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William H. Robinson · Michelle M. Tutt Landolfi
Jane R. Parnes

Allele-specific expression of the mouse B-cell surface protein CD72 on T cells

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Abstract CD72 is a 45000 M_r mouse B-cell surface glycoprotein involved in B-cell proliferation and differentiation. Expression of mouse CD72 is thought to be restricted to the B-cell lineage. We recently demonstrated that the monoclonal antibodies K10.6 and B9.689, previously defined as recognizing the mouse lymphocyte alloantigens Ly-19.2 and Ly-32.2, respectively, recognize specific alleles of *CD72*. Early studies using antibody-mediated cytotoxicity assays demonstrated that K10.6 and B9.689 react with B cells, several T-cell lines, and a subset of peripheral T cells. These findings led us to consider the possibility that CD72 might also be expressed on a subset of T cells. In this report we demonstrate that CD72 is constitutively expressed on a fraction of peripheral T cells isolated from strains of mice expressing the *CD72^b* allele, but not the *CD72^a* or *CD72^c* alleles. Three days after activating T cells with concanavalin A or plate-bound CD3-specific mAb, CD72 is expressed on a larger fraction of peripheral T cells as well as a fraction of thymocytes from mouse strains expressing the *CD72^b* allele. CD72 is expressed on both the CD4$^+$ and CD8$^+$ thymocyte and peripheral T-cell subsets. No CD72 expression is detected on activated thymocytes or peripheral T cells from mouse strains expressing the *CD72^a* or *CD72^c* alleles. Expression of *CD72^b* on peripheral T cells was confirmed by northern blot analysis demonstrating *CD72* mRNA expression. These results demonstrate that CD72 expression is not restricted to B lineage cells in mouse strains expressing the *CD72^b* allele; instead, a population of T lineage cells in these mice also expresses CD72.

Introduction

CD72 is a 45000 M_r glycoprotein that belongs to a family of type II integral membrane proteins that contain C-type lectin-like domains (Beavil et al. 1992; Nakayama et al. 1989; von Hoegen et al. 1990). CD72 is expressed in pre-B and mature B cells, but not in immunoglobulin-secreting plasma cells (Nakayama et al. 1989; von Hoegen et al. 1990; Sato et al. 1976; Tung et al. 1977; Dorken et al. 1990; Schwarting et al. 1990). CD72-specific monoclonal antibodies (mAb) stimulate proliferation and enhance class II major histocompatibility complex (MHC) cell surface expression in resting and activated B cells (Subbarao and Mosier 1982, 1983, 1984; Pezzutto et al. 1990; Kamal et al. 1991; Polla et al. 1988). In mouse B cells cultured in lipopolysaccharide (LPS) and IL-4, CD72-specific mAb inhibit production of IgG1 but not IgG2b or IgG3 (Yakura et al. 1988; Ogimoto et al. 1992). CD72-specific mAb stimulation of human peripheral blood B cells triggers tyrosine phosphorylation of several cellular proteins (Kamal et al. 1993). The antibody response to T-cell-dependent antigens is inhibited by CD72-specific mAb (Subbarao and Mosier 1982; Yakura et al. 1981). CD72 has been reported to bind to CD5, a cell surface glycoprotein expressed on peripheral T cells and a subpopulation of B cells (Van de Velde et al. 1991; Luo et al. 1992). The CD72-CD5 interaction may play an important role in regulating B- and T-cell function by delivering contact-dependent signals during B-cell-helper T-cell interactions.

We have demonstrated by isolation and sequencing of the cDNAs encoding mouse *CD72^a*, *CD72^b*, and *CD72^c* that the alleles of mouse *CD72* are highly polymorphic in their membrane-distal extracellular domain (Robinson et al. 1992). Using transfectants expressing these cDNAs we have shown that mAb K10.6, previously defined as recognizing the mouse lymphocyte alloantigen Ly-19.2, recognizes mouse CD72^a and CD72^b (Tada et al. 1981; Robinson et al. 1993). We also demonstrated that mAb B9.689, previously defined as recognizing the mouse lymphocyte alloantigen Ly-32.2, recognizes CD72^b (Tada et al. 1987; Robinson et al. 1993). Others, using antibody-mediated cytotoxicity assays, had demonstrated that K10.6 and B9.689 bound not only B cells, but also a small population of peripheral T cells, a large fraction of splenic concanavalin A (Con A) blasts, and a few of the T-cell lines tested (Tada et al. 1981, 1987). These data suggest that

W. H. Robinson · M. M. Tutt Landolfi · J. R. Parnes (✉)
Division of Immunology and Rheumatology, Department of Medicine, Medical School Lab Surge Building, Room P306, Stanford University School of Medicine, Stanford, CA 94305 USA

mouse CD72 expression may not be restricted to B-lineage cells as was previously thought on the basis of antibody-mediated cytotoxicity assays and northern blot analysis (Nakayama et al. 1989; Sato et al. 1976; Tung et al. 1977). In order to resolve this apparent discrepancy in the expression pattern of CD72, we examined potential differences in the tissue-specific expression of the *CD72a*, *CD72b*, and *CD72c* alleles.

Materials and methods

Mice and mAbs

Mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Hybridoma cells producing CD72.4-specific mAb K10.6 (which recognizes both CD72a and CD72b; Tada et al. 1981) and ascites fluid containing CD72.3-specific mAb 82-9.2 (which recognizes CD72c) were generously provided by U. Hammerling (Sloan-Kettering Cancer Center, New York, NY). Ascites fluid containing CD72.2-specific mAb B9.689 [Tada et al. 1987; which recognizes CD72b (Robinson et al. 1993)] was a gift from N. Tada (Tokai University School of Medicine, Japan). Hybridoma cells producing CD72.1-specific mAb 10.1.D2 and CD3-specific mAb yCD3.1 (Portoles et al. 1989) were gifts of D. E. Moiser (Medical Biology Institute, La Jolla, CA) and C. A. Janeway, Jr. (Yale University School of Medicine, New Haven, CT), respectively. Hybridoma cells producing mAb, 2–169 [which recognizes the CD72.2 or a similar epitope unique to CD72b (W. H. Robinson, L. Reininger, and J. R. Parnes, unpublished results)] were a generous gift of L. Reininger (INSERM U 291, Montpellier, France). Hybridoma cells producing mAb 2.4G2 (specific for the mouse Fc-γ receptor and used for blocking Fc-γ receptor binding of specific mAb), mAb OKT 4 (which recognizes human CD4 and was used as an IgG2b isotype-matched control for K10.6 and 10.1.D2), and mAb OKT 3 (which recognizes human CD3 and was used as an IgG2a isotype-matched control for 82-9.2) were obtained from the American Type Culture Collection (Rockville, MD).

Preparation and culture of lymphocytes

Single-cell suspensions were prepared from inguinal, axillary, and mesenteric lymph nodes. Immunoglobulin(Ig)-negative peripheral T cells were then isolated by two rounds of panning on plates coated with affinity-purified goat antiserum specific for mouse IgG, IgM, and IgA (Cappel, West Chester, PA). The isolated lymphocyte population was greater than 95% pure for T cells by FACS analysis using CD3-, B220-, and Ig-specific mAb (PharMingen, San Diego, CA). The isolated T cells were cultured at 10^6/ml in a 24-well plate (Costar, Cambridge, MA) in RPMI 1640 with 10% fetal calf serum (as previously described (Robinson et al. 1993) in the presence of 3 μg/ml Con A; (Sigma, St. Louis, MO) and supplemented with 10 units/ml IL-2 (a generous gift of Cetus Corp., Emeryville, CA) after 24 h. Bulk single-cell suspensions of thymocytes were cultured in similar conditions except that 10 units/ml IL-2 was added immediately.

Immunofluorescence analysis

Resting and activated lymph node T cells (isolated as described above) and thymocytes were incubated with culture supernatant containing the mAb 2.4G2 for 30 min to block Fc-γ receptor binding of the specific mAb. One million cells were then stained for 45 min with fluorescein isothiocyanate (FITC)-conjugated CD3-, CD4-, or CD8-specific mAb (PharMingen, San Diego, CA), followed by 0.5 μg of biotinylated protein A-purified CD72-specific mAb or Ig isotype-matched control mAb, followed by phycoerythrin (PE)-conjugated streptavidin (Caltag, South San Francisco, CA). Immunofluorescence was determined by analysis on a modified FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Northern blot analysis

Total RNA was isolated using RNAzol (Cinna/Biotecx, Friendswood, TX) according to the manufacturer's instructions. Peripheral T cells were isolated and cultured with Con A and IL-2 as described above. Before total RNA preparation from activated peripheral T-cell populations, dead cells were removed using Lympholyte-M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) according to the manufacturer's instructions. Total thymic and splenic RNA was isolated from a DBA/2 mouse. Northern blotting and hybridization in formamide with a ^{32}P-labeled *CD72a* probe were performed as previously described (Nakayama et al. 1989; Zamoyska et al. 1985). Comigrated duplicate RNA samples were stained with ethidium bromide to demonstrate the quantity of 18S and 28S rRNA loaded for each sample.

Results

Allele-specific CD72 expression on resting peripheral T cells

Purified resting lymph node T cells from mouse strains expressing the *CD72a* allele (DBA/2, CBA, C58, C57L and SWR), the *CD72b* allele [C57BL/6 (B6) and Balb/c], and the *CD72c* allele (SJL and AKR) were screened for CD72 expression using immunofluorescence staining. Figure 1 shows FACS analysis of T cells isolated from strains of mice representing each *CD72* allele using the appropriate CD72-specific mAb and Ig isotype-matched control mAb. A population of T cells isolated from B6 mice, representing mouse strains expressing the *CD72b* allele, stained positively with the CD72-specific mAb K10.6 as compared with the Ig isotype-matched control mAb OKT 4 (Fig. 1C,

Table 1 Allele-specific expression of CD72 on mouse T cells[a]

		CD72 expression			
		Lymph node T cells		Thymocytes	
Mouse strains tested	Allele expressed	Constitutive	Day 3-activated	Constitutive	Day 3-activated
DBA/2, C57L, CBA, C58, SWR	CD72a	–	–	–	–
C57BL/6, Balb/c	CD72b	+	++	–	+
AKR, SJL	CD72c	–	–	–	?[b]

[a] FACS analysis was used to determine the level of CD72 expression (–, +, ++) on resting and day 3 con A-activated lymph node T cells or thymocytes

[b] Because the CD72c-specific mAb 82-9.2 non-specifically stained activated T cells (not shown) no result was obtained for day 3-activated thymocytes from mouse strains expressing CD72c. The (–) resulted reported for activated lymph node T cells from mouse strains possessing CD72c is based on the lack of detectable CD72 mRNA in northern blot analysis (Fig. 4, lanes 5 and 6)

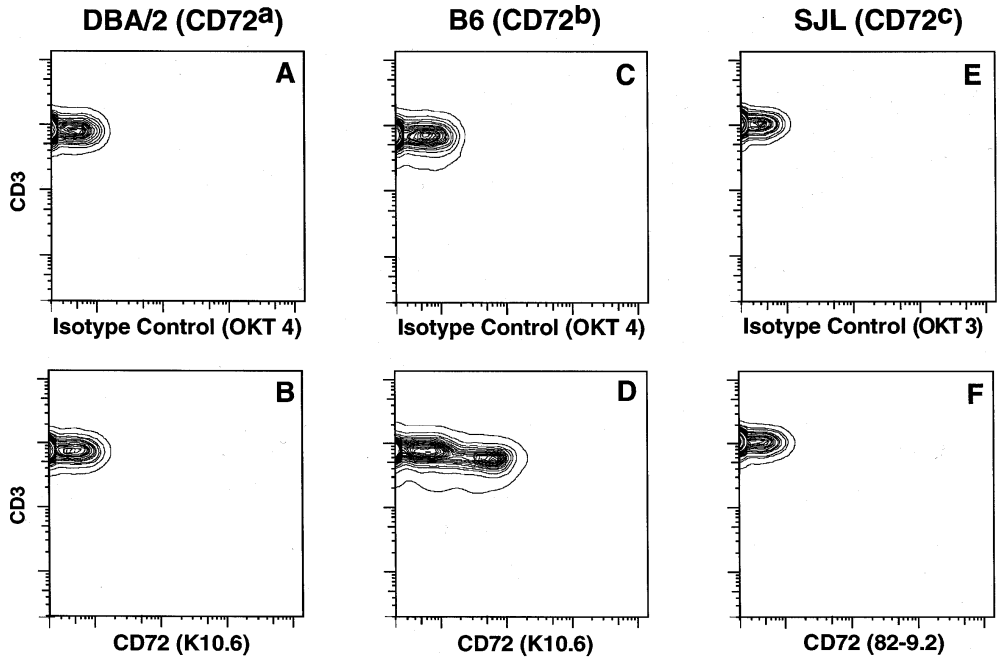
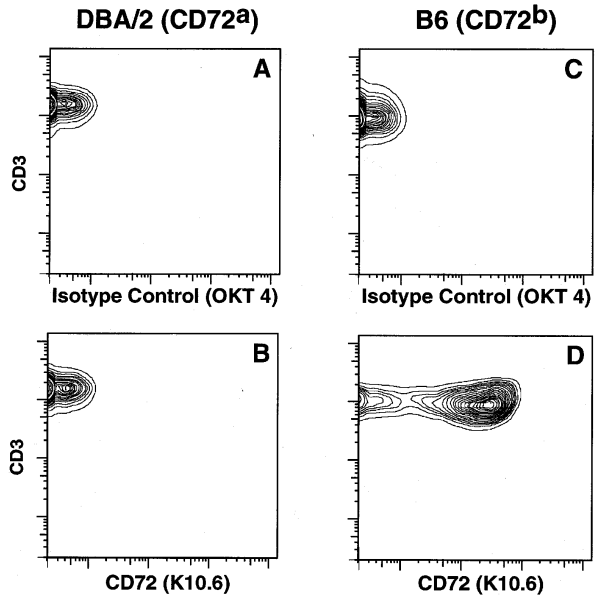


Fig. 1A–F FACS analysis demonstrating expression of CD72b, but not CD72a or CD72c, on purified resting lymph node T cells. Vertical columns of plots represent immunofluorescence staining of T cells isolated from a DBA/2 mouse representing mouse strains expressing CD72a (**A** and **B**), a B6 mouse representing mouse strains expressing CD72b (**C** and **D**), and an SJL mouse representing mouse strains expressing CD72c (**E** and **F**). The isolated resting peripheral T cells were stained with FITC-conjugated CD3-specific mAb and either biotinylated CD72-specific mAb [K10.6 (**B** and **D**) or 82-9.2 (**F**)] or the appropriate biotinylated Ig isotype-matched control mAb [OKT 4 (**A** and **C**) or OKT 3 (**E**)], followed by PE-conjugated streptavidin. Samples were analyzed by FACS analysis and data are displayed as contour plots of correlated PE and FITC fluorescence on four-decade log scales

Fig. 2A–D FACS analysis demonstrating expression of CD72b, but not CD72a, on purified lymph node T cells 3 days after activation. Purified lymph node T cells were cultured with 3 μg/ml Con A and supplemented with 10 units/ml IL-2 after 1 day. Vertical columns of plots represent immunofluorescence staining of day 3-activated T cells isolated from a DBA/2 mouse representing mouse strains expressing CD72a (**A** and **B**) and a B6 mouse representing mouse strains expressing CD72b (**C** and **D**). Day 3-activated peripheral T cells were stained with FITC-conjugated CD3-specific mAb and either biotinylated CD72-specific mAb K10.6 (**B** and **D**) or biotinylated Ig isotype-matched control mAb OKT 4 (**A** and **C**), followed by PE-conjugated streptavidin. Samples were analyzed by FACS analysis and data are displayed as contour plots of correlated PE and FITC fluorescence on four-decade log scales



D). This subset includes $CD4^+$ and $CD8^+$ T cells (data not shown). In contrast, resting peripheral T cells from a DBA/2 mouse, representing mouse strains expressing the *CD72a* allele, were not stained by the CD72-specific mAb K10.6 as compared with the Ig isotype-matched control mAb OKT 4 (Fig. 1A, B). T cells isolated from an SJL mouse, representing mouse strains expressing the *CD72c* allele, were also not stained by CD72-specific mAb 82-9.2 as compared with the Ig isotype-matched control mAb OKT 3 (Fig. 1E, F). Similar results were obtained using splenic lymphocytes as well as the CD72a-specific mAb 10.1.D2 and CD72b-specific mAb 2-169 (data not shown). Thus, as summarized in Table 1, a population of resting peripheral T cells isolated from mouse strains expressing the *CD72b* allele, but not the *CD72a* or *CD72c* alleles, expressed CD72. Similar results were obtained in all strains of mice examined expressing a particular allele of mouse *CD72* (Table 1).

Increased allele-specific CD72 expression on activated T cells

To determine whether T-cell activation causes increased CD72b expression and/or induces CD72a or CD72c expression, we used FACS analysis to examine activated peripheral T cells. Purified T cells were incubated with 3 μg/ml

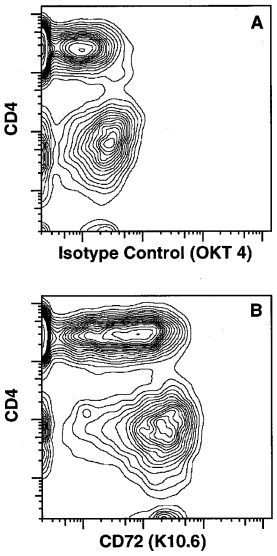


Fig. 3 A, B FACS analysis demonstrating expression of CD72^b on day 3-activated CD4$^+$ lymph node T cells. Purified lymph node T cells from a Balb/c mouse were cultured with 3 μg/ml Con A and supplemented with 10 units/ml IL-2 after 1 day. T cells 3 days after activation were stained with FITC-conjugated CD4-specific mAb and **A** either biotinylated Ig isotype-matched control mAb OKT 4 or **B** biotinylated CD72-specific mAb K10.6, followed by PE-conjugated streptavidin. Samples were analyzed by FACS analysis and data are displayed as contour plots of correlated PE and FITC fluorescence on four-decade log scales

Con A and supplemented with 10 units/ml IL-2 after 24 h. T cells were examined using FACS analysis 1, 2, 3, 4, and 5 days following activation to determine the level of CD72 expression. A significant increase in the level of CD72 expression on T cells isolated from mouse strains expressing CD72^b was detected 3 days after activation, with $>80\%$ of the T cells expressing CD72 (Fig. 2 C, D; Table 1). T cells from mouse strains expressing CD72^b analyzed 4 and 5 days after activation continued to express high levels of CD72, although the level and percentage of cells expressing CD72 gradually decreased (data not shown). No CD72 expression was observed at any of these points on activated peripheral T cells obtained from the five mouse strains tested that express CD72^a (Fig. 2 A, B; Table 1).

Activated peripheral T cells and thymocytes from mouse strains expressing CD72^c also appeared to express CD72, on the basis of positive immunofluorescence staining with the CD72^c-specific mAb 82-9.2. However, similar levels of staining with this mAb were also observed using activated T cells from mouse strains expressing CD72^a and CD72^b, even after blocking with the Ig isotype-matched control mAb OKT 3 (data not shown). Since the mAb 82-9.2 specifically recognizes the CD72^c molecule as previously demonstrated by its staining of *CD72^c* cDNA transfectants, but not *CD72^a* or *CD72^b* cDNA transfectants, this mAb should not recognize any gene product present in mouse strains that are homozygous for the *CD72^a* or *CD72^b* alleles (Robinson et al. 1992). Thus we conclude that the 82-9.2 mAb staining of activated T cells is non-specific. *CD72* mRNA expression was not detected in activated peripheral T cells isolated from an SJL mouse which expresses CD72^c (see below). This result supports the conclusion that the observed positive immunofluorescence staining of activated T cells by the mAb 82-9.2 is non-specific and indicates that activated T cells from mouse strains expressing CD72^c do not express CD72.

Similar to resting T cells, both CD4$^+$ and CD8$^+$ activated peripheral T cells from mouse strains expressing CD72^b expressed CD72 (Fig. 3). The activated T cells that stained positively with CD72-specific reagents also expressed T cell-specific markers including TcR β chain, CD3, and CD4 or CD8 (Figs. 2, 3; data not shown). The cells did not

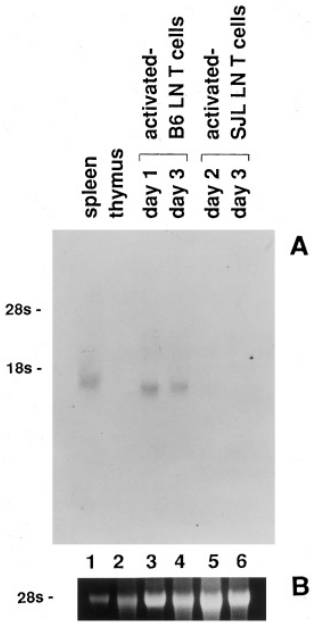
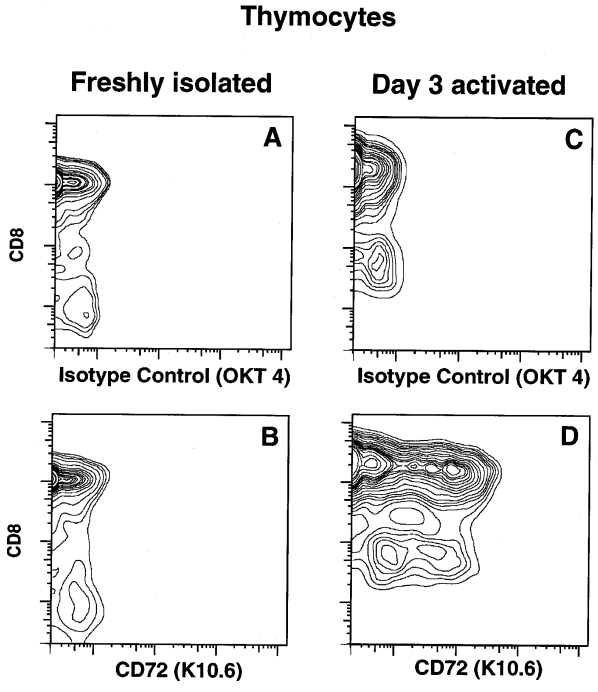


Fig. 4 A Northern blot analysis demonstrating *CD72^b* mRNA expression in peripheral T cells. Purified lymph node T cells were cultured with 3 μg/ml Con A and supplemented with 10 units/ml IL-2 after 1 day. Total RNA isolated from spleen (lane 1), thymus (lane 2), purified B6 lymph node T cells 1 day (lane 3) and 3 days (lane 4) following activation, and purified SJL LN T cells 2 days (lane 5) and 3 days (lane 6) following activation were electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled *CD72^a* cDNA. The migration position of 18S and 28S rRNA (based on ethidium bromide staining of comigrated samples) are shown in the *left* margin. **B** The relatve amounts of 28S rRNA present in comigrated duplicate samples stained with ethidium bromide

Fig. 5 A–D FACS analysis demonstrating expression of CD72^b on day 3-activated but not resting thymocytes. Vertical columns of plots represent immunofluorescence staining of freshly isolated thymocytes (**A** and **B**) and thymocytes 3 days following activation with Con A (**C** and **D**). Thymocytes were stained with FITC-conjugated CD8-specific mAb and either biotinylated CD72-specific mAb K10.6 (**B** and **D**) or the biotinylated Ig isotype-matched control mAb OKT 4 (**A** and **C**), followed by PE-conjugated streptavidin. Samples were analyzed by FACS analysis and data are displayed as contour plots of correlated PE and FITC fluorescence on four-decade log scales



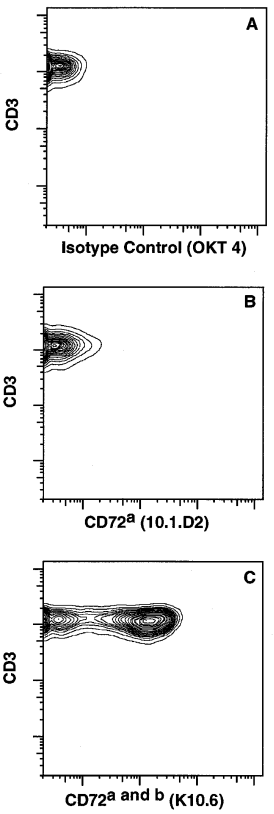


Fig. 6 FACS analysis demonstrating expression of CD72^b but not CD72^a on activated peripheral T cells isolated from (CD72^a × CD72^b)F$_1$ mice. Purified lymph node T cells from B6D2F1 [(C57BL/6 × DBA/2)F$_1$] mice were cultured with 3 μg/mg Con A and supplemented with 10 units/ml IL-2 after 1 day. Three days following activation the T cells were stained with FITC-conjugated CD3-specific mAb and either **A** biotinylated Ig isotype matched control mAb OKT 4, **B** biotinylated CD72^a-specific mAb 10.1.D2, or **C** biotinylated CD72$^{a\ and\ b}$-specific mAb K10.6, followed by PE-conjugated streptavidin. Samples were analyzed by FACS analysis and data are displayed as contour plots of correlated PE and FITC fluorescence on four-decade log scales

stain with mAb specific for B cell markers including IgM, B220, CD22, and CD23 (data not shown). Similar increases in CD72^b expression were observed following activation with immobilized CD3-specific mAb yCD3.1 (data not shown).

CD72^b mRNA is expressed by activated peripheral T cells

To demonstrate that anti-CD72-specific mAb staining of peripheral T cells represents expression of CD72 synthesized by those T cells, we performed northern blot analysis on total mRNA from thymus, spleen, and activated peripheral T-cell populations (Fig. 4A). As described in Materials and methods, peripheral T cells were isolated by panning out B cells using Ig-specific antisera and were >95% pure for T cells by FACS analysis. As previously reported (Nakayama et al. 1989), *CD72* mRNA was detected in total splenic RNA but not in total thymic RNA (Fig. 4A, lanes 1 and 2). Day 1 and 3 Con A-activated peripheral T cells from B6 mice expressed *CD72^b* mRNA (Fig. 4A, lanes 3 and 4), supporting the conclusion that the staining of peripheral T cells with the CD72-specific mAb K10.6 represents CD72 expression. No *CD72^c* mRNA expression was detected in day 2- and 3-activated peripheral T cells isolated from SJL mice (Fig. 4A, lanes 5 and 6), indicating that no significant B-cell contamination was present in the purified peripheral T-cell populations. Similar amounts of total RNA were present in each of the lanes representing activated B6 and SJL LN T cells, based on the similar amounts of 28S rRNA observed by ethidium bromide staining of comigrated duplicate samples (Fig. 4B).

Activation-induced CD72^b expression on thymocytes

Immunofluorescence staining analysis was performed to determine whether CD72 is expressed on thymocytes. No CD72 expression was detected on resting or activated thymocytes isolated from mouse strains expressing either the *CD72^a* or *CD72^c* alleles (Table 1). In addition, no CD72 expression was detected on resting thymocytes isolated from a B6 mouse which expresses the *CD72^b* allele (Fig. 5A, B). Nevertheless, a significant fraction of day 3 Con A-activated thymocytes from a B6 mouse stained positively for CD72 expression (Fig. 5C, D; Table 1).

CD72^b but not CD72^a is expressed by activated peripheral T cells from (CD72^a × CD72^b)F$_1$ mice

In order to examine whether expression of CD72 on T cells is dominant, T cells isolated from (C57BL/6 × DBA/2)F$_1$ (B6D2F$_1$) mice, which express both CD72^a and CD72^b, were analyzed for cell surface CD72 expression. CD72^b but not CD72^a was expressed on both resting and activated peripheral T cells from B6D2F$_1$ mice, indicating that expression of CD72^b on T cells is dominant (Fig. 6; data not shown).

Discussion

Using transfectants expressing the mouse *CD72^a*, *CD72^b*, and *CD72^c* cDNA, we have already demonstrated that the mAbs K10.6 and B9.689, originally described as recognizing the mouse lymphocyte differentiation antigens Ly-19.2 and Ly-32.2, in fact recognize *CD72* alleles (Robinson et al. 1993). This result provided us with two mAbs that recognize CD72^b, for which there was previously no identified specific mAb. In light of reports based on antibody-mediated cytotoxicity assays that the K10.6 and B9.689 mAbs exhibit reactivity with a small population of resting peripheral T cells, a larger population of activated peripheral T cells, and several T cell lines (Tada et al. 1981, Tada et al. 1987), we wanted to examine whether CD72 is expressed on a population of T cells and whether such expression might differ among the *CD72* alleles. Our results indicate that CD72 expression is restricted to the B-cell lineage for CD72^a and CD72^c, but that a population of peripheral T cells and activated thymocytes from mouse strains expressing the *CD72^b* allele express CD72. These data reconcile the previously observed differences in the tissue distribution pattern of CD72 [expressed only on immature and mature B cells (Nakayama et al. 1989; von Hoegen et al. 1990; Sato et al. 1976; Tung et al. 1977; Dorken et al. 1990; Schwarting et al. 1990)] as compared with Ly-19.2 and Ly-32.2 [expressed on B cells and a subset of T cells (Tada et al. 1981, 1987)]. Although K10.6 recognizes both CD72^a and CD72^b, Tada and coworkers (1981) only reported studies of tissues from mice expressing CD72^b, and not CD72^a, in analyzing the tissue distribution of Ly-19.2 expression. It was not realized that in mouse strains expressing CD72^a, expression of Ly-19.2 is restricted to the CD72-expressing B-cell subset. On the

basis of our results, the previous discrepancies in the tissue distribution pattern of Ly-19 and Ly-32 as compared with CD72 were found not to be discrepancies but instead to reflecte the expression of CD72^b on a population of peripheral T cells.

In B6D2F$_1$ mice, which possess both the *CD72^a* and *CD72^b* genes, we found that resting and activated lymph node T cells and thymocytes express CD72^b but not CD72^a. This indicates that expression of CD72^b in T cells is dominant. A possible explanation for the allelic differences in CD72 expression may be polymorphism in the promoter regions of the allelic forms of the gene, resulting in transcription of *CD72^b*, but not *CD72^a* or *CD72^c*, in T lineage lymphocytes. In support of this possibility, DNA sequence analysis of the 500 base pair region upstream of the start site in the allelic forms of the *CD72* gene revealed 11 base pair changes in *CD72^b* when compared with the consensus sequence of *CD72^a* and *CD72^c* (H. Ying and J. R. Parnes, manuscript in preparation).

It is unclear what the function of CD72 on peripheral T cells and activated thymocytes might be. On the basis of the observation that CD72-specific mAb stimulate proliferation of resting and preactivated B cells (Subbarao and Mosier 1982, 1983, 1984; Pezzutto et al. 1990; Kamal et al. 1991), we performed proliferation assays using CD72-specific mAb in an attempt to stimulate either resting or activated peripheral T cells isolated from mouse strains expressing CD72^b. We did not observe increased T cell proliferation in response to mAb stimulation of CD72 alone or in conjunction with CD3-specific mAb (data not shown). Further studies will be necessary to better understand the possible effect of CD72 on T cells.

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