. 7). Their number was notably increased in the PALS 6 hr after SEB injection (Fig. 8). From day 1 of SEB administration, the number of MIDC-8$^+$ DCs decreased gradually (Fig. 9). So, from day 4 of SEB treatment, their number and distribution pattern appeared to be similar to those of control mice (Fig. 10). Thus, it was demonstrated that the NLDC-145 mAb and MIDC-8 mAb exhibited almost the same staining pattern both in control and SEB-treated mice.

Immunocytochemical localization of CD86 with the GL1 mAb revealed strong positive staining in the marginal zone where the intensely stained cells were predominantly localized in the inner region of the marginal zone, constituting a single, continuous layer around the PALS in control mice (Fig. 11). In addition, the GL1 mAb also exhibited weaker reactivity in the red pulp and PALS of control mice (Fig. 11). Interestingly, 6 hr after SEB injection, the level of CD86 expression was increased in many cells within the PALS, whereas the level of CD86 expression in the marginal zone was somewhat reduced and that of CD86 staining in the other areas of the spleen was not significantly altered (Fig. 12). Immunostaining of the serial sections and double-staining revealed that most of the cells in the PALS showing upregulated CD86 expression were the N418$^+$ DCs and those cells in the red pulp were the F4/80$^+$ red pulp macrophages (Fig. 13). From day 1 of SEB administration, the level of CD86 expression on DCs in the PALS was decreased again and the expression pattern became similar to that of control mice. From day 4 of SEB treatment, the level of CD86 expression in the marginal zone was increased again and the expression pattern became similar to that of control mice (Fig. 14).

Immunostaining of the serial sections and by double-labeling also showed that the intensely stained CD86$^+$ cells, located around the PALS and distributed in the inner region of the marginal zone between the marginal zone and PALS in control mice, coincided with the Mac-

Table 1. Monoclonal antibodies used for immunostaining.

ANTIBODY	SPECIFITY	SOURCE	DILUTION
500A2	CD3$^+$ T cells	Pharmingen (USA)	1:1000
H129.19	CD4$^+$ T cells	Pharmingen (USA)	1:1000
53-6.7	CD8$^+$ T cells	Pharmingen (USA)	1:1000
KJ16	TCR V 8.1, 8.2$^+$ T cells	Serotec (UK)	1:200
1D3	CD19$^+$ B cells	Pharmingen (USA)	1:500
N418	CD11c (integrin α^x) mainly expressed on dendritic cells	Serotec (UK)	1:300
NLDC-145	Interdigitating dendritic cells and thymic epithelium	Serotec (UK)	1:50
MIDC-8	Interdigitating dendritic cells	Serotec (UK)	1:50
M5/114	Ia (MHC class II) molecule on dendritic cells, B cells and some macrophages	Beringer-Menheim (Germany)	1:1000
3/23	CD40 molecule	Serotec (UK)	1:100
16-10A1	CD80 (B7-1) molecule	Pharmingen (USA)	1:50
GL1	CD86 (B7-2) molecule	Pharmingen (USA)	1:500
C1:A3-1	F4/80 antigen on mature macrophages	Serotec (UK)	1:500
MOMA-1	Metallophilic macrophages	Serotec (UK)	1:100
M3/84	Mac-3 antigen expressed on mouse mononuclear phagocytes, tissue-resident macrophages and mature dendritic cells	Pharmingen(USA)	1:500

3^+ cells but did not coincide with the F4/80$^+$ cells. Only a few of the CD86$^+$ cells coincided with the N418$^+$ DCs and MOMA-1$^+$ metallophilic macrophages (Figs. 15-18).

Immunostaining of the serial sections and double-staining with several mAbs, including the 3/23 mAb and N418 mAb, revealed that the CD40$^+$ cells were the N418$^+$ DCs and CD19$^+$ B cells both in control and SEB-treated mice (Figs. 19, 20). Immunocytochemical localization of CD40 with the 3/23 mAb exhibited that a few CD40$^+$ DCs were scattered within the PALS and most of the B cells located in the lymphoid follicles were weakly stained with the 3/23 mAb in control mice (Fig. 21). Characteristically, the CD40$^+$ DCs in the PALS were remarkably increased both in number and staining intensity 6 hr later (Fig. 22). This increased CD40 expression on DCs in the PALS was most conspicuous 24 hr after SEB injection (Fig. 23). The CD40$^+$ DCs were decreased both in number and staining intensity 2 days after SEB administration. From day 4 of SEB treatment, the CD40 expression pattern of DCs in the PALS became similar to that of control mice (Fig. 24). However, B cells did not exhibit any significant alteration in the CD40 expression pattern throughout all the time sequences (Figs. 21-24) .

Immunocytochemical localization of CD80 with the 16-10A1 mAb revealed few immunoreactive cells in the marginal zone of splenic sections from control mice (Fig. 25). However, the CD80 antigen was not detected in the PALS of control mice (Fig. 25). The number of CD80$^+$ cells was slightly increased in the marginal zone 2 hr after SEB injection (Fig. 26). Remarkably, the CD80$^+$ cells were increased in number, size and staining intensity in the PALS 6 hr after SEB administration (Fig. 27). From day 1 of SEB treatment, the CD80 staining disappeared in the PALS (Fig. 28).

Numerous CD3$^+$, CD4$^+$ and CD8$^+$ T cells were located in the PALS of splenic sections from control mice. These T cells were gradually increased in number after SEB injection, reaching a peak level 48 hr later and from day 4 of SEB treatment, their number became similar to that of control mice. Especially, many TCR Vß8$^+$ T cells were located in the PALS of splenic sections from control mice (Fig. 29) and they were also gradually increased in number after SEB injection, reaching at a peak level 48 hr later. From day 4 of SEB treatment, their number was gradually decreased (Fig. 30). However, B cells did not exhibit any significant alteration in number after SEB injection.

Discussion

In the present study, it was demonstrated that systemic administration of SEB induces the migration of most splenic DCs from the marginal zone to the PALS within 6 hr. This movement paralleled a maturation process, as assessed by upregulation of costimulatory molecule expression, which was conspicuous only in DCs in contrast to other APCs such as macrophages and B cells which did not show any significant alterations in their costimulatory molecule expression. These observations indicate that SAgs are a potent and effective activator of DCs *in vivo* and that DCs are the principal type of APCs which mediate T cell activation by SAg *in vivo* among multiple cell types of APCs.

In control mice, DCs in the marginal zone of spleen were detected only by the N418 mAb, whereas DCs in the PALS of spleen, interdigitating dendritic cells (IDCs)

Fig. 1. N418$^+$ dendritic cells (DCs) in the spleen of a control mouse. Many DCs are observed in the marginal zone (M) and PALS (P). x 125

Fig. 2. N418$^+$ DCs in the spleen 6 hr after SEB treatment. Note the DCs which are greatly increased in number within the PALS (P). Asterisks: central arteries. x 125

Fig. 3. N418$^+$ DCs in the spleen 1 day after SEB treatment. The number of N418$^+$ DCs in the PALS (P) is fewer than that of the PALS from the splenic sections 6 hr after SEB treatment, but still larger than that of the PALS of control mice. x 125

Fig. 4. N418$^+$ DCs in the spleen 15 days after SEB treatment. The number and distribution pattern of N418$^+$ DCs in the PALS (P) and marginal zone (M) appear to be similar to those of the splenic sections from control mice. x 125

Fig. 5. NLDC-145$^+$ DCs in the spleen of a control mouse. The NLDC-145 mAb stains DCs only in the PALS (P) of control mice. x 125

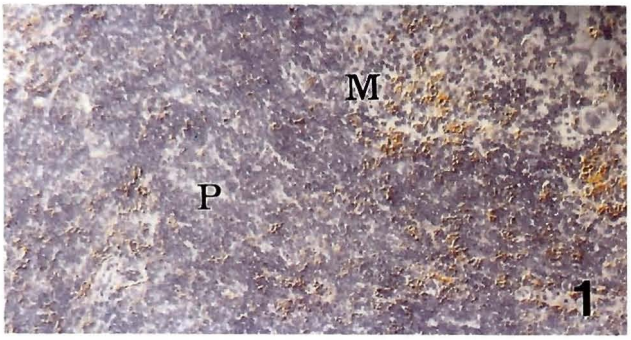
Fig. 6. NLDC-145$^+$ DCs in the spleen 6 hr after SEB treatment. The number of NLDC-145$^+$ DCs is notably increased in the PALS 6 hr after SEB injection. x 125

Fig. 7. MIDC-8$^+$ DCs in the spleen of a control mouse. The MIDC-8 mAb stains DCs only in the PALS (P) of control mice. x 125

Fig. 8. MIDC-8$^+$ DCs in the spleen 6 hr after SEB treatment. The number of MIDC-8$^+$ DCs is notably increased in the PALS (P) of spleens 6 hr after SEB injection. x 125

Fig. 9. MIDC-8$^+$ DCs in the spleen 24 hr after SEB treatment. The number of MIDC-8$^+$ DCs in the PALS (P) is decreased in comparison with that of MIDC-8$^+$ DCs in the PALS (P) of spleens 6 hr after SEB injection. x 125

Fig. 10. MIDC-8$^+$ DCs in the spleen 4 days after SEB treatment. The number and distribution pattern of MIDC-8$^+$ DCs in the PALS (P) appear to be similar to those of control mice. x 125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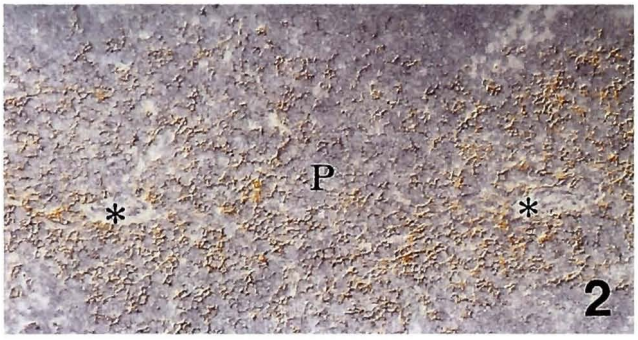
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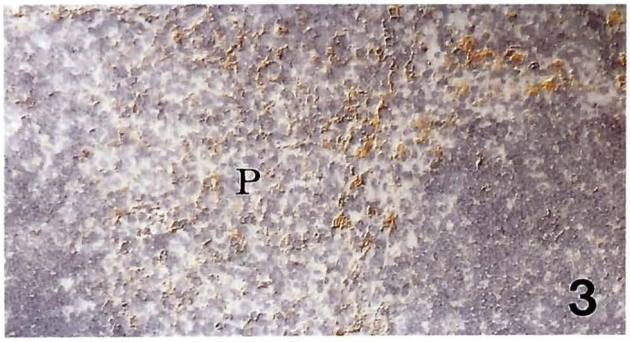
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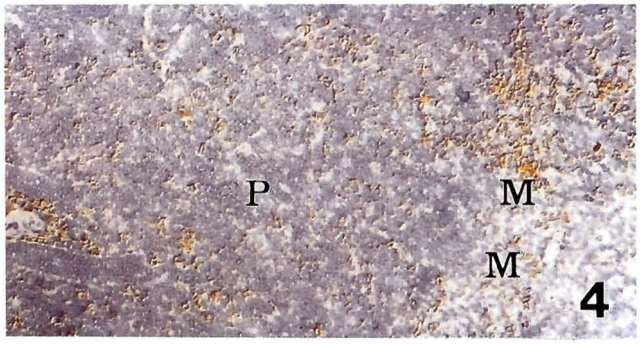
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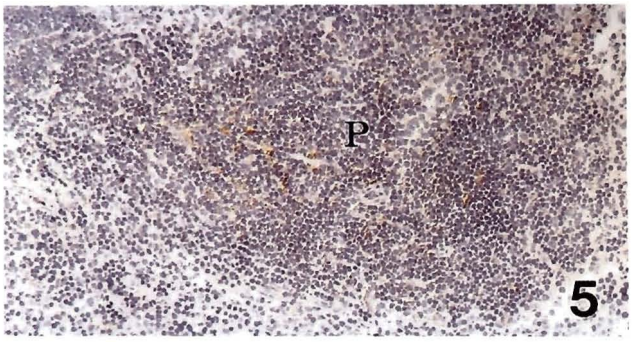
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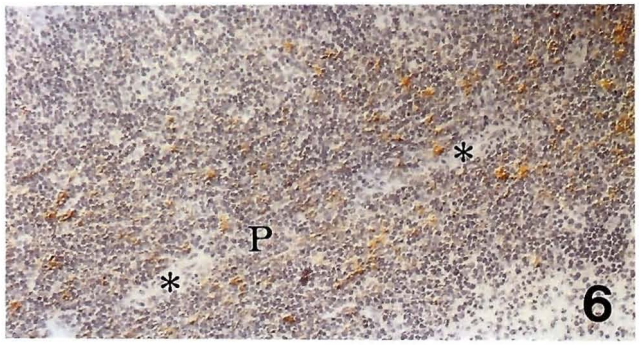
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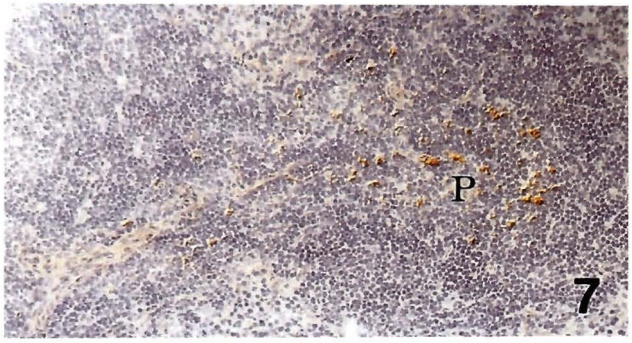
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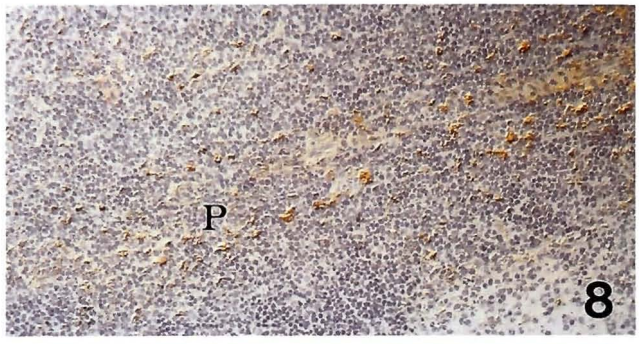
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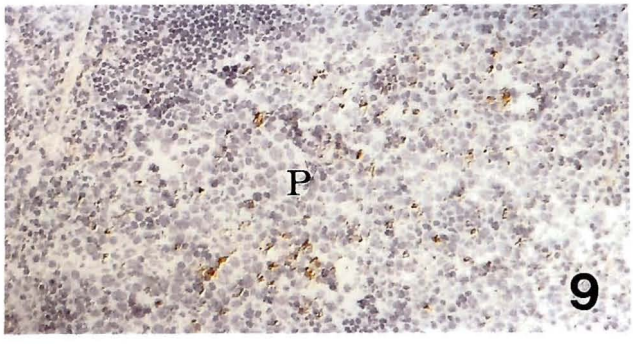
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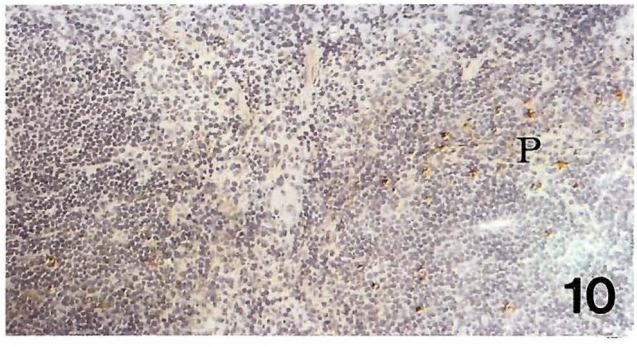
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were stained with the N418, NLDC-145 and MIDC-8 mAbs. The hamster N418 mAb reacts with an epitope of the $ß_2$ integrin family heterodimer p150/95 (CD11c), which is found primarily on DCs in the mouse (Metlay et al., 1990). Thus, it has been widely used as a selective marker of murine DCs (Hart, 1997). The rat NLDC-145 and MIDC-8 mAbs stain DCs in the splenic PALS but not the marginal zone DCs (Hart, 1997; Steinman et al., 1997). The marginal zone DCs may represent circulating blood-derived DCs more equivalent to blood DCs and thus, they are functionally immature (Hart, 1997). In contrast, DCs in the PALS (IDCs) are functionally mature. In the present study, the conversion of immature to mature DCs *in vivo* was demonstrated since splenic DCs shifted location from the marginal zone to the PALS within 6 hr after SEB administration. These results are consistent with a previous study demonstrating that lipopolysaccharide (LPS) induces the marginal zone DCs to mature and migrate into the PALS within 6 hr after administration (De Smedt et al., 1996). Similarly, systemic administration of LPS also makes epidermal DCs leave the skin and intestinal DCs enter the lymph (MacPherson et al., 1995; Roake et al., 1995). In the present study, it was also found that the temporal changes in number of DCs in the spleen after SEB administration were essentially the same regardless of the types of mAbs to DCs used in this study. In particular, after a profound increase in number of IDCs following SEB treatment, their number decreased on day 2 and appeared to be similar to those of control mice from day 4. Apoptotic cell death of DCs could be involved in the mechanism of this decrease in number of DCs observed 2 days after *in vivo* administration of SEB injection since De Smedt et al. (1998) found that DCs rapidly die by apoptosis once they have entered the splenic PALS and increasing numbers of apoptotic DCs accumulate in the T cell area after LPS-induced maturation, and suggested that DCs that have undergone maturation and migration induced by inflammatory stimuli are programmed to die unless they receive a signal from Ag-specific T cells. They proposed that the regulation of DC survival may be a mechanism aimed at controlling the initiation and the termination of the immune response (De Smedt et al., 1998).

The tissue distribution of CD40, CD80 and CD86 costimulatory molecules has been studied in normal mouse, rat and human tissues (Vandenberghe et al., 1993; Inaba et al., 1994; Reiser and Schneeberger et al., 1994; Damoiseaux et al., 1998; Vyth-Dreese et al., 1998). In control mice, CD86 staining was observed in many cells in the marginal zone, PALS and red pulp. Immunostaining of the adjacent serial sections and by double labeling revealed that most of the intensely stained $CD86^+$ cells located in the inner region of the marginal zone between the marginal zone and PALS, exhibited $Mac\text{-}3^+$ and $F4/80^-$ immunoreactivity. Only a few of the $CD86^+$ cells coincided with the $N418^+$ DCs and $MOMA\text{-}1^+$ metallophilic macrophages. The M3/84 mAb can detect Mac-3 antigen not only on mouse tissue macrophages, thioglycolate-elicited peritoneal macrophages, and some myeloid cell lines but also on DCs and endothelial cells in sections of murine spleen, thymus, lymph nodes and gut-associated lymphoid tissues (Springer, 1981; Flotte et al., 1983; Ho and Springer, 1983). The C1:A3-1 mAb can detect F4/80 antigen on mature mouse macrophages but not on mature DCs (Hume et al., 1983; Lee et al., 1985; Gordon et al., 1986). The MOMA-1 mAb recognizes the marginal metallophilic macrophages in the mouse spleen localized at the marginal sinus forming a ring around the

Fig. 11. $CD86^+$ cells in the spleen of a control mouse. Strong positive staining in the marginal zone is noted where the intensely stained cells are predominantly localized in the inner region of the marginal zone (M), constituting a single, continuous layer around the PALS (P) in control mice. In addition, the GL1 mAb also exhibits weaker reactivity in the PALS and red pulp of control mice (arrow heads). x 125

Fig. 12. $CD86^+$ cells in the spleen 6 hr after SEB treatment. Interestingly, the level of CD86 expression is increased in many cells within the PALS (P), whereas the level of CD86 expression in the marginal zone (M) is somewhat reduced and that of CD86 staining in the other areas of the spleen is not significantly altered. x 125

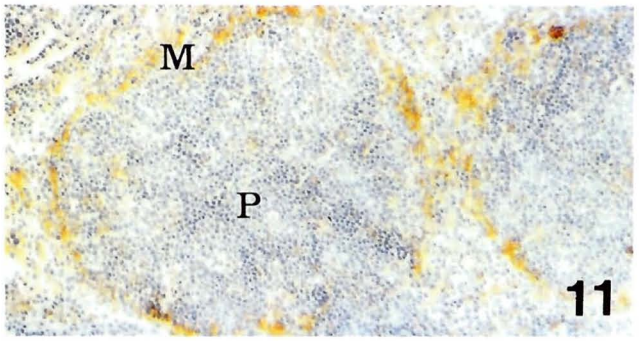
Fig. 13. Two-color immunostaining of $CD86^+$ cells (brown color) and $N418^+$ cells (blue color) in the PALS of the spleen 6 hr after SEB treatment. Double-staining reveals that most of the $N418^+$ DCs express CD86 antigen. x 250

Fig. 14. $CD86^+$ cells in the spleen 4 days after SEB treatment. The level of CD86 expression in the marginal zone (M) is increased again and the expression pattern is similar to that of control mice. x 125

Figs. 15 and 16. Immunostaining of CD86 (Fig. 15) and Mac-3 (Fig. 16) in the 3 μm-thick, adjacent serial sections of control mice. The intensely stained $CD86^+$ cells (arrow heads), which surround the PALS (P) and are distributed in the inner region of the marginal zone, coincide with the $Mac\text{-}3^+$ cells (arrow heads). x 250

Figs. 17 and 18. Immunostaining of CD86 (Fig. 17) and MOMA-1 (Fig. 18) in the 3 μm-thick, adjacent serial sections of control mice. Most of the intensely stained $CD86^+$ cells (arrow heads), located around the PALS (P) and distributed in the inner region of the marginal zone, do not coincide with the $MOMA\text{-}1^+$ cells (arrow heads). Only a few $CD86^+$ cells (arrows) coincide with the $MOMA\text{-}1^+$ cells (arrows). x 250

Figs. 19 and 20. Two-color immunostaining of $N418^+$ cells (brown color) and $CD40^+$ cells (blue color) in the spleen 6 hr after SEB treatment. Double-staining reveals that most of the $N418^+$ DCs ex press CD40 antigen in the PALS. Fig. 19, x 125; Fig. 20, x 250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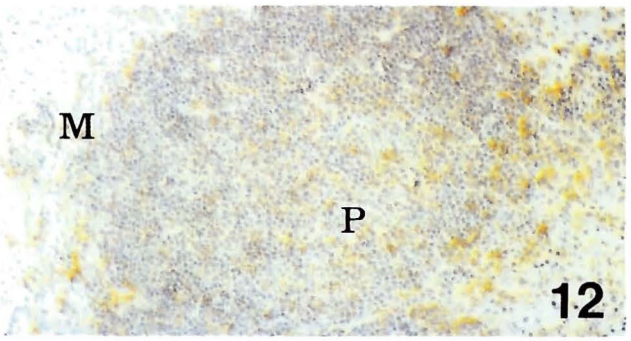
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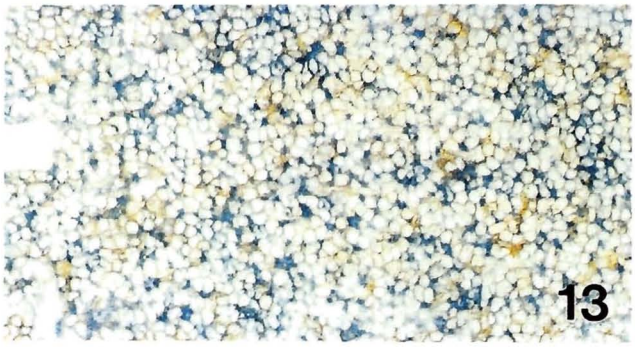
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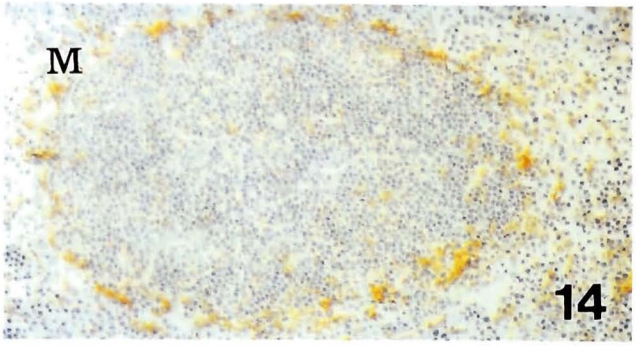
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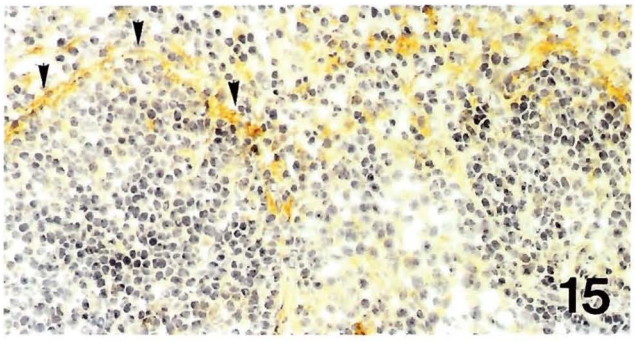
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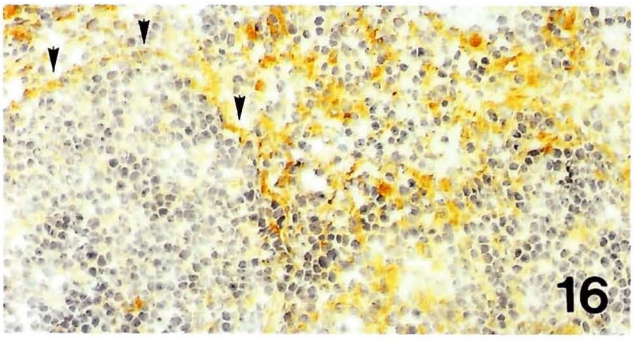
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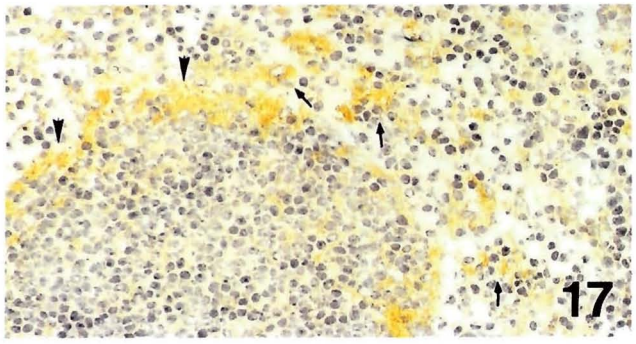
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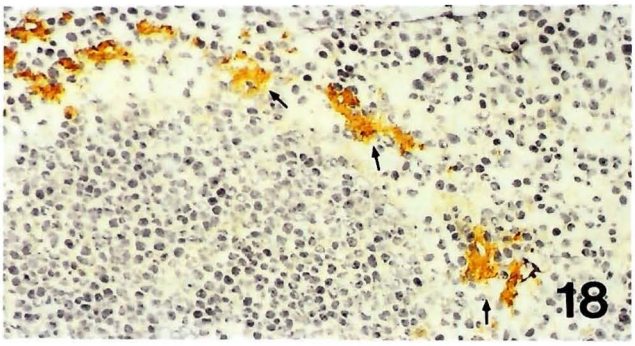
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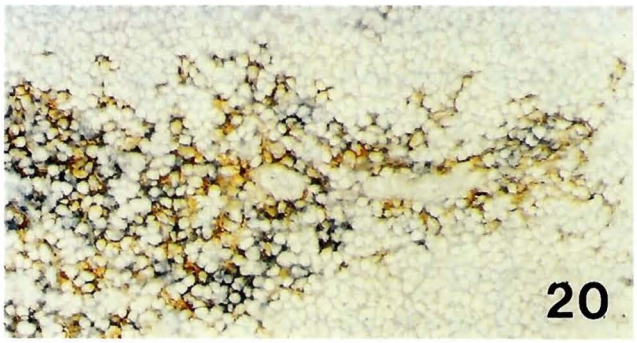
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PALS and lymphoid follicles at the inner side of the marginal zone but do not recognize the marginal zone macrophages (Kraal and Janse, 1986). Thus, most of the $CD86^+$ cells are believed to be a certain subpopulation of macrophages and DCs located in the inner region of the marginal zone. Immunostaining of the serial sections and double-staining of the same section also demonstrated that most of the $CD86^+$ cells in the PALS are IDCs and those in the red pulp are the red pulp macrophages. CD86 expression was upregulated notably in the IDCs among multiple cell types expressing CD86 antigen 6 hr after SEB injection. At the same time, CD86 expression on the cells in the marginal zone was somewhat reduced. It is difficult to speculate on the exact mechanism of this at the moment. However, this finding may suggest that the $CD86^+$ DCs in the marginal zone migrate into the PALS to become IDCs, relating with a maturation effect of DCs.

CD40 expression was detected in the IDCs and B cells in control and SEB-treated mice. In agreement with the CD86 expression pattern, CD40 staining revealed an upregulated expression only in the IDCs within 6 hr after SEB administration, although B cells did not show any significant alteration in their CD40 expression in SEB-treated mice. Interestingly, however, CD40 expression on DCs lasted longer than the CD86 and CD80 expression, since the upregulation of CD40 expression reached a peak level 24 hr after SEB injection in contrast to the CD80 and CD86 expression which reached a peak level 6 hr after SEB injection and exhibited a considerably reduced level 24 hr after SEB administration. The CD40 molecule, a member of the TNF-α receptor family, is a receptor for CD40 ligand which is expressed on T cells within a few hours of their activation (Fanslow et al., 1994). Retrograde signaling via CD40 enhances the costimulatory abilities of DCs (McLellan et al., 1996). Recently, it was shown that CD40 activation, in the presence of SAg, not only promotes $CD4^+$ and $CD8^+$ T cell clonal expansion but also delays the subsequent death of SAg-stimulated T cells *in vivo* and thus, CD40 enhances an immune response *in vivo* by increasing the number of effector T cells and delaying their subsequent deletion (Maxwell et al., 1999).

In control mice, the $CD80^+$ DCs were not detected in the splenic PALS, which is consistent with the results obtained in previous studies using murine and rat spleens (Inaba et al., 1994; Damoiseaux et al., 1998). Interestingly, many $CD80^+$ cells were found in the splenic PALS 6 hr after SEB injection. Although it is difficult to confirm the exact nature of the $CD80^+$ cells in contrast to the $CD86^+$ and $CD40^+$ cells, however, the immunohistochemical analysis of the 3 μm-thick serial sections suggested that these $CD80^+$ cells in the PALS after SEB treatment may be the $N418^+$ IDCs. Further studies may provide more insight into this matter.

Interestingly, IDCs showed longer expression of CD40 than the costimulatory molecules B7.1 and B7.2 after SEB injection in the present study. Muraille et al. (1997) demonstrated that *in vivo*, SEB induces an early and transient but profound state of unresponsiveness affecting both T cell and APC functions, and suggested that the defective APC functions of the spleen cell populations from SEB-treated mice may be related to a selective decrease in splenic DC number. In addition to the decrease in splenic DC number, our finding observed

Fig. 21. $CD40^+$ cells in the spleen of a control mouse. Few $CD40^+$ DCs are scattered within the PALS (P) and most of B cells located in the lymphoid follicles are weakly stained with the 3/23 mAb. x 125

Fig. 22. $CD40^+$ cells in the spleen 6 hr after SEB treatment. Characteristically, $CD40^+$ DCs in the PALS (P) are notably increased both in number and staining intensity. However, B cells do not exhibit any significant alteration in the CD40 expression pattern. x 125

Fig. 23. $CD40^+$ cells in the spleen 24 hr after SEB treatment. The increased CD40 expression on DCs in the PALS (P) is most conspicuous 24 hr after SEB injection. However, B cells do not exhibit any significant alteration in the CD40 expression pattern. x 125

Fig. 24. $CD40^+$ cells in the spleen 4 days after SEB treatment. The CD40 expression pattern on DCs in the PALS (P) is similar to that of control mice. However, B cells do not exhibit any significant alteration in the CD40 expression pattern. x 125

Fig. 25. $CD80^+$ cells in the spleen of a control mouse. The CD80 antigen is not detected in the PALS (P). Few immunoreactive cells are seen in the marginal zone of the splenic section. x 125

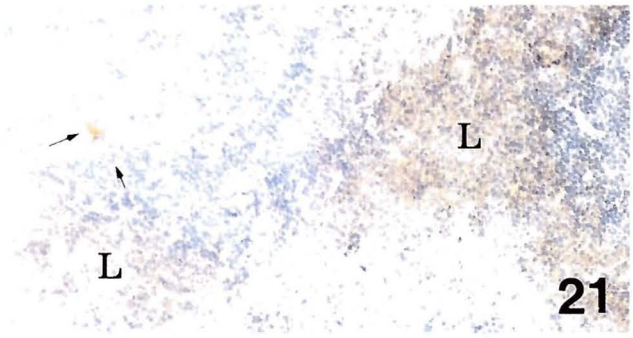
Fig. 26. $CD80^+$ cells in the spleen 2 hr after SEB treatment. The $CD80^+$ cells are slightly increased in number in the marginal zone and red pulp (arrows). x 125

Fig. 27. $CD80^+$ cells in the spleen 6 hr after SEB treatment. Remarkably, $CD80^+$ cells are increased in number, size and staining intensity in the PALS (P). x 125

Fig. 28. $CD80^+$ cells in the spleen 4 days after SEB treatment. The CD80 staining is not detected again in the PALS (P) as in control mice. x 125

Fig. 29. T cell receptor (TCR) $V\beta8^+$ T cells in the spleen of a control mouse. Many TCR $V\beta8^+$ T cells are located in the PALS (P). x 125

Fig. 30. TCR $V\beta8^+$ T cells in the spleen 2 days after SEB treatment. These T cells are greatly increased in number in the PALS (P). x 125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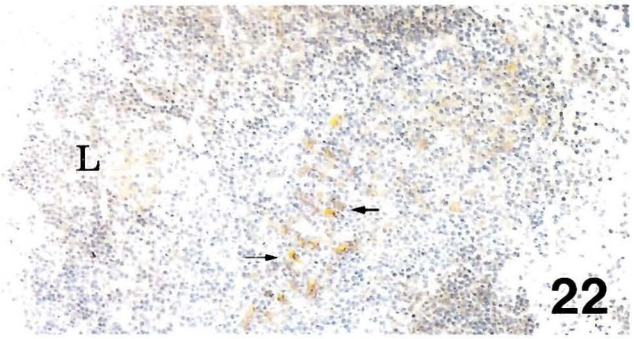
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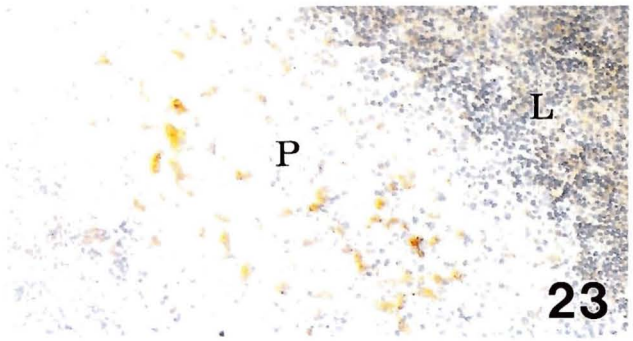
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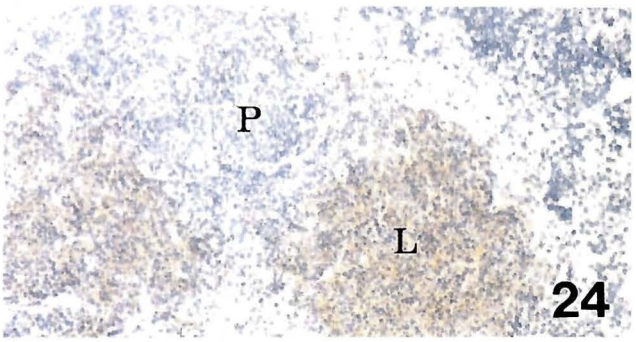
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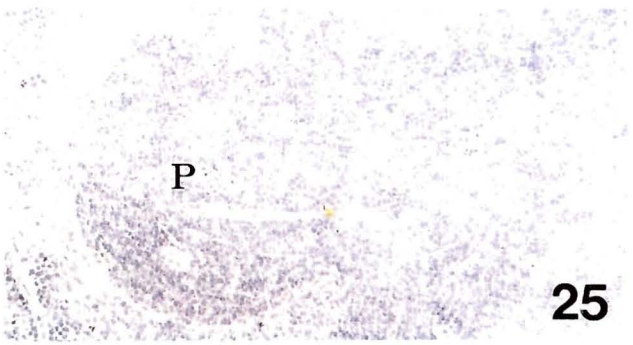
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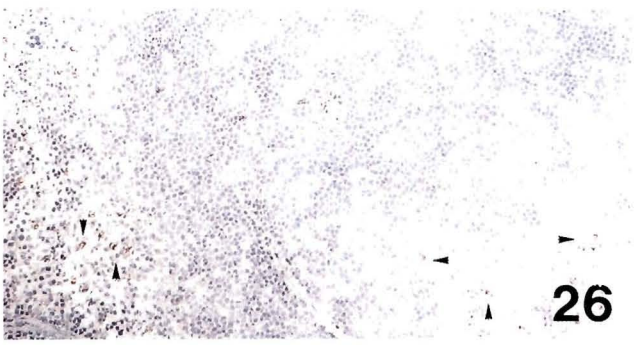
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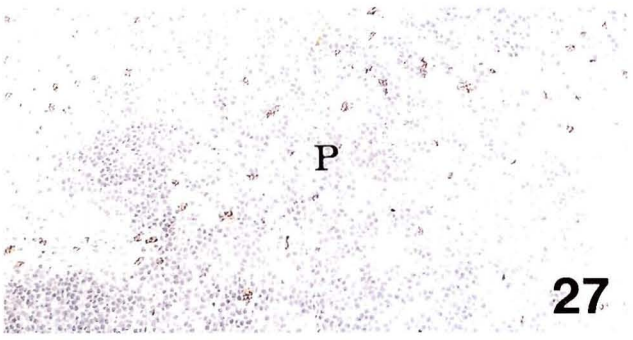
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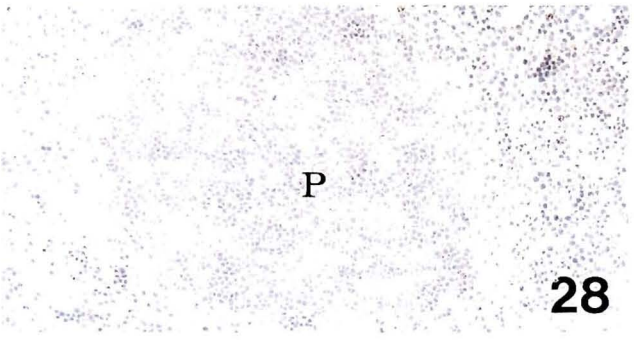
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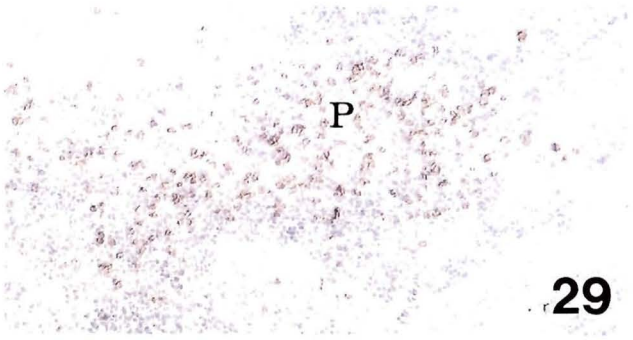
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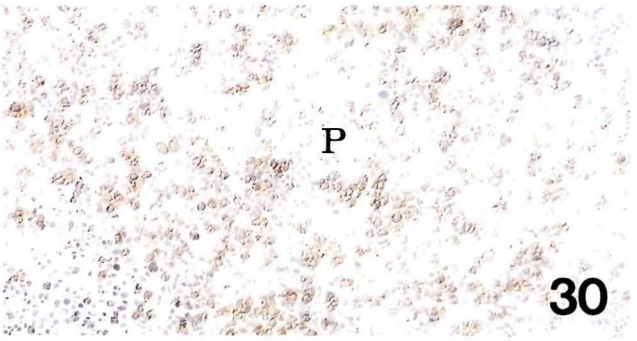
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in this study on the discrepancy between the costimulatory molecule expression pattern on DCs with time after SEB treatment may provide a new insight into the mechanism by which SEB affects APC function *in vivo*.

In recent years, much attention has been paid to the contribution of SAgs to the pathogenesis of various human diseases including septic shock and autoimmune disorders (Litton et al., 1994; Schiffenbauer et al., 1998; Muraille et al., 1999). Moreover, it was suggested that APC activation may lead to alterations in antigen processing, with the subsequent production and presentation of autoantigens and cryptic epitopes to auto-reactive or cross-reactive T cells (Schiffenbauer et al., 1998). Thus, the present study may shed some light on the understanding of the possible mechanisms relevant to the role of DCs in various SAg-related diseases since our data have clearly demonstrated the *in vivo* maturation and activation of DCs by SAg.

In conclusion, we demonstrated the *in vivo* DC response to SEB in the mouse spleen, especially a potent stimulative effect of SEB on DCs *in vivo*, a temporal distribution pattern of DCs as well as T cells including TCR Vß8$^+$ T cells, and a differential expression pattern of costimulatory molecules on the activated DCs. The results of the present study indicate that DCs are the principal type of APCs which mediate T cell activation by SAg *in vivo*, and that each costimulatory molecule may have different role in the activation of DCs by SAg. Thus, it is plausible to speculate that DCs play a critical role in the T cell clonal expansion by SAgs and other SAg-induced immune responses *in vivo*.

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