The Tissue Distribution of the B7-2 Costimulator in Mice: Abundant Expression on Dendritic Cells In Situ and During Maturation In Vitro

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Summary

B7-2 is a recently discovered, second ligand for the CTLA-4/CD28, T cell signaling system. Using the GL-1 rat monoclonal antibody (mAb), we monitored expression of B7-2 on mouse leukocytes with an emphasis on dendritic cells. By cytofluorography, little or no B7-2 was detected on most cell types isolated from spleen, thymus, peritoneal cavity, skin, marrow, and blood. However, expression of B7-2 could be upregulated in culture. In the case of epidermal and spleen dendritic cells, which become highly immunostimulatory for T cells during a short period of culture, the upregulation of B7-2 was dramatic and did not require added stimuli. Lipopolysaccharide did not upregulate B7-2 levels on dendritic cells, in contrast to macrophages and B cells. By indirect immunolabeling, the level of staining with GL-1 mAb exceeded that seen with rat mAbs to several other surface molecules including intercellular adhesion molecule 1, B7-1, CD44, and CD45, as well as new hamster mAbs to CD40, CD48, and B7-1/CD80. Of these accessory molecules, B7-2 was a major species that increased in culture, implying a key role for B7-2 in the functional maturation of dendritic cells. B7-2 was the main (>90%) CTLA-4 ligand on mouse dendritic cells. When we applied GL-1 to tissue sections of a dozen different organs, clear-cut staining with B7-2 antigen was found in many. B7-2 staining was noted on liver Kupffer cells, interstitial cells of heart and lung, and profiles in the submucosa of the esophagus. B7-2 staining was minimal in the kidney and in the nonlymphoid regions of the gut, and was not observed at all in the brain. In the tongue, only rare dendritic cells in the oral epithelium were B7-2$^+$, but reactive cells were scattered about the interstitial spaces of the muscle. In all lymphoid tissues, Gl-1 strongly stained certain distinct regions that are occupied by dendritic cells and by macrophages. For dendritic cells, these include the thymic medulla, splenic periarterial sheaths, and lymph node deep cortex; for macrophages, the B7-2–rich regions included the splenic marginal zone and lymph node subcapsular cortex. Splenic B7-2$^+$ cells were accessible to labeling with GL-1 mAb given intravenously. Dendritic cell stimulation of T cells (DNA synthesis) during the mixed leukocyte reaction was significantly (35–65%) blocked by GL-1. The block could be enhanced by adding 1G10 anti-B7-1 or by using CTLA-4 Ig, a ligand for both B7-1 and B7-2. We conclude that B7-2, like other accessory molecules, is expressed by many types of antigen-presenting cells. However, the regulation and extent of B7-2 expression seems to differ among cell types. Dendritic cells express very high levels, in several sites in vivo and after maturation into strong accessory cells in culture.

CD28 and CTLA-4 are closely related molecules (1, 2) that are expressed on most T cells. CD28 was first identified using monoclonals that were comparably mitogenic to anti-TCR mAbs, when administered together with PMA (3–5). The simultaneous triggering of CD28/CTLA-4 and CD3/TCR, without PMA, is sufficient to stimulate strong T cell proliferative responses (6–13). The costimulation provided via CD28CTLA-4 operates at the level of height-

ened IL-2 mRNA levels, either by increased transcription (14, 15) or mRNA stability (16).

Initially, the B7/BB1 molecule was identified as the ligand for CD28 and CTLA-4. It is now evident that there are at least two ligands, the original B7-1 or CD80 (2, 10, 17–21) and the newly recognized B7-2 (22–26). These molecules, when expressed by antigen-presenting B cells or by transfection into cell lines, markedly enhance the capacity of the APC to induce the production of IL-2 from T cells.

Early on it was realized that strong T cell growth factor production was especially characteristic of responses that could be elicited by antigen-presenting, dendritic cells (27, 28). Dendritic cells were found to be reactive with reagents that bind to BB1 and B7-1 (29–31, and Pope, M., M. Betjes, H. Hirmand, L. Hoffman, and R. M. Steinman, manuscript submitted for publication), and accessory function was reduced either by soluble CTLA-4 Ig fusion protein (6) or by anti-CD28 mAb (29). The possibility of B7-2 expression must now be evaluated on dendritic cells and on other primary populations.

Here we describe indirect immunolabeling evidence that B7-2 is abundant on dendritic cells, apparently more abundant than most other known surface molecules such as B7-1/CD80, intercellular adhesion molecule 1 (ICAM-1)[1]/CD54, LFA-3 equivalent/CD48, CD40, and CD44. No other leukocyte in culture expresses such high levels of B7-2 as the fully mature dendritic cell, whereas B7-1 is much less abundant. Immunocytochemical studies for B7-2 expression in many different organs indicate that this costimulator is expressed at high levels on many populations of macrophages and dendritic cells in situ.

Materials and Methods

Mice. BALB/c, (BALB/C × DBA/2) F_1, C3H (HeSlc, HeJ, and HeN) 8-wk-old female mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). (BALB/C × DBA/2)F_1 (Taconic Farms Inc., Germantown, NY), (BALB/C × C57BL/6)F_1 (The Jackson Laboratory, Bar Harbor, ME), and (C57BL/6 × DBA/2)F_1 (Trudeau Institute, Saranac Lake, NY) mice were also used.

Cell Suspensions. Cell suspensions from spleen, thymus, lymph node, blood, marrow, skin, and peritoneal cavity were studied immediately upon isolation or after a period of culture. The medium was RPMI-1640 supplemented with 5% FCS, 50 μM 2-ME, and 20 μg/ml gentamicin. In some cases, LPS was added as a stimulus to upregulate expression of B7 and other accessory molecules. Peritoneal, spleen (32), and epidermal (33) suspensions were cultured in bulk, and then the component B cells, macrophages, and dendritic cells were identified with select mAbs as indicated below and in Results. Dendritic cells also were derived from bone marrow progenitors by culture in rGM-CSF as described (34).

Cytofluorography. A panel of mAbs was selected to identify several molecular species that contribute to APC function, especially the APC-T cell interaction. The rat anti-mouse mAbs were directed to anti-B7-2 (GL-1 [22]); ICAM-1/CD54 (YN1/1.7.4; American Type Culture Collection [ATCC], Rockville, MD, #CRL1878); heat stable antigen (HSA) (M1/69.16.11, ATCC TIB125); MHC class II (B21-2, ATCC TIB229, and M5/114, ATCC TIB120); B7-1 (1G10 [35]); pgp-1/CD44 (2D2C [36]); CD45 (M1/9.3.4; ATCC TIB122); NLDC-145 (anti-dendritic cell [37]); B220 (RA3-6B2 [38]); macrophage sialoadhesin (SER-4 [39]); and 110-10, a nonreactive IgG2a isotype control. All the rat mAbs except the anti-class II reagents are IgG2as. The hamster anti-mouse mAbs were directed to MHC class II (N22, ATCC HB225); CD48 (HM48-1 [40]) CD40 (HM40-3, to be described elsewhere by Yagita, H. et al., raised by immunizing Armenian hamsters with WEHI 231 cells and screening for reactivity with CHO cells transfected with mouse CD40 cDNA); B7-1/CD80 (16-10A1 [21] and HMB7, to be described elsewhere by Azuma, M. et al. and raised by immunizing Armenian hamsters with P815 cells transfected with mouse B7 cDNA). Biotin-modified mouse anti-$I-A^d$ (mouse IgG2b) was from PharMingen (San Diego, CA), and control rat IgG2a and hamster Ig were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Primary mAbs were applied at saturating levels either as hybridoma supernatants or as pure Igs (1 μg/ml GL-1 and 1G10) to cells for 30 min in PBS-1%, FCS-0.02% azide on ice, washed, stained with FITC-mouse anti-rat Ig (FITC-MAR; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 min on ice. Normal rat Ig was added at 500 μg/ml to quench, and then populations were stained with a second mAb to identify specific cell types. The second mAbs were either biotin anti-B220 (B cells [38]; Pharmigen), biotin anti-MHC class II (Pharmingen), biotin N418 anti-CD11c (dendritic cells [41, 42]; Endogen, Inc., Boston, MA), PE-anti TCR-α/β or PE-antiRB6-8C5 (T cells and granulocytes; Pharmingen). PE-streptavidin was from Tago, Inc. (Burlingame, CA). For staining with CTLA-4 human Ig fusion protein, 10 μg/ml followed by FITC anti-human Ig was used, with CD7 human Ig or polyclonal human IgG1 as controls.

T Cell Stimulation. T cells were purified from mixtures of spleen and lymph node by passage over nylon wool plus treatment with anti-MHC class II, anti-B220, and J11d monoclonals plus rabbit complement. T cells were added at a dose of 3×10^5 (allogeneic MLR, syngeneic MLR, and Mls responses) or 2×10^5 (stimulation with 2C11 hamster anti-mouse CD3 hybridoma supernatant at 5% vol/vol) in flat-bottomed microtest wells (Nunc, Naperville IL; Corning, Corning, NY). The stimulator cells were either adherent spleen cells or LPS-stimulated B cells that had been cultured overnight before use and were treated with mitomycin-C (40 μg/ml for 30 min at 37°C). To measure DNA synthesis, [^{3}H]thymidine was added at 4 μCi/ml or 148 KBq/ml (222 GBq/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO; 183 GBq/mmol, New England Nuclear, Boston, MA) at 36–48 h (anti-CD3) and 84–96 h (MLR and Mls). To measure IL-2 release, aliquots of culture supernatant were assayed by ELISA (Endogen, Inc.).

Immunocytochemistry. Organs were removed and immediately frozen in OCT medium. Cryostat sections were cut at 10 μm and applied to 10-well multiwell slides (Carlson Scientific Inc., Peotone, IL). The sections could be stored at −20°C or stained immediately. The slides were fixed in absolute acetone for 10 min, rinsed in PBS, and stained with mAbs followed by peroxidase-conjugated $F(ab')_2$ donkey anti-rat Ig (Jackson ImmunoResearch Laboratories). Diaminobenzidine was the chromogen. In some cases, the sections were counterstained with Gill's hematoxylin (Fisher Scientific, Fair Lawn, NJ). Several experiments were first performed using lymphoid tissues (spleen and thymus primarily), and then a systematic tour of 12 different organs (see Results) was done in two mice.

[1] *Abbreviation used in this paper:* ICAM-1, intercellular adhesion molecule 1.

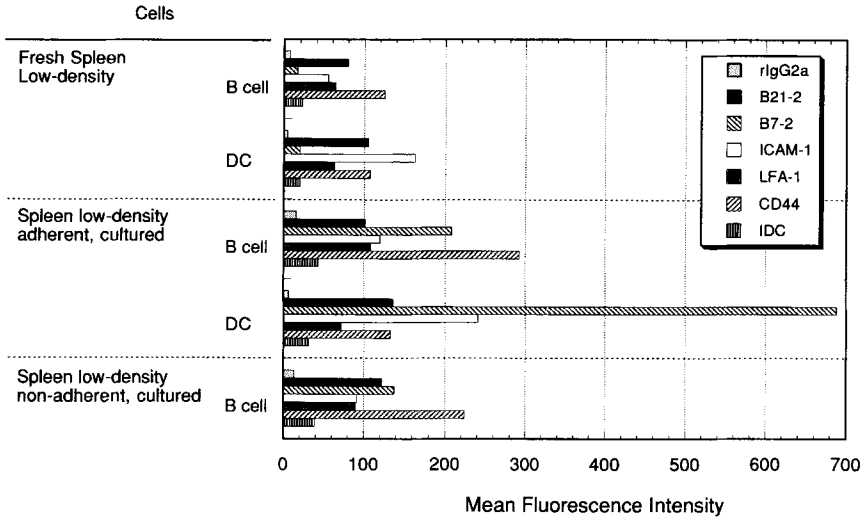

Figure 1. B7-2 antigen is upregulated on cultured B cells and dendritic cells. A fraction of spleen with a low buoyant density, which is enriched in dendritic cells and activated B cells (32, 42), was stained with a panel of rat anti-mouse mAbs as indicated and analyzed on a FACS®. The cells were stained either fresh or after overnight culture. Dendritic cells were identified with an N418 double label and were the major cell type in the plastic adherent fraction. B cells were identified with a B220 double label and were present in both adherent and nonadherent fractions.

Results

B7-2 Expression on Isolated Leukocytes: Cytofluorography. To gain information on the tissue distribution of the new B7-2 costimulator, especially on immunostimulatory dendritic cells, we utilized the mAb GL-1 that has been raised to this molecule (22). Spleen was studied first since this organ is the most frequently used for analyzing the immune functions of mouse leukocytes, including dendritic cells. To enrich for the trace subset of dendritic cells, a fraction with a low buoyant density (5–10% of spleen) was prepared (32, 42) and analyzed immediately using anti-B220 and anti-CD11c (41–43) monoclonals to selectively label B and dendritic cells, respectively. Both B and dendritic cells expressed MHC class II, as well as several adhesion molecules including ICAM-1/CD54, LFA-1/CD11a, and pgp-1/CD44; however, only trace levels of the B7-2 costimulator were detected (Fig. 1, *top*).

The spleen cells were then separated into adherent and nonadherent fractions, the former containing most of the dendritic cells but some B cells as well, and cultured overnight. For each of the cultured populations, there was some upregulation in the levels of MHC class II, ICAM-1, LFA-1, and CD44. However, the increase in B7-2 was particularly dramatic on dendritic cells (Fig. 1, *middle*) where the staining was greater than that seen with anti-MHC class II. Whereas GL-1 was an IgG2a mAb, and the anti-class II an IgG2b mAb, the secondary FITC-mouse anti-rat Ig reacted better with IgG2b than IgG2a primary mAbs as assessed with anti-CD44 mAbs of the two isotypes (data not shown). B7-1 also was upregulated during culture, but the level of staining with the isotype-matched 1G10 mAb was <10% of than that seen with GL-1 anti-B7-2 (data not shown in Fig. 1, but see Fig. 4).

Expression of B7-2 in spleen cells was compared with that of cells from several other organs. Peritoneal B cells and macrophages expressed very low levels of B7-2, but substantial levels of CD54 and CD44 (Fig. 2, *PECs*). Fresh spleen and

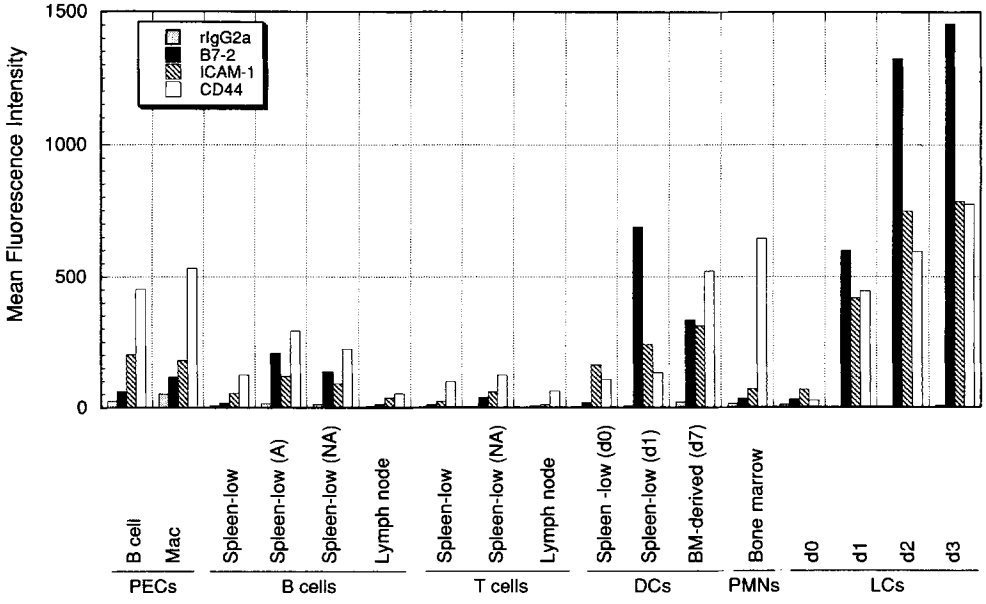

Figure 2. B7-2 antigen expression is abundant on dendritic cells. Cell suspensions were prepared from a variety of different sites as indicated on the x-axis. PECs are freshly isolated peritoneal cells in which B cells and macrophages were distinguished by light scattering. Spleen and lymph node cells were stained fresh or after culture as in Fig. 1. B cells were double stained with B220 mAb, and T cells with anti-TCR mAb. Neutrophils (*PMNs*) were identified with an RB6 mAb in fresh bone marrow. Dendritic cells were studied from three tissues: GM-CSF–stimulated marrow cultures as described (34), fresh and cultured spleen low density cells as in Fig. 1, and epidermal dendritic cells identified by double labels with anti-MHC class II in skin cultures maintained for 0, 1, 2, or 3 d (33).

Table 1. *LPS Upregulates B7-2 on Macrophages and B Lymphocytes, but not Dendritic Cells*

Cell type	Mouse strain	LPS	Level of B7-2 Expt. 1	Expt. 2
Epidermal	C3H/HeN	–	483	510
dendritic		+	ND	510
cells	C3H/HeJ	–	481	487
		+	ND	486
Peritoneal	C3H/HeN	–	136	69
macrophages		+	205	121
	C3H/HeJ	–	132	49
		+	80	48
Peritoneal	C3H/HeN	–	101	63
B cells		+	211	163
	C3H/HeJ	–	87	21
		+	101	27

Three different cell types from LPS responsive (C3H/HeN) and nonresponsive (C3H/HeJ) strains were cultured 24 h ± LPS and then analyzed for B7-2 expression on the FACS®. The mean fluoresxence of B7-2 on fresh epidermal isolates was <40 as in Fig. 2. Data are mean fluorescence intensity minus background staining with nonreactive IgG2a antibody (background was about 4 for dendritic cells and B cells, and 40 for macrophages). In experiment 1, LPS was at 30 μg/ml whereas in experiment 2, LPS was at 1 μg/ml. In experiment 1, LPS was accidentally omitted from the dendritic cell cultures (*ND*), but there was no increase in B7-2 in companion cultures with CxD2 F_1 epidermal dendritic cells.

lymph node B cells expressed little or no surface B7-2, but the low density subpopulation of B cells could upregulate B7-2 upon culture (Fig. 2, *B cell* adherent and nonadherent part of histogram). Splenic and lymph node T cells, and bone marrow neutrophils, had little or no B7-2 (Fig. 2). Dendritic cells from several different sites could express high levels of B7-2, but only upon culture (Fig. 2). This was true of dendritic cells from spleen, skin (epidermal Langerhans cells [*LCs*] and bone marrow after 7-d growth of precursors using GM-CSF as described (34).

We were concerned that sufficient LPS might have been present in the cultures to upregulate B7-2 expression. Hathcock et al. (44) have shown that LPS upregulates B7-2 expression, and we confirmed this with peritoneal B cells and macrophages (underlined, Table 1). We chose to study the LPS-responder and nonresponder strains, C3H/HeN and HeJ, for B7-2 expression before and after culture. B7-2 was markedly upregulated (increase in mean fluorescence from <40 to >400) when either C3H/HeN or C3H/HeJ dendritic cells from spleen and skin were cultured overnight (Table 1). The FACS® (Becton Dickinson & Co., Mountain View, CA) signal for B7-2 on cultured epidermal dendritic cells was 100 × background, and for macrophages, 2–5 × background (Table 1).

In additional experiments, we looked for expression of B7-2 on fresh isolates of leukocytes from blood, marrow, and thymus. Little or no B7-2 staining over the background IgG2a isotype control was observed, except for a weak level of stain on the $N418^+$ subset of thymic dendritic cells (43) (which could increase markedly in culture, data not shown). We conclude that B7-2 is expressed at very low levels on standard suspensions of freshly isolated mouse leukocytes, but that the costimulator can be upregulated in culture, particularly on dendritic cells.

Comparison of Dendritic Cell B7-2 Expression with other Surface Molecules, including B7-1. The increase of GL-1 staining on cultured dendritic cells was monitored in spleen and epidermal dendritic cells, as in Fig. 2, however, GL-1 staining was compared to a large panel of other mAbs, including recently isolated hamster mAbs to mouse CD40, CD48, and CD80/B7-1 (see Materials and Methods). The epitopes recognized by these mAbs, like all of the epitopes seen by the rat mAbs except for 2D2C anti-CD44 and NLDC-145, were affected minimally by the level of trypsin that was used to prepare the epidermal dendritic cells (data not shown). For the rat mAbs, B7-2 was the accessory molecule that was most strikingly upregulated during overnight culture (Fig. 3). CD54 or ICAM-1 also increased substantially. HSA, CD44, and CD45 were upregulated minimally, if at all (CD44 is trypsin sensitive).

For the hamster mAbs (Fig. 3, *bottom*), CD40 and B7-1 (mAbs HM40-3 and HMB7) increased in culture, but the absolute levels were relatively low. CD48, the ligand for mouse CD2 detected with mAb HM48-1 (40), was not upregulated on cultured dendritic cells. To compare the extent of staining with hamster vs rat mAbs, one can use the data with the hamster and rat anti-MHC class II reagents.

Expression of B7 molecules frequently is assessed using a fusion protein generated by a construct in which the external domains of CTLA-4 have been fused to the Ig-C domains of human Igγ (2). CTLA-4 Ig binds to B7-2 and to B7-1. CTLA-4 binding was markedly upregulated in culture (Fig. 3, *bottom left*), confirming the prior work of Larsen et al. (6). The binding of CTLA-4 Ig closely paralleled binding of the B7-2 mAb, GL-1.

To further assess the relative expression of B7-1 and B7-2, we compared staining by FACS® analyses using the 1G10 and GL-1 isotype-matched mAbs. GL-1 anti-B7-2 staining was at least 10 times greater than 1G10 anti-B7-1 staining on both splenic and epidermal dendritic cells (Fig. 4, *left*). We then did blocking studies to show that CTLA-4 Ig at 1 μg/ml totally blocked the binding of B7-2 mAb to dendritic cells, and B7-2 mAb blocked CTLA-4 binding >90% (Fig. 4, *right*).

We conclude that dendritic cells express several adhesion and costimulatory molecules, but B7-2 appears to be the most abundant and most dependent upon culture for its upregulation. Given the fact that the immunostimulatory properties of dendritic cells rise markedly in culture (33, 45, 46), the results suggest that B7 expression is a critical element of maturation. These findings also emphasized a need to assess the expression of B7-2 and B7-1 on cells in situ.

Expression of B7-2 in Tissue Sections of Several Nonlymphoid Organs. The binding of B7-2 to cryostat sections was visual-

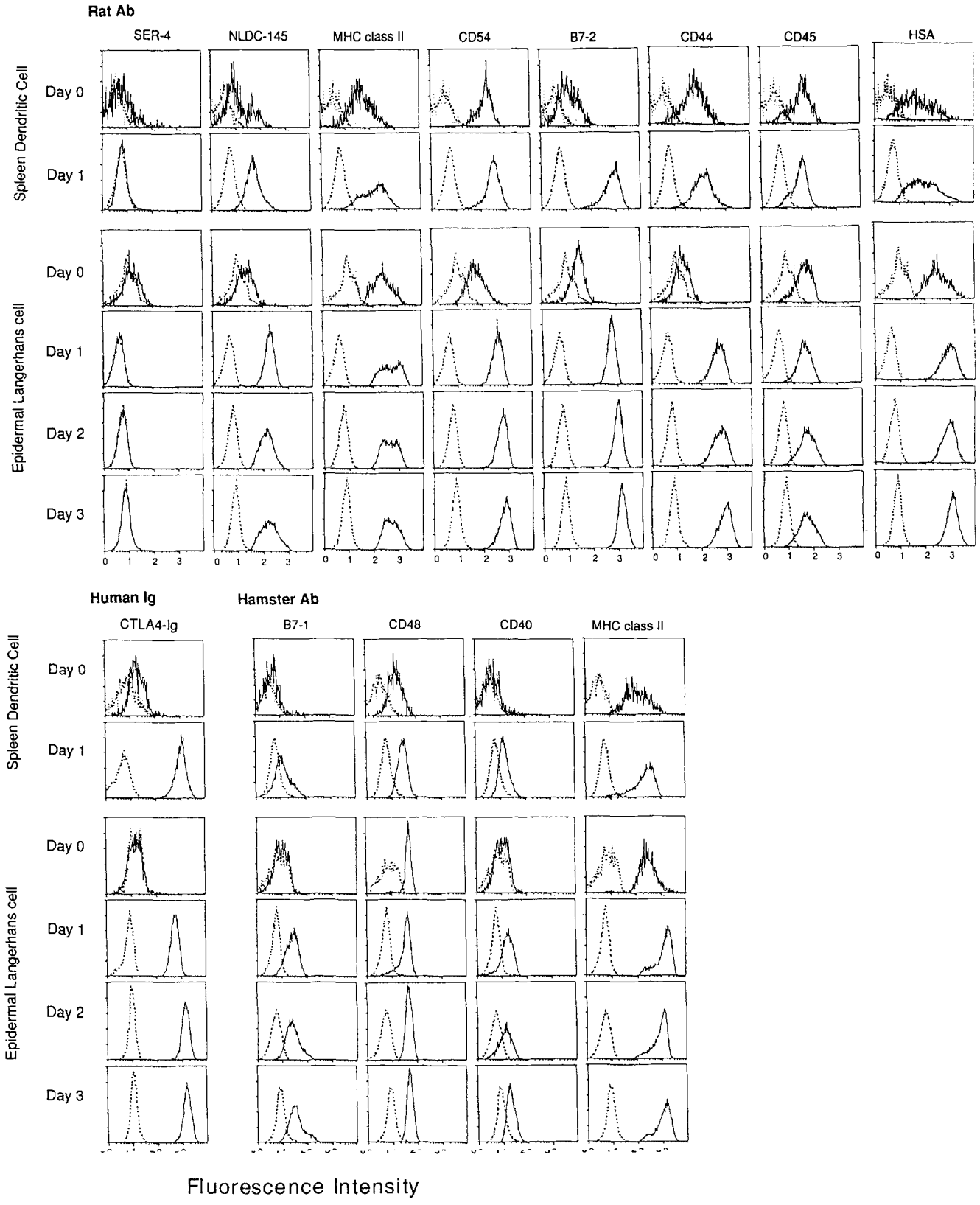

Figure 3. B7-2 on dendritic cells is abundant relative to other cell surface molecules. Spleen or epidermal dendritic cells were stained with a panel of mAbs, fresh (*day 0*), or after 1–3 d in culture. The mAbs (see Materials and Methods) were of rat, hamster, or human (*CTLA4-Ig*) origin.

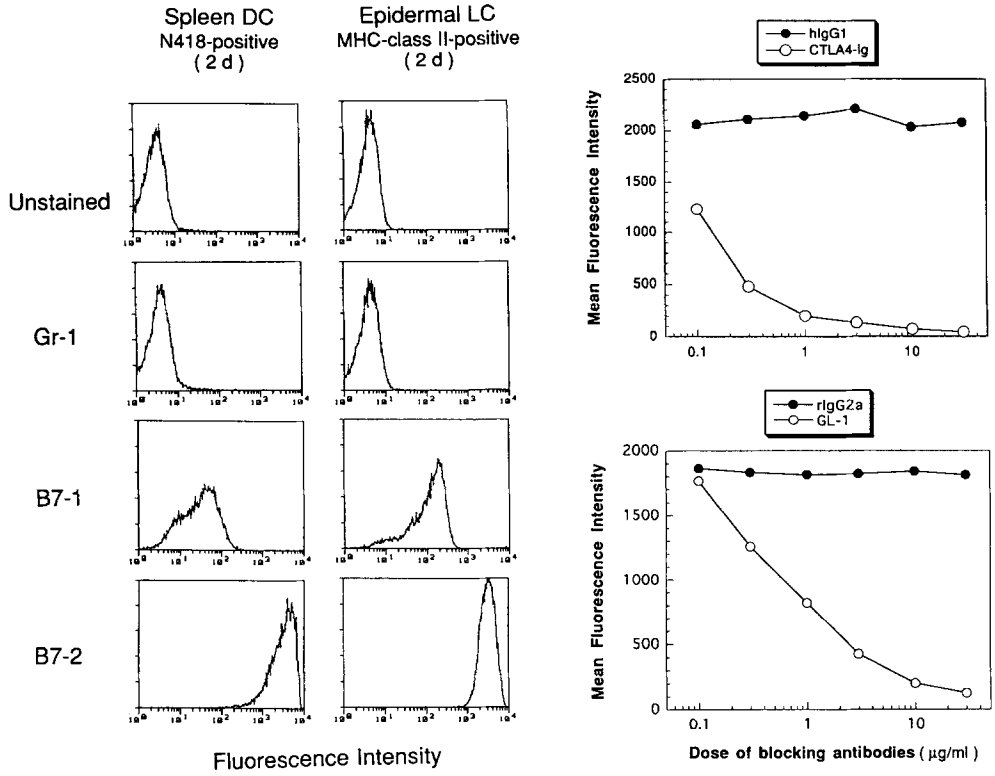

Figure 4. Relative expression of the CTLA-4 ligands B7-1 and B7-2 on dendritic cells. (*Left*) 2-d cultures of splenic and epidermal dendritic cells were stained with a nonreactive antigranulocyte antibody (*Gr*), or with 1G10 anti-B7-1 and GL-1 anti-B7-2 mAbs. (*Right*) Cultures of epidermal dendritic cells were stained with GL-1 anti-B7-2 in the presence of graded doses of blocking CTLA-4 Ig (*top*) or vice versa (*bottom*) to show that B7-2 is the major CTLA-4Ig binding species on dendritic cells.

ized with a peroxidase $F(ab')_2$ donkey anti–rat Ig. Other mAbs (see Materials and Methods) were applied to adjacent sections to localize B cells, T cells, macrophages, and dendritic cells. Shown here are data comparing staining with GL-1 anti-B7-2 with antimacrophage and anti-MHC class II mAbs. The 1G10 and 16-10A1 anti-B7-1 mAbs did not stain any of the tissues we studied, even though strong staining was observed on COS cells transfected with B7-1 cDNA and fixed similarly to the tissue sections (data not shown).

In liver, GL-1 staining paralleled that seen with antimacrophage and anti-MHC class II mAbs; however, GL-1 staining was weaker (Fig. 5, *top*). The B7-2 was expressed by sinusoidal lining profiles, presumably Kupffer cells, the mononuclear phagocytes of the liver.

In the heart, GL-1 staining again paralleled that seen with antimacrophage and anti-MHC class II antibodies (Fig. 5, *middle*). As in liver, the GL-1 staining was not as strong as the staining with mAbs to macrophages and MHC class II. For each mAb, the reactive cells were found in the interstitial spaces throughout the cardiac muscle. We did not perform double labeling studies, but prior work by Spencer and Fabre (47) has shown that in the rat, separate macrophage (MHC class II weak) and dendritic cell (MHC class II strong) populations occupy the interstitium of rat heart.

In the tongue (Fig. 5, *bottom*), anti-MHC class II stained the dendritic cells found in a suprabasal position within the stratified squamous epithelium (particularly the dorsal aspect of the tongue shown here). Class II^+ cells also were found beneath the epithelium and scattered throughout the interstitial spaces of the tongue muscle. SER-4 antimacrophage did not stain the stratified squamous epithelium, but did stain numerous profiles just below the epithelium (Fig. 5, *bottom left*) and throughout the interstitial spaces of the tongue muscle (data not shown). GL-1 stained a few epithelial dendritic cells strongly (Fig. 5, *bottom*), and weakly stained profiles beneath the epithelium and throughout the muscle, in a distribution similar to that of the antimacrophage mAb. The findings with esophagus (data not shown) were similar to those with tongue. For example, anti-MHC class II stained dendritic cells in the stratified squamous epithelium, whereas GL-1 anti-B7-2 stained but a few, and SER-4 antimacrophage none. In constrast, submucosal profiles stained strongly with antimacrophage and less strongly with anti-B7-2. Therefore, most dendritic cells in the stratified squamous epithelium have little B7-2, as is the case in epidermal cell suspensions (Figs. 2 and 3).

In several other organs (data not shown), the profiles that stained with GL-1 anti-B7-2 were found in the same regions as $SER\text{-}4^+$ macrophages, but the number and intensity of B7-2 profiles were much less. $B7\text{-}2^+$ profiles were noted in the interstitium of the lung, particularly around vessels and bronchi, in small numbers in the interstitium of the kidney and salivary glands, and at the base of the lamina propria and submucosa of the small intestine. None of the mAbs (SER-4, GL-1, and M5/114) stained profiles in the brain parenchyma, where microglia are abundant.

Expression of B7-2 in Tissue Sections of Lymphoid Organs. More abundant staining with GL-1 was noted in lymphoid relative to nonlymphoid organs, even though suspensions isolated from these organs expressed so little antigen by FACS® analyses (Fig. 2). Therefore, we suspect that the bulk of the

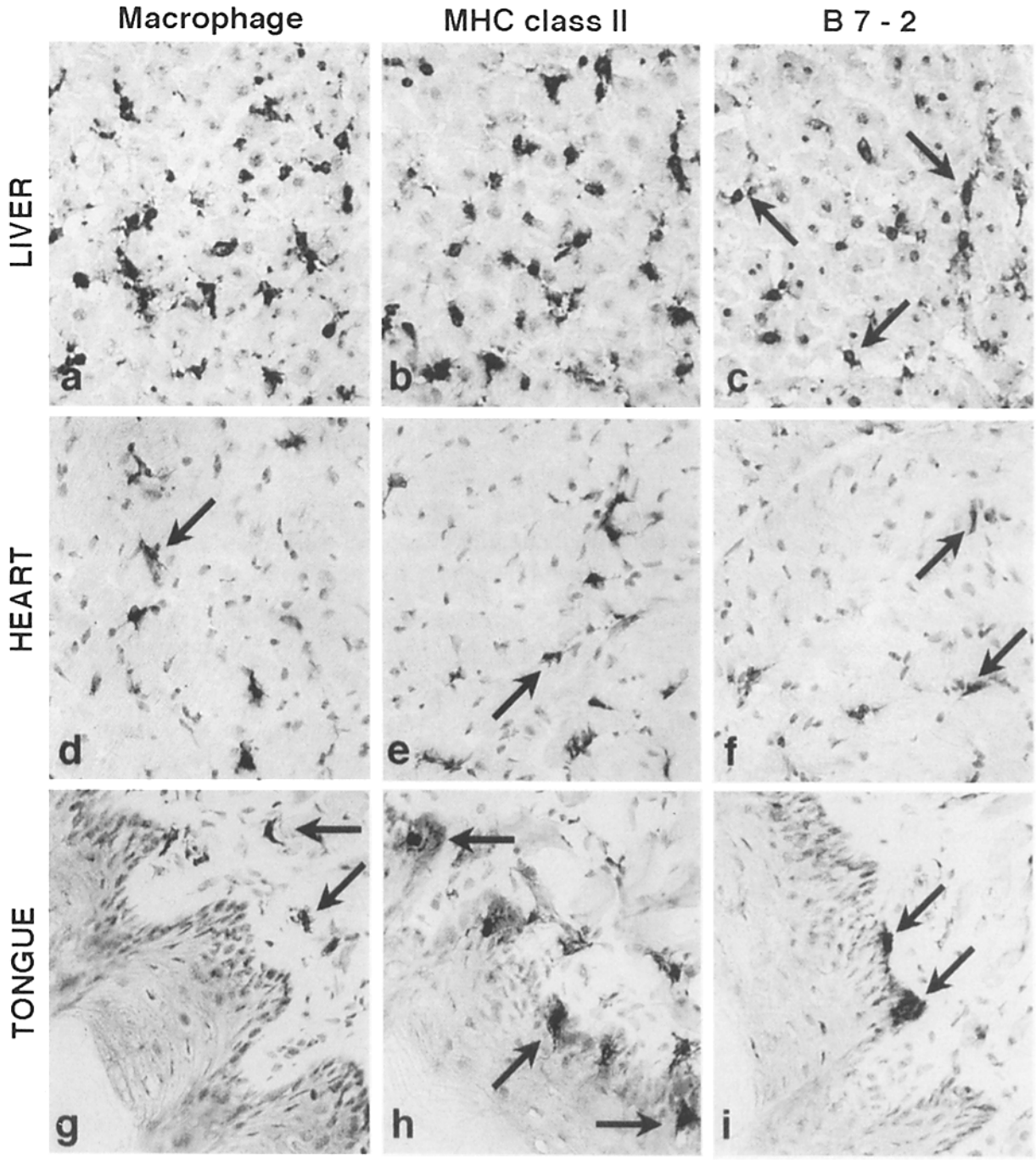

Figure 5. Distribution of B7-2 antigen in nonlymphoid tissues. Sections from nonlymphoid organs (*liver, heart,* and *tongue*) were stained with the GL-1 anti-B7-2 mAb (*c, f,* and *i*). Adjacent sections were stained with mAbs that help localize B and T cells, macrophages, and dendritic cells. Shown here on the left is anti-macrophage mAb (FA-11 anti-CD68 [56] for liver or SER-4 antisialoadhesin [39] for heart and tongue) and in the middle, M5/114 anti MHC class II. In the liver, sinusoidal lining macrophages (*arrows*) are stained with all three mAbs. In heart, interstitial cells (*arrows*) are stained throughout the myocardium. In tongue, SER-4 positive macrophages lie beneath the oral epithelium (*arrows*), whereas MHC class II positive dendritic cells are found in a suprabasal location within the epithelium (*arrows*). GL-1 stains occasional epithelial dendritic cells as shown here (*arrows*), and also stains subepithelial profiles weakly.

staining to be described in lymphoid tissues is present on stromal elements that are difficult to release into suspension (see Discussion). These B7-2 strong elements were in most cases confined to regions known to be rich in either macrophages or dendritic cells, as verified by staining adjacent sections with appropriate mAbs, e.g., SER-4, FA-11, and F4/80 for macrophages, and NLDC-145 and N418 for dendritic cells (for an example see reference 42).

In lymph node, GL-1 staining was strong in the subcapsular sinus, a region that is rich in SER-4$^+$ macrophages

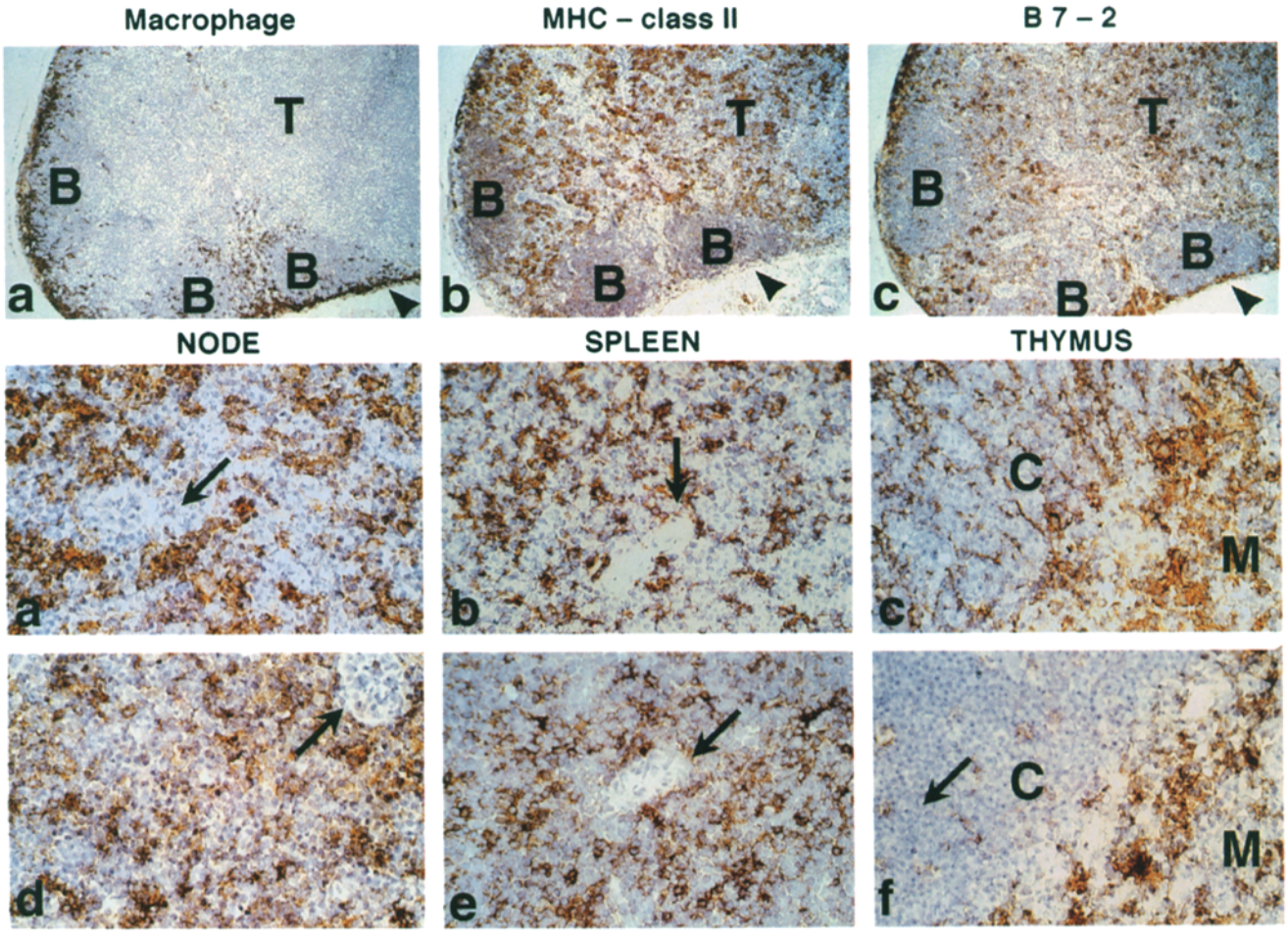

Figure 6. (*Top*) Distribution of B7-2 antigen in lymph node. Sections from lymph node are shown to contrast the distribution of stain with SER-4 antimacrophage, M5/114 MHC class II, and GL-1 anti-B7-2. Not shown is the lack of staining with 1G10 anti-B7-1. In lymph node, the B cell follicles and T cell areas are marked, whereas an arrowhead points to the macrophage-rich, subcapsular region.

Figure 7. (*Bottom*) Distribution of MHC class II and B7-2 antigens in regions of lymphoid organs that are known to be enriched in dendritic cells. (*a–c*) MHC class II stains; (*d–f*) B7-2 stains. In lymph node (*a* and *d*), the T cell area contains the postcapillary venules and many irregular profiles that stain strongly for MHC class II and B7-2. In spleen (*b* and *e*), the T cell area surrounds the central artery (*arrow*), and stains similarly to lymph node. In thymus (*c* and *f*), MHC class II and B7-2 profiles are in the medulla (*M*). The cortex (*C*) contains abundant epithelium that stains for class II but not B7-2. B7-2 weakly stains scattered cortical profiles (*arrow*), and these profiles have the same distribution as macrophages.

(compare Fig. 6, *a* and *c*, *arrowheads*). It is interesting to note that this macrophage-rich region could express relatively little MHC class II (Fig. 6 *b*, *arrowhead*). The B cell follicles stained with anti-MHC class II, but only weakly for the B7-2 antigen. Dendritic cells are known to be concentrated in the T cell–rich, deep cortical areas (37, 48–50). These areas contained many dendritic profiles that were rich in both MHC class II and B7-2, but that lacked macrophage sialoadhesin (Fig. 6, Fig. 7, *a* and *d*).

In spleen, GL-1 again stained certain macrophages strongly, particularly the marginal metallophils that surround the white pulp nodule and are identified with SER-4 in adjacent sections (data not shown). Both MHC class II and B7-2 were expressed strongly on dendritic profiles in the T cell regions that occupy the center of the white pulp (Fig. 7, *b* and *e*). These periarterial regions are a known depot of dendritic cells (37, 42, 49–51). Staining of the B cell follicles for B7-2 was weak, with the exception of scattered profiles, possibly macrophages (data not shown).

In Peyer's patch (data not shown), GL-1 showed staining beneath the dome of the epithelium, on scattered profiles in the B cell follicles, and on many dendritic profiles in the interfollicular T cell areas.

In thymus, a network of abundant B7-2 and MHC class II antigen was evident in the medulla, the known location for most thymic dendritic cells (50, 52–54) (Fig. 7, *c* and *f*). B7-2 weak profiles were scattered throughout the cortex (*arrow*, Fig. 7 *f*), in a pattern similar to that exhibited by cortical macrophages. MHC class II was abundant on thymic cortical epithelium, where B7-2 could not be detected (Fig. 7, *c* and *f*).

Since isolated leukocytes stained so weakly with GL-1, we

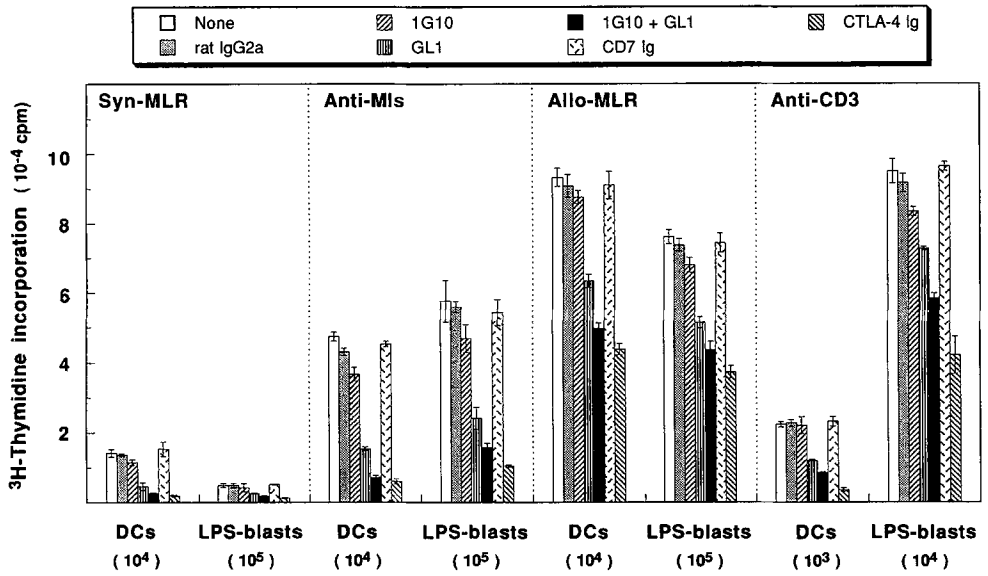

Figure 8. Blocking effects of GL-1 mAb on T cell proliferative responses to dendritic cells or LPS-induced B blasts. C57BL/6 × DBA/2 F_1 (H-2 bxd) APCs were irradiated and used to stimulate syngeneic T cells (*syn MLR*), MHC-disparate BALB/c × DBA/2 F_1 (H-2 dxd) T cells (*Allo-MLR*), Mls-1 disparate BALB/c × C57BL/6 T cells (Mls), or syngeneic T cells in the presence of 2C11 anti-CD3 mAb. Shown here are data with two doses of APCs in the absence of blocking mAb or in the presence of control rat IgG2a, GL-1 anti-B7-2, 1G10 anti-B7-1, CTLA-4 Ig, and CD7 Ig, each at 1 μg/ml. DNA synthesis was used as the measure of the T cell response.

considered the possibility that the GL-1 staining in tissue sections reflected intracellular biosynthetic pools rather than cell surface antigen. To prove that GL-1 was in fact expressed on the surface of macrophages and dendritic cells in situ, we injected 50 μg of GL-1 Ig intravenously and 50 μg of a non-reactive IgG2a mAb. 16 h later, the spleens were removed for sectioning and staining with anti-Ig. No staining was seen if control IgG2a had been injected, but after GL-1 injection, the antibody was distributed in a pattern identical to that seen with direct application of GL-1 to sections, and nearly as strong (data not shown). Therefore, the B7-2 antigen is very likely to be expressed at the cell surface to be able to selectively trap the corresponding monoclonal, GL-1.

Blocking Dendritic Cell Function at the Level of the B7 Costimulator System. T cell responses to B7-2–bearing APCs were monitored with three different types of ligands for the TCR (allogeneic and syngeneic MHC, the Mls-1^a superantigen, and anti-CD3). GL-1 anti-B7-2 significantly reduced stimulation by both dendritic cells and B blasts over a broad range of APC doses (Fig. 8). The block of dendritic cell function in four experiments ranged from 35 to 65% and was comparable using doses of 1 to 5 μg/ml. 1G10 anti-B7-1 exerted a weak blocking effect and this was additive when 1G10 and GL-1 were tested together. Likewise, CTLA-4 Ig blocked T cell stimulation better than anti-B7-2, and CTLA-4 would be expected to block both B7-1 and B7-2 costimulators (Fig. 8).

When IL-2 production was measured (data not shown), we again observed a much better block with GL-1 anti-B7-2 (≤70%) than 1G10 anti-B7-1 (≤20%), and that CTLA-4 Ig blocked better than GL-1. We conclude that B7-2 is the most prevalent CTLA-4/CD28 ligand on dendritic cells and accounts for the costimulation provided by this pathway during several in vitro immune responses.

Discussion

Prior work has shown that dendritic cells in certain sites are immunologically immature, i.e., the cells do not act as potent APCs for several T cell responses. Functional immaturity is evident in dendritic cells isolated from mouse spleen (43) and skin (33, 45, 46), and from human blood (30). Potent T cell stimulatory function rapidly develops when the dendritic cells are cultured, most likely the result of cytokines, particularly GM-CSF (45, 46) but also IL-1 (46, 55). Since GM-CSF is made by made by many cell types, e.g., the keratinocytes of the skin (45), it is possible that whenever one dissociates a tissue, sufficient GM-CSF is released to trigger the maturation of dendritic cells that are subsequently placed in culture.

Recent work (44) has described the regulated expression of B7-2, a newly recognized costimulator of IL-2 production (see Introductory section). B7-2 expression is induced on B cells by LPS or by anti-Ig, and on macrophages by LPS or by IFN-γ. Here we have focused on dendritic cells. B7-2 is difficult to detect on freshly isolated dendritic cells but when placed in culture, B7-2 increases dramatically, much more than on any other leukocyte (Figs. 1–3). The levels of B7-2 appear to be as high or even higher than most other known surface components of the dendritic cell including the HSA, pgp/CD44, leukocyte common antigen/CD45, LFA-3 analogue/CD48, ICAM-1/CD54, and even MHC class II (Fig. 3).

The factors that regulate B7-2 on dendritic cells remain to be identified. We have achieved only partial decreases in B7-2 by applying anti-GM-CSF antibodies to skin cultures but as mentioned, the requisite cytokines may have been released during the preparation of the skin cells. LSP however, does not seem to upregulate B7-2 expression as occurs with macrophages and B cells (Table 1, and 44). Likewise, dendritic cells upregulate B7-2 to a much greater extent than other APCs, when cells are placed in culture. Therefore, the control of B7-2 expression in dendritic cells seems to differ from the controls in other APCs.

B7-1 expression, as assessed by 1G10 rat and HMB7 hamster mAbs, also is upregulated on spleen and skin dendritic cells in culture, but the levels of staining are much lower than with antibody to B7-2 (Figs. 3 and 4). B7-2 accounts for

most of the CTLA-4/CD28 ligand activity on dendritic cells (Fig. 4, *right*). The block of dendritic cell function by GL-1 anti-B7-2 was not as great as with CTLA-4 Ig, and the block was increased by adding the 1G10 anti-B7-1 reagent (Fig. 8). Whereas B7-1 may contribute less than B7-2 to the function of dendritic cells in the assays we studied, there may be other functions for B7-1 including distinct effects at the level of the dendritic cell rather than the T cell.

Whereas leukocytes isolated by standard methods lack B7-2, the molecule was readily visualized in tissue sections of lymphoid organs. Again, B7-2 was easily detected, but B7-1 was not, using two anti-B7-1 mAbs. Most of the B7-2$^+$ cells in situ appear to be macrophages and T cell area dendritic cells. We presume that these B7-2$^+$ cells are difficult to release into suspension. Prior evidence exists that the dendritic cells one releases from spleen by current methods are not identical to the cells that occupy the central periarterial sheaths. Most of the dendritic cells that are released from spleen lack a granular antigen recognized by mAb M342, but this antigen is upregulated with a day in culture (50). In situ, the M342 antigen is found within dendritic cells in the periarterial T cell areas of spleen, the deep cortex of the lymph node, and the thymic medulla (50). This suggests that isolated splenic dendritic cells are not identical to the M342$^+$ "interdigitating cells" in the periarterial T areas (but may give rise to them). Instead, freshly isolated dendritic cells likely correspond to intensely N418$^+$ (CD11c$^+$ [41]) profiles within the marginal zone, at points where the periarterial sheaths of T cells enter the white pulp (41, 42). These regions represent "doors" where T cells access the central white pulp. Likewise, there is very little B7-2 reactivity in stratified squamous epithelia, the location of LC (Fig. 5, *bottom*), and isolated epidermal dendritic cells have little B7-2 (Figs. 2 and 3). Therefore, the dendritic cells which one isolates from spleen and skin express little B7-2 in situ but upregulate the costimulator during culture.

We were concerned that the B7-2 antigen in tissue sections might represent an intracellular biosynthetic pool. However, B7-2 was accessible to GL-1 mAb given intravenously.

Beginning with the studies of Linsley et al. (2), a good deal of evidence has implicated CD28/CTLA-4 ligands in the control of IL-2 production. Hathcock et al. (44) recently showed that GL-1 anti-B7-2 did not block induction of the IL-2 receptor in stimulated T cells, but greatly reduced IL-2 synthesis. For dendritic cells, the block of dendritic cell function by anti-B7-2 reagents is incomplete (Fig. 8). It has been consistently observed that mAbs to a single accessory molecule on dendritic cells fail to exert a total block of APC function (29). Part of the difficulty may relate to the high levels of expression of these ligands on dendritic cells, but most likely, each of the accessory molecules are playing significant roles in initiating IL-2 production and DNA synthesis in T cells.

Whereas the potency and other properties of different APCs (B cells, macrophages, and dendritic cells) are distinct, qualitative differences in the expression of many adhesion and costimulator molecules have yet to be encountered. For example, CD11a, CD44, CD54, CD48/58, CD80, and B7-2 all can be expressed on each of these APCs. What does appear to differ are the relative levels of the accessory molecules and the regulation of this expression. The mean fluorescence intensity of the B7-2 stain on cultured splenic dendritic cells is much higher than that on B cells, including B cells stimulated 24 h with LPS or anti-IgM, or on peritoneal macrophages, including macrophages stimulated with LPS or IFN-γ (e.g., Table 1, Fig. 1). As mentioned, the upregulation of B7-2 on cultured epidermal dendritic cells takes place within 16 h in culture, is long lasting (at least 3 d in our studies; Figs. 2 and 3); and occurs without any additions to the medium. LPS does not seem responsible, since upregulation takes place equally well in LPS-unresponsive and -responsive, C3H/HeJ and C3H/HeN strains. Therefore, many accessory molecules do not vary qualitatively amongst different APCs, but there are differences relating to content and cues. The dendritic cell is posed to upregulate many accessory molecules, and to high levels and for prolonged periods, upon minimal provocation.

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