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Inhibition of the function of the FcγRIIB by a monoclonal antibody to thymic shared antigen-1, a Ly-6 family antigen

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SUMMARY

Thymic shared antigen-1 (TSA-1) is a member of the Ly-6 family of glycosyl-phosphatidylinositol (GPI)-linked proteins. While it has been proposed that TSA-1 may play a role in thymic development, a physiological ligand for this antigen has not been identified. Here we report that a monoclonal antibody (mAb) to TSA-1, generated by immunizing a hamster with CD40 ligand (CD40L)-activated B cells, interferes with the function of FcγRIIB on splenic B cells and the B-cell lymphoma cell line, M12, by binding to TSA on the same cells. The interaction of anti-TSA with FcγRIIB resulted in an inhibition of the ability of the FcγRIIB to cross-link and/or aggregate soluble anti-CD3 or soluble anti-Cβ T-cell receptor (TCR), leading to an inhibition of induction of expression of CD25 and CD69, interleukin (IL)-2 production and proliferation of naive T cells. Cross-blocking studies with mAbs strongly suggested that a physical association exists between TSA-1 and the FcγRIIB on the surface of activated B cells and favour the view that a functional intermolecular association exists between these two distinct membrane antigens.

INTRODUCTION

The murine Ly-6 gene complex encodes a family of cell-surface proteins, which are anchored to the plasma membrane through a C-terminal glycosyl-phosphatidylinositol (GPI) moiety.[1] The antigens of this family include Ly-6A/E, Ly-6C, Ly-6F, Ly-6G, ThB and thymic shared antigen-1 (TSA-1), and are widely expressed in a number of tissues and haematopoietic cells.[2,3] A large number of functional studies using monoclonal antibodies (mAbs) to the Ly-6A/E antigens have suggested that these antigens play an important role in the regulation of T-cell activation. Surprisingly, mAbs to Ly-6A/E can both induce interleukin (IL)-2 production in the presence of T-cell receptor (TCR) stimulation[4] and inhibit IL-2 production by T-cell hybridomas when stimulated with soluble anti-CD3.[5–7] Thus, the Ly-6 antigens on T cells may either transduce a positive signal which synergies with the signal generated by TCR ligation to activate IL-2 production, or transduce a negative signal which inhibits anti-CD3-mediated TCR signalling. The delivery of positive signals via Ly-6 antigens is dependent on their GPI anchor and the expression of the ζ-chain of the TCR complex, while delivery of the negative signals can be mediated by Ly-6 molecules engineered to express a conventional transmembrane segment and does not require expression of the TCR ζ-chain.[6] The positive effects of engagement of Ly-6E by specific mAbs appear to be mediated by induction of transcription factor activities, including nuclear factor (NF)-κB and activator protein-1 (AP-1) binding activities, and increasing activity of nuclear factor of activated T cell (NF-AT).[8]

The TSA-1 antigen was initially identified as a thymic differentiation antigen that appeared to be selectively expressed on the least mature thymocytes as well as thymic epithelial cells.[9] cDNA cloning of TSA-1[10,11] revealed that it is identical to stem cell antigen-2 (Sca-2), a differentiation antigen expressed on early thymic precursor cells.[12] Although TSA-1 is also expressed on most peripheral B cells, functional studies

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Abbreviations: AC, accessory cells; CD40L, CD40 ligand; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; TCR, T-cell receptor; TSA-1, thymic shared antigen-1.

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have focused on its potential role in T-cell development and activation. Addition of antibody to TSA-1 (anti-TSA-1) to fetal thymic organ cultures resulted in marked inhibition of thymocyte differentiation post-$CD3^-$ $CD4^-$ $CD8^-$ T cells[13] or in skewing the progression of early $CD4^+$ $CD8^+$ double-positive thymocytes to the mature CD8 lineage.[14] This study raised the possibility that TSA-1, on either T cells or thymic epithelial cells, played a critical role in the process of T-cell differentiation. Recent studies have also suggested that TSA-1 may also play a role on more mature T cells, as a hamster anti-TSA-1 mAb has been demonstrated to inhibit IL-2 production by a T-cell hybridoma stimulated with soluble anti-CD3 in a manner analogous to that seen with mAbs to other members of the Ly-6 family.[15] This inhibitory effect was proposed to be mediated by TSA-1 expressed on the T-cell hybridomas rather than on accessory cells (AC). In addition to the inhibition of IL-2 production, tyrosine phosphorylation of the CD3 ζ-chain following TCR stimulation by anti-CD3 was also markedly reduced by anti-TSA-1.[16] A cDNA encoding human (h)TSA-1 has recently been isolated and hTSA-1 mRNA has been detected in a variety of tissues including brain, heart, liver, lung, kidney, spleen and ovary.[17]

During the process of identification of activation antigens induced on B cells through CD40/CD40 ligand (CD40L) interaction, we generated a hamster mAb, C2F8, which is specific for TSA. We demonstrated that C2F8 recognizes the TSA-1 antigen and inhibits T-cell proliferation and IL-2 production by blocking AC-mediated delivery of the TCR signal. Both hamster mAb C2F8 and a second anti-TSA mAb, MTS35, inhibited the interaction of mAb 2.4 G2 with its ligand, FcγRIIB. This study demonstrates that TSA-1 may physically and/or functionally associate with the FcγRIIB. Therefore, members of the Ly-6 family of GPI-linked proteins may play novel roles in immunoregulation by associating with transmembrane proteins involved in the signal transduction cascade.[18]

MATERIALS AND METHODS

Animals

Six- to 8-week old female BALB/c and C57BL/6 mice, and mice that express a transgenic TCR specific for ovalbumin $(OVA)_{323–339}$ in the context[19] of $I\text{-}A^d$, were obtained from the Animal Production Service, National Institutes of Health (Bethesda, MD). $CD28^{-/-}$ mice backcrossed eight times onto C57BL/6 mice were originally provided by Dr C. Thompson and then bred in our colony. Armenian hamsters were purchased from Cytogen Research and Development (West Roxbury, MA).

Antibodies and reagents

Unconjugated and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IA^d, FcγRII, human serum albumin (HSA) and TSA-1 mAbs, and hamster anti-mouse Cβ TCR mAb, were purchased from PharMingen (La Jolla, CA). Mouse anti-human FcγRII mAb was purchased from MEDAREX, Inc. (Princeton, NJ). Sheep anti-mouse immunoglobulin G (IgG)-conjugated beads (Dynabeads) were purchased from Dynal (Oslo, Norway). The CL-HRP substrate system was purchased from Pierce (Rockford, IL). HAT and phosphatidylinositol-specific phospholipase C (PI-PLC) were purchased from Sigma (St. Louis, MO). Mitomycin C was obtained from Calbiochem (La Jolla, CA).

Production of mAb

The M12 B-cell line was cultured with L-cells that had been transfected for 48 hr with human CD40L and human FcγRII[20] at a ratio of 1 : 10; the L-cells were then depleted from the culture by preincubation with mouse anti-human FcγRII mAb followed by separation with anti-mouse IgG-coated magnetic beads. Activated M12 cells (5×10^6) were injected intraperitoneally into hamsters three times at intervals of 2–3 weeks. Spleen cells of immunized hamsters were fused with SP2/0 myeloma cells, according to standard procedure,[21] and the resulting hybridomas were selected with HAT. The supernatants from HAT-resistant clones were initially screened by fluorescence-activated cell sorter (FACS) analysis for reactivity with M12 cells, and all positive supernatants were then screened for their ability to inhibit T-cell proliferation to anti-CD3 in the presence of M12 cells or CD40L-activated normal B cells as ACs.

Cell purification

T cells were purified from spleen and lymph nodes of normal BALB/c, C57BL/6, $CD28^{-/-}$, or TCR transgenic mice by passing over nylon wool columns followed by antibody-mediated depletion of $B220^+$ and $I\text{-}A^+$ cells, or by using T-cell purification columns (R&D Systems, Minneapolis, MN). T-depleted spleen cells and thioglycollate-induced peritoneal macrophages were isolated as described previously.[22] The TSA-1-transfected Jurkat cells have been described previously.[16]

Cell culture

The purified T cells were cultured (1×10^6/ml) with 3000 rads-irradiated T-depleted spleen cells, lipopolysaccharide (LPS)- or CD40L-activated spleen B cells, or with mitomycin C-treated M12 cells as ACs in the presence of soluble anti-CD3, anti-mouse TCR Cβ, or OVA peptide,[21] for 62 hr. [^{3}H]Thymidine ([^{3}H]TdR) was added during the last 14 hr of incubation, and [^{3}H]TdR incorporation was determined as previously described.[22]

Production of $F(ab')_2$ fragments of C2F8

C2F8 was purified from culture supernatants on protein A columns (Pharmacia Biotech, Uppsala, Sweden), digested with immobilized pepsin at 37° overnight and the $F(ab')_2$ fragments were separated from any residual intact IgG by passage over a protein A column. The $F(ab')_2$ fragment was then purified using the ImmunoPure $F(ab')_2$ Preparation Kit (Pierce). The activity of the $F(ab')_2$ of C2F8 was documented by its ability to block the binding of intact anti-C2F8 to TSA-1-transfected Jurkat cells.

Immunoprecipitation

M12 cells (2×10^7) were washed with phosphate-buffered saline (PBS) three times and suspended in 2 ml of PBS (pH 8·4). NHS-LC biotin (5 mg) was then added to the cell suspension, mixed immediately and incubated on ice for 5 min with gentle rotation. The biotin-labelled cells were then washed with 10 ml of complete medium and then with 50 ml of complete medium

containing glycine (1 mg/ml) and PBS, and then lysed with 1 ml of lysis buffer containing 10% Nonidet P-40 (NP-40), 12 mM CHAPS, 150 mM Tris, 0·1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg/ml of aprotinin. The whole-cell lysate was absorbed with pansorbin (Calbiochem) for 4 hr and with protein A-conjugated sepharose 4B beads overnight at 4°. The cell lysate was then subjected to immunoprecipitation for 1 hr, at 4°, with C2F8 conjugated to protein A–sepharose 4B beads. The immunoprecipitate was washed twice with 1 mM NaCl, once with 150 mM NaCl, dissolved in 40 μl of sample buffer containing 2% sodium dodecyl sulphate (SDS), and then heated at 95° for 5 min with shaking. The immunoprecipitate (20 μl) was then applied to each lane of a 4–12% gradient SDS gel. After electrophoresis, the gel was transferred to PVDF membrane by using semidry transfer. The membrane was incubated with I-block at room temperature for 1 hr, with avidin–protein A at room temperature for 1 hr and then washed five times with PBS containing 0·05% Tween-20. The immunoprecipitates were revealed by incubation of the membrane with chemiluminescent substrate solution for 5 min and development of the film.

PI-PLC treatment

Cells were incubated with PI-PLC (2·5 U/ml; Sigma) at 37° for 30 min, washed, then stained with mAb.

FACS analysis

Cells were incubated with directly conjugated mAbs for 30 min at 4° and washed three times; alternatively, cells were incubated with unlabelled mAbs for 30 min at 4°, washed, then stained with FITC-conjugated protein A (FITC-PA). Analysis was performed on a Becton-Dickinson FACscan using CellQuest software (Becton-Dickinson, San Jose, CA). For competition binding experiments, the cells were preincubated at 4° with the antibodies indicated in figure legends, washed and then stained with the FITC-labelled second antibody.

RESULTS

Tissue distribution of the antigen identified by C2F8

Our initial screening protocol involved testing the hybridoma supernatants for reactivity with the immunogen, CD40L-activated M12 cells, by FACS staining. Positive supernatants were then tested for their capacity to inhibit anti-CD3-induced T-cell activation. One cloned mAb, C2F8, was isolated which stained CD40L-activated M12 cells and produced marked inhibition of the T-cell proliferative response to soluble anti-CD3 (see below). FACS analysis was then performed to determine the tissue distribution of the antigen identified by C2F8. A major subpopulation of thymocytes and a fraction of $B220^+$ spleen cells were reactive with the mAb, as were the majority of thioglycollate-induced peritoneal macrophages (Fig. 1). The expression of the antigen identified by C2F8 could be up-regulated by stimulation of B cells with CD40L-transfected L-cells (Fig. 2b). Although resting T cells were not stained by C2F8, stimulation with anti-CD3 induced a time-dependent increase in the level of expression with maximalinduction observed at 24 hr (Fig. 2a). M12 cells, BW5147 cells and several T-cell hybridomas derived from fusion with BW5147 were also strongly constitutively reactive with C2F8 (Fig. 3 and data not shown). The reactivities for M12 cells of C2F8, MTS35 (rat anti-TSA-1) and J11d (anti-HSA), but not AMS32.1 (anti-I-A^d), were significantly diminished after PI-PLC treatment, demonstrating that the antigen recognized by the C2F8 mAb is a GPI-linked protein (Fig. 3). Similar results were observed both with normal spleen cells and T-cell hybridomas.

C2F8 reacts with TSA-1

C2F8 precipitated a molecule with a molecular mass (M_r) of ≈11 kDa under both reducing and non-reducing conditions (Fig. 4). The tissue distribution, M_r and GPI linkage of the antigen recognized by C2F8 strongly suggested that it is a member of the Ly-6 family, probably TSA-1. Indeed, Jurkat cells which had been transfected with murine TSA-1 were strongly reactive with C2F8, while control Jurkat cells were not stained (Fig. 5). Similarly, the reactivity of rat mAb MTS35 with the TSA-1-transfected Jurkat cell could be completely inhibited by C2F8 (data not shown).

Inhibition of T-cell activation by C2F8

When C2F8 was added to cultures of $CD4^+$ T cells from wild-type C57BL/6 mice or $CD28^{-/-}$ mice activated by soluble anti-CD3 in the presence of T-depleted spleen cells or CD40L-activated B cells, a marked inhibition of T-cell proliferation was observed (Fig. 6a, 6b, 6c, 6d). While the proliferative responses to a second soluble antibody to the TCR, H57-97 (which recognizes TCR Cβ), were substantially inhibited

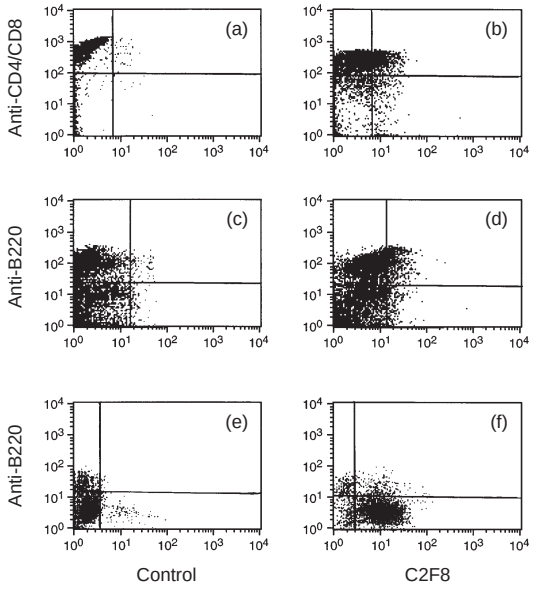

Figure 1. Expression of thymic shared antigen-1 (TSA-1) on normal cells. Single-cell suspensions of thymus (a) and (b), spleen (c) and (d), and thioglycollate-stimulated peritoneal exudate cells (e) and (f), were stained with phycoerythrin (PE)-conjugated anti-CD4/8 (a) and (b) or PE-conjugated anti-B220 (c), (d), (e) and (f), followed by normal hamster immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-conjugated protein A (FITC-PA) (a), (c) and (e) or C2F8 and FITC-PA (b), (d) and (f).

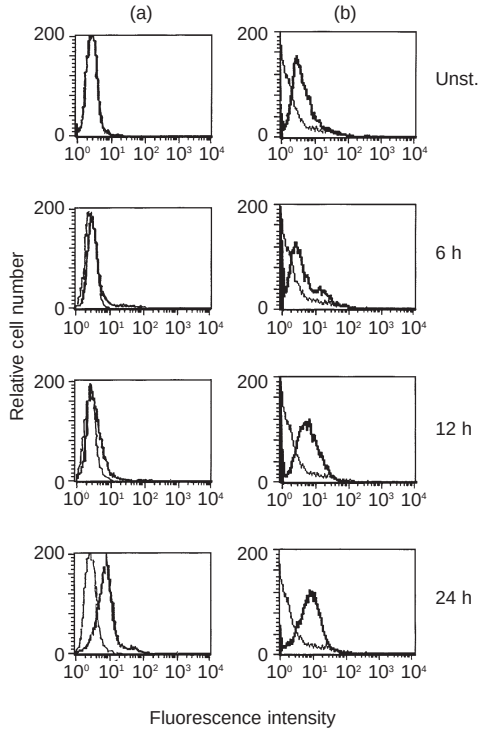

Figure 2. Expression of C2F8 on activated $CD4^+$ T and B cells. (a) Spleen cells were stimulated with anti-CD3 (1 μg/ml) *in vitro* for the time-periods shown. The cells were then harvested and stained with phycoerythrin (PE)-conjugated anti-CD4 and control hamster immunoglobulin G (IgG)/fluorescein isothiocyanate (FITC)-conjugated protein A (FITC-PA) (thin line) or C2F8/FITC-PA (thick line). The expression of C2F8 on gated $CD4^+$ T cells is shown. (b) T-depleted spleen cells were cultured with CD40 ligand (CD40L)-transfected L-cells (10 : 1 ratio) for the time-periods shown. The cells were then harvested and stained with PE-anti-B220 and C2F8/FITC-PA (thick line) or with normal hamster IgG/FITC-PA (thin line). The expression of C2F8 on gated B220-positive cells is shown.

(Fig. 6e), C2F8 did not inhibit the antigen-specific responses of T cells from animals that expressed a transgenic TCR specific for $OVA_{323–339}$ in the context of $I\text{-}A^d$. Similarly, C2F8 failed to inhibit T-cell proliferation induced by concanavalin A, allogeneic stimulator cells, or plate-bound anti-CD3 (data not shown). One simple explanation for these findings is that the Fc domain of C2F8 competes with the anti-TCR mAb for binding the FcγRII. Although we routinely use a polyclonal preparation of normal hamster IgG as a control in these studies, it was imperative to rule out this explanation. $F(ab')_2$ fragments of C2F8 were prepared and tested for their ability to inhibit T-cell activation by soluble anti-CD3. The $F(ab')_2$ fragment was shown to be active as it could readily inhibit the binding of MTS35, the rat anti-TSA-1 mAb, to TSA-1-transfected Jurkat cells. Addition of $F(ab')_2$ to cultures resulted in 50–70% inhibition of the response to anti-CD3, which was similar to the degree of inhibition seen with the intact mAb (Fig. 6f).

C2F8 inhibits AC function

Previous studies have reported that anti-TSA-1 mAb and mAbs to other Ly-6 antigens inhibited IL-2 production by T-cell hybridomas by acting directly on the responder T cells. However, unlike T-cell hybridomas which constitutively express TSA-1, naive T cells do not express TSA-1, and TSA-1 expression on T cells can only be detected 20 hr after anti-CD3 stimulation. To elucidate the mechanism and cellular site of action of C2F8 on resting T-cell proliferation, we treated T-depleted spleen cells or the responder T cells with C2F8 for 30 min and then washed all the populations of cells prior to stimulation with anti-CD3. Pretreatment of the ACs, but not the responder T cells, resulted in a significant inhibition of T-cell proliferation, although somewhat greater inhibition was seen in the continuous presence of C2F8 (Fig. 7). Addition of C2F8 to the cultures after 2·5 hr resulted in a similar degree of inhibition of T-cell proliferation to that seen when antibody was added at culture initiation, while addition of C2F8 after 24 hr failed to inhibit T-cell proliferation (data not shown). Taken together, these studies strongly support the view that the cellular site of action of C2F8 is the AC and not the resting responder T cell.

C2F8 inhibits the delivery of the TCR signal

The failure of C2F8 to inhibit all modes of T-cell activation, other than those induced by soluble mAbs to the TCR/CD3 complex in the presence of ACs, raised the possibility that C2F8 inhibits the delivery of the signal to the TCR delivered by cross-linking of the stimulatory mAb by FcγRIIB on the AC. Indeed, the addition of IL-2 to the cultures failed to restore the proliferative responses to soluble anti-CD3 (data not shown). As these results suggested that C2F8 inhibited both IL-2 production and IL-2 receptor expression, we measured induction of IL-2R α-chain (CD25) expression in the presence of C2F8. High levels of CD25 were induced on T cells cultured in the presence of normal hamster IgG and anti-CD3, while C2F8 markedly inhibited the induction of CD25 (Fig. 8). More importantly, the induction of the very early activation antigen, CD69, was also inhibited by C2F8. As CD69 expression is relatively costimulation independent under these conditions,[23] it is highly probable that the inhibition seen in the presence of C2F8 is secondary to inhibition of the delivery of the TCR signal.

TSA-1 and FcγRIIB are associated on the cell surface

These results suggest that engagement of TSA-1 with the C2F8 mAb results in inhibition of the function of the FcγRIIB, which in turn results in defective delivery of the stimulatory signal to the TCR and raises the possibility that the two molecules are physically associated on the cell surface. Preincubation of M12 cells with either C2F8 or MTS35 resulted in an ≈50% inhibition of the staining of these cells by the anti-FcγRIIB mAb, 2.4G2. Two other hamster mAbs, 2C11 and H1.2F3 (anti-CD69), did not inhibit the binding of 2.4G2 (Fig. 9a, 9b, 9c, 9d). Conversely, preincubation of M12 cells with 2.4G2 markedly inhibited the binding of MTS35 (Fig. 9e, 9f); although the intensity of staining of M12 cells by C2F8/FITC-PA was lower than that observed with the directly FITC-conjugated MTS35, preincubation of M12 cells with 2.4G2 only slightly inhibited the binding of C2F8 (Fig. 9g, 9h). These results strongly suggest that TSA-1 and the FcγRIIB

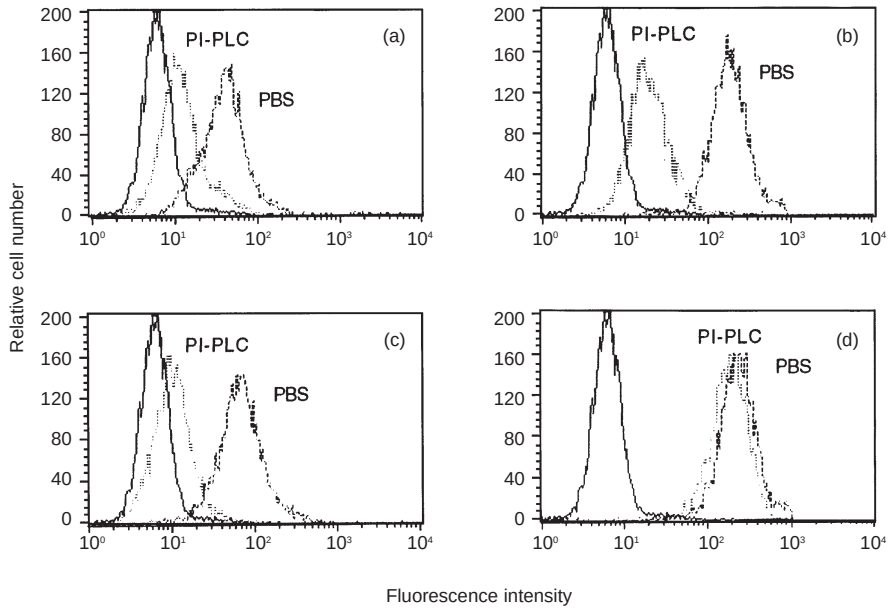

Figure 3. C2F8 reacts with a glycosyl-phosphatidylinositol (GPI)-linked protein. M12 cells were treated with phosphate-buffered saline (PBS) or phosphatidylinositol-specific phospholipase (PI-PLC) and then stained with C2F8/fluorescein isothiocyanate (FITC)-conjugated protein A (FITC-PA) (a), FITC-J11.D (b) (anti-HSA), FITC-MTS35 (c) (anti-thymic shared antigen-1 [anti-TSA-1]), or FITC-AMS32.1 (d) (anti-I-A^d).

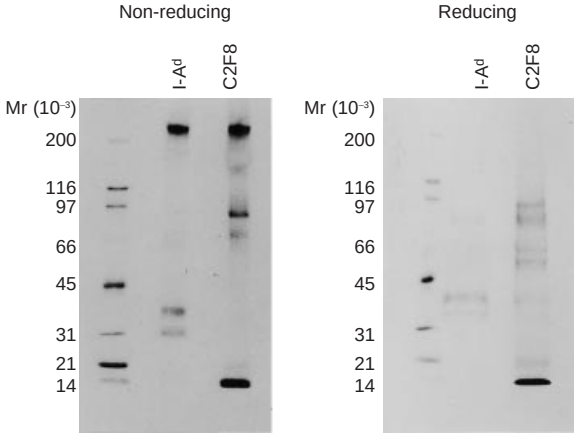

Figure 4. Immunoprecipitation by C2F8. M12 cells were biotin-labelled, lysed and immune precipitated by C2F8 and AMS32 (anti-I-A^d), as described in detail in the Materials and methods. The precipitates were resolved by electrophoresis through a 4–20% sodium dodecyl sulphate (SDS) gel under both reducing and non-reducing conditions.

are associated on the cell surface and that C2F8 and MTS35 recognize related, but not identical, epitopes on this molecule.

DISCUSSION

Although the B7 family (CD80/CD86) of molecules on ACs has been shown to play the most critical role in providing T-cell costimulation for all T-cell functions,[24–32] studies with $CD28^{-/-}$[23] mice have raised the possibility that other cell-surface molecules can also, albeit to a weaker extent than the B7 family, provide a costimulatory signal for the activation of both $CD4^+$ and $CD8^+$ T cells. We[18] and others[33] have

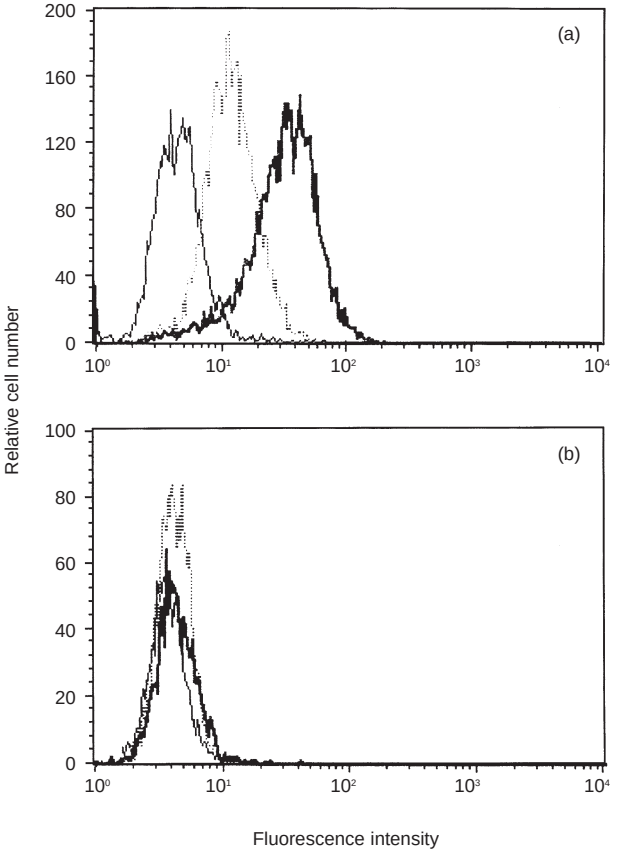

Figure 5. C2F8 reacts with thymic shared antigen-1 (TSA-1). TSA-1 transfected Jurkat cells (a) or non-transfected Jurkat cells (b) were stained with normal hamster immunoglobulin G (IgG)/fluorescein isothiocyanate (FITC)-conjugated protein A (FITC-PA) (solid line), C2F8/FITC-PA (dotted line), or FITC-MTS35 (thick line).

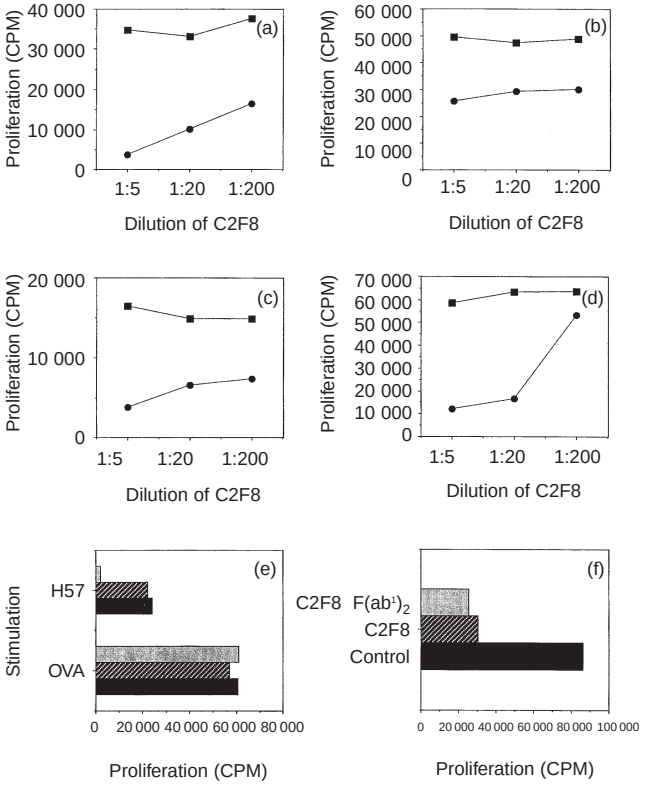

Figure 6. Inhibition of T-cell proliferation by C2F8 (a), (b), (c) and (d). Purified T cells from C57BL/6 (a) and (b) or $CD28^{-/-}$ mice (c) and (d) were stimulated with soluble anti-CD3 in the presence of T-depleted spleen cells (a) and (c) or with CD40 ligand (CD40L)-activated B cells (b) and (d) *in vitro* for 72 hr in the presence of control hamster IgG (■) or C2F8 hybridoma supernatant (●). [^{3}H]Thymidine ([^{3}H]TdR) was added for the last 18 hr of culture. Results are expressed as mean counts per minute (c.p.m.) of [^{3}H]TdR incorporated. The mean c.p.m. values of triplicate cultures are shown. The standard error of the mean was rarely higher than 10% of the mean value and has been omitted for simplicity. (e) Purified T cells from DO-11-10 T-cell receptor (TCR) transgenic mice were stimulated with anti-Cβ TCR (H57, 10 μg/ml) or ovalbumin $(OVA)_{323–339}$ (1 μM) *in vitro* in the absence (solid bar) or the presence of hamster IgG (hatched bar) or C2F8 (dotted bar). [^{3}H]TdR incorporation was expressed as described above. (f) Purified T cells from C57BL/6 mice were stimulated with soluble anti-CD3 and T-depleted spleen cells in the presence of intact C2F8, the $F(ab)'_2$ fragment of C2F8, or normal hamster IgG. Proliferation was measured as described above.

suggested that activation of B cells via CD40L/CD40 interactions may be a highly effective means for induction of the CD28/B7-independent costimulatory pathway. Studies by Guo *et al.*[34] have demonstrated that one candidate molecule on the CD40L-activated B cell, which plays a prominent role in the activation of T cells from normal and $CD28^{-/-}$ mice, is CD44H. We employed a similar strategy to that used by Wu *et al.*[33] to generate mAbs to CD40L-activated B cells and to select for mAbs that were capable of inhibiting the activation of $CD4^+$ T cells both from normal mice and from $CD28^{-/-}$ mice.

The mAb selected for intensive study initially appeared to meet the major criteria for a molecule involved in the delivery of CD28/B7-independent costimulation. C2F8 reacted with resting B cells, the expression of its target antigen was up-regulated upon CD40L/CD40 interaction, and it suppressed the proliferative response to stimulation with soluble anti-CD3 in the presence of ACs. However, the inability of this mAb to suppress all other forms of T-cell activation, including alloantigen-, soluble protein antigen- and mitogen-induced activation, suggested that its inhibitory properties were completely unrelated to inhibition of costimulation. Furthermore, the capacity of C2F8 to inhibit the costimulatiory-independent induction of the expression of the early activation antigen, CD69, indicated that the inhibitory properties of the mAb were mediated by blocking the delivery of the stimulus to the CD3/TCR complex (i.e. Signal 1). The inhibition seen in the presence of the C2F8 was not secondary to direct inhibition of the binding of the Fc of the anti-CD3 to the FcγRIIB on the ACs, as $F(ab')_2$ fragments of C2F8 were as effective as the intact mAb in suppressing the response to soluble anti-CD3.

Detailed studies of the tissue distribution and biochemical properties of the antigen identified by C2F8 strongly demonstrated that it recognized the TSA-1 molecule, a member of the Ly-6 gene family. The target antigen of C2F8 was definitely shown to be TSA-1 because C2F8 reacted strongly with human Jurkat cells which had been transfected with the murine TSA-1 gene and owing to its capacity to inhibit the binding of a well-characterized anti-TSA-1 mAb, MTS35, to the transfected Jurkat cells. mAbs to multiple members of the Ly-6 family (Ly-A/E, Ly-6C, Ly-6G and chimeric Ly-6E/G) have been shown to be capable of inhibiting the activation of both normal resting T cells and T-cell hybridomas to stimulation by soluble, but not plate-bound, anti-CD3. Kosugi and colleagues[16] have recently demonstrated that a unique anti-TSA-1 mAb generated in their laboratory can inhibit anti-CD3-induced activation in a manner identical to that seen with mAbs to the other Ly-6 antigens. They could definitively demonstrate that the target cell for the inhibitory effects of anti-TSA-1 on T-cell proliferation was the T cell and not the AC, as only Jurkat cells transfected with murine TSA-1 were susceptible to inhibition when stimulated with anti-hCD3 in the presence of mouse ACs. No studies of the effects of anti-TSA-1 on other forms of T-cell activation were reported in these studies. The anti-TSA-1 mAb also produced modest inhibition of the activation of normal T cells by anti-CD3; the level of inhibition was less than that seen with the T-cell hybridomas and these investigators concluded that this was because normal T cells do not express TSA-1 in the absence of activation, while TSA-1 is constitutively expressed on the T-cell hybridomas.

In contrast, our studies strongly support the view that the inhibitory effects of C2F8 on anti-CD3-induced proliferation of resting T cells were mediated by its action on ACs. First, brief exposure of the AC, but not the responder T cells, to C2F8 resulted in the inhibition of T-cell proliferation. Second, marked inhibition of T-cell proliferation was produced by the $F(ab')_2$ fragment of C2F8, while inhibition of T-cell activation by anti-Ly-6 required intact antibody.[5]

The target cell for the inhibitory activity of the anti-TSA-1 mAb in our studies is probably the B cell, as proliferative responses in the presence of M12 cells (a B-cell tumour line), as well as highly purified small resting B cells that were activated by CD40L-transfected L-cells, were markedly inhibited. It is also possible that the function of macrophages (which are strongly TSA-1 positive) was also blocked when we used mixed populations of ACs. Previous studies[35,36] of the effect of

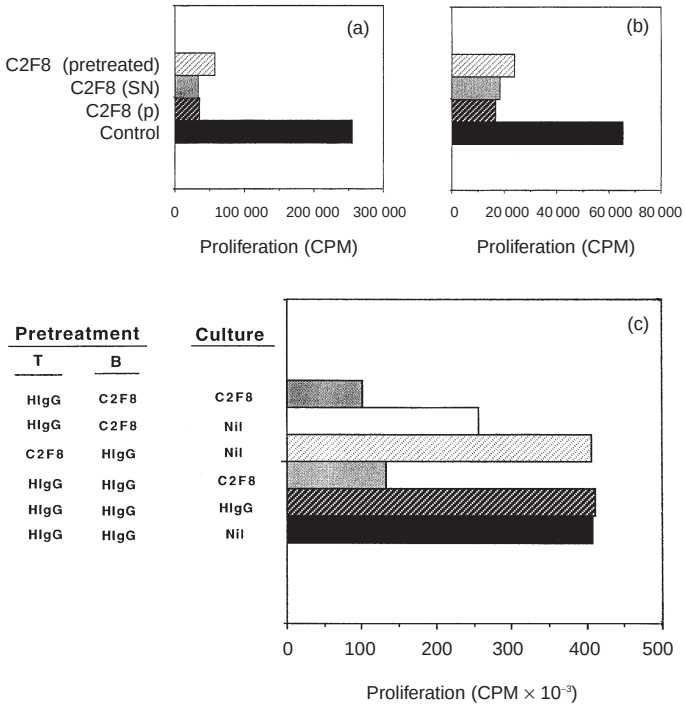

Figure 7. C2F8 acts on accessory cells (ACs). (a) and (b) Purified T-depleted spleen cells were preincubated with purified C2F8 (50 μg/ml) and washed. Pretreated T-depleted spleen cells were then mixed with purified T cells and cultured in the presence of soluble anti-CD3. The purified T cells were stimulated with soluble anti-CD3 in the presence of T-depleted spleen cells and either the supernatant (SN) of C2F8 (20%), or purified (p) C2F8 (10 μg/ml), or control hamster immunoglobulin G (IgG). [^{3}H]Thymidine ([^{3}H]TdR) was added for the last 18 hr of culture. Results are expressed as mean counts per minute (c.p.m.) of [^{3}H]TdR incorporated. The mean c.p.m. values of triplicate cultures are shown. (c) Purified T cells or T-depleted spleen cells were preincubated with C2F8 (50 μg/ml) or normal hamster IgG on ice for 30 min and washed. Pretreated populations were then mixed as indicated on the figure and cultured in the presence of soluble anti-CD3. Some cultures were performed in the continuous presence of C2F8. [^{3}H]TdR incorporation was determined as described above. The standard errors of the means of all the data presented in panel (c) were less than 10% of the mean values and were omitted for simplicity.

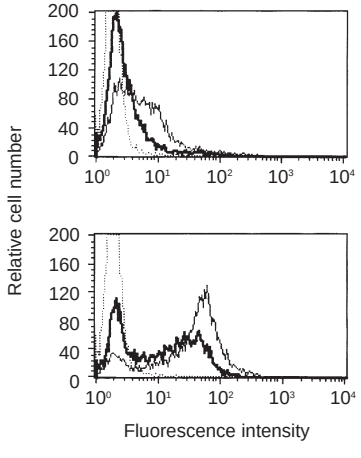

Figure 8. Effect of C2F8 on induction of CD25 and CD69 expression on naive CD4$^+$ T cells. Whole spleen cells from C57BL/6 mice were stimulated with soluble anti-CD3 *in vitro* for 20 hr in the presence of control hamster immunoglobulin G (IgG) (thin lines) or C2F8 (thick lines). The cells were harvested and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD25 or with FITC-anti-CD69 and phycoerythrin (PE)-anti CD4.

mAbs to members of the Ly-6 family on B-cell function have shown that anti-Ly-6A/E mAbs were capable of: inducing a large and sustained increase of [Ca^{2+}] in interferon-γ (IFN-γ)-treated resting B cells; inducing B-cell proliferation in the presence of IL-4 and IFN-γ; reversing the reduced responses when B cells were cultured in the presence of anti-IgM and IFN-γ; and causing up-regulation of Ly-6A/E in the presence of IFN-γ. Although both the intact antibody and its F(ab′)$_2$ fragment caused up-regulation of Ly-6A/E expression, only the intact mAb was capable of inducing proliferation. Ly-6A/E capped independently after cross-linking, which suggests that the effects of the anti-Ly-6 mAbs are not caused by the modulation of mouse immunoglobulin (mIg).

A number of studies have indirectly suggested that a complex physical and/or functional association exists between members of the Ly-6 family and the CD3/TCR complex. When an antigen-specific T helper 2 (Th2) clone or normal antigen-primed T cells were transiently transfected with an antisense primer complementary to the 5′ end of the mRNA encoding the Ly-6A protein,[18] the responses of both T-cell populations were markedly impaired and both cell-surface expression of Ly-6A and surface expression of the TCR were markedly suppressed. Similar results were observed when this same T-cell clone was stably transfected with the antisense primer.[37] When the Ly-6A antisense transfectant was subsequently transfected with Ly-6E, normal levels of TCR

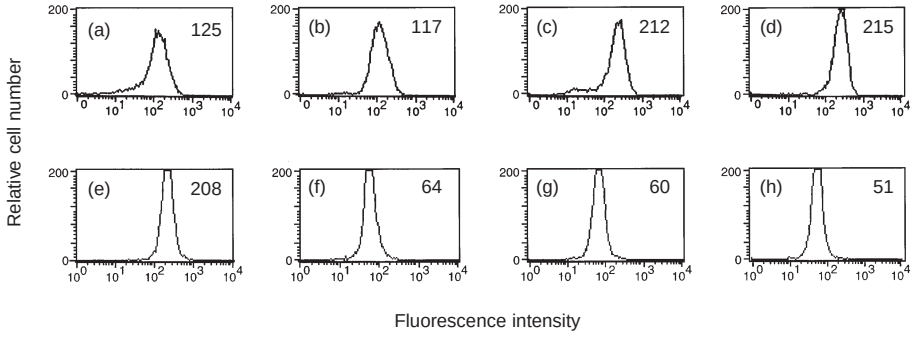

Figure 9. Thymic shared antigen-1 (TSA-1) is associated with the FcγRII. Upper panels: M12 cells were preincubated on ice for 30 min with MTS35 (a), C2F8 (b), H1.2F3 (c) or 2C11 (d), washed and then stained with fluorescein isothiocyanate (FITC)-conjugated 2.4G2. The mean fluorescence intensity (MFI) for 2.4G2 on untreated M12 was 237 and for the negative control was 4. Lower panels: M12 cells were preincubated for 30 min at 40° with control rat immunoglobulin G (IgG) (e) and (g) or with 2.4G2 (f) and (h), washed, and then stained with fluorescein isothiocyanate (FITC)-conjugated MTS35 (e) and (f) or C2F8/FITC-conjugated protein A (FITC-PA) (g) and (h). The number in the upper right hand corner of each panel indicates the MFI.

expression and functional activity were restored. However, when surface TCR expression was reconstituted and Ly-6 levels were still low, activation of the T-cell clone continued to be impaired. In addition, surface expression of Ly-6A was required for optimal *in vitro* fyn kinase activity, irrespective of expression of the TCR.

Although the above studies strongly favour the view that a functional association exists between the members of the Ly-6 family and the signal transducing TCR and B-cell receptors (BCR), it has proven exceedingly difficult to demonstrate a physical association between Ly-6 proteins and either of the antigen–receptor complexes in co-capping and immunoprecipitation studies. Our results are most consistent with a model where engagement of TSA-1 by mAb interferes with the function of the FcγRIIB in cross-linking the stimulatory anti-TCR mAb to create a sufficient level of aggregation which would lead to T-cell activation. Our cross-blocking studies strongly suggested that a physical association exists between TSA-1 and the FcγRIIB on the surface of the B cell, as two distinct anti-TSA-1 (C2F8 and MTS35) mAbs could inhibit by ≈50% the binding of the anti-FcγRIIB mAb, 2.4G2, and 2.4G2 markedly inhibited the binding of MTS35. Although these experiments are in favour of the possibility that an intermolecular association exists between TSA-1 and the FcγRIIB, we have thus been unable to demonstrate complexes between these two molecules in immunoprecipitation studies. A similar association has recently been demonstrated[38] between another GPI-anchored protein, the urokinase-type plasminogen activator receptor (uPAR), and members of the integrin family. The uPAR was shown to co-localize with integrins at sites of cell adhesion, and this interaction promoted the adhesion of cells to vitronectin via a binding site on the uPAR and simultaneously suppressed the normal adhesive function of the integrins.

The mechanism by which engagement of TSA-1 inhibits FcγRIIB function remains to be determined. Cross-linking of the FcγRIIB with the antigen receptor on B cells results in delivery of a signal that aborts B-cell activation[39] because the cytoplasmic domain of the FcγRIIB contains the SH2 recognition motif, YSSL, which interacts with the protein tyrosine phosphatase SHP-1[40] and the inositol polyphosphate 5-phosphatase, SHIP.[41] It would be of interest to determine whether simultaneous engagement of TSA-1 by mAb and the FcγRIIB, by the Fc of the stimulatory anti-TCR mAb, in some manner activates or inhibits one of the enzymatic pathways mediated by SHP-1 or SHIP, which leads to down-regulation of the capacity of the FcγRIIB to aggregate antibodies.

Although lymphocyte activation by mAbs to GPI-anchored proteins was first noted[42] in the mid-1980s and interpreted as perhaps mimicking the interaction of these proteins with a physiological ligand, ligands for GPI-anchored proteins have been very difficult to define.[43,44] Indeed, it has been proposed that the cellular activation observed after engagement of GPI-anchored proteins is merely a by-product of their mode of attachment in the membrane which leads to their association with multiple src family tyrosine kinases that co-localize with GPI-anchored proteins in calveolae and can be co-precipitated with almost all GPI-anchored proteins. However, lymphocytes do not have calveolae, so other mechanisms, perhaps an association with G-protein α-subunits,[45] are needed to explain the requirement of the GPI anchor for mAb-induced activation of T and B cells. On the other hand, the delivery of a suppressive signal for activation of T-cell hybridomas appears to be a unique property of the Ly-6 family, which does not require the GPI anchor. We have not yet determined whether the GPI anchor is required for delivery of the suppressive signal to the FcγRIIB by engagement of TSA-1. One intriguing possibility is that distinct physiological ligands mediate the induction of the activating and suppressive pathways.

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