

## **A Role for CD9 Molecules in T Cell Activation**

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### **Summary**

Costimulation mediated by the CD28 molecule plays an important role in optimal activation of T cells. However, CD28-deficient mice can mount effective T cell-dependent immune responses, suggesting the existence of other costimulatory systems. In a search for other costimulatory molecules on T cells, we have developed a monoclonal antibody (mAb) that can costimulate T cells in the absence of antigen-presenting cells (APC). The molecule recognized by this mAb, 9D3, was found to be expressed on almost all mature T cells and to be a protein of ~24 kD molecular mass. By expression cloning, this molecule was identified as CD9. 9D3 (anti-CD9) synergized with suboptimal doses of anti-CD3 mAb in inducing proliferation by virgin T cells. Costimulation was induced by independent ligation of CD3 and CD9, suggesting that colocalization of these two molecules is not required for T cell activation. The costimulation by anti-CD9 was as potent as that by anti-CD28. Moreover, anti-CD9 costimulated in a CD28-independent way because anti-CD9 equally costimulated T cells from the CD28-deficient as well as wild-type mice. Thus, these results indicate that CD9 serves as a molecule on T cells that can deliver a potent CD28-independent costimulatory signal.

Full activation of the T cell has been shown to require two independent signals (1). The first signal is provided by antigen-specific T cell receptor (TCR) interacting with processed antigen peptides plus major histocompatibility complex (MHC) molecules on APC. This signal leads to an effective T cell response only when accompanied by a second costimulatory signal(s) presented by the APC. The lack of costimulation not only prevents activation but also induces tolerance called anergy (1). Identifying molecules capable of delivering costimulatory signals has been the subject of a large number of recent investigations (2–5). CD28 expressed on T cells was found to be a receptor for the costimulatory molecules CD80 and CD86 on APC (6). CD28 engagement, by either anti-CD28 mAb or ligands (CD80/CD86), has been shown to costimulate T cells in the absence of APC, resulting in T cell activation (7–9). Conversely, the blocking of CD28-ligand interactions induced substantial inhibition of T cell activation (2). These observations indicated that the CD28-CD80/CD86 interaction functions as a critical pathway of T cell costimulation. Nevertheless, recent studies have revealed that CD28-deficient mice can develop normal *in vivo* immune responses (10) and that T cells from these mice mount APC-dependent responses for T cell activation *in vitro* although the

response is reduced compared to T cells from wild-type mice (10, 11). Thus, these results strongly suggest that there may exist other molecules capable of providing costimulatory activity.

In this report, we have developed a rat IgG mAb (9D3) by immunization with cells of a murine thymic stromal clone (12). This mAb recognized a protein of ~24 kD that is expressed on immunizing thymic stromal cells as well as murine T cells. By cDNA expression cloning, the antigen recognized by 9D3 was identified as CD9. We found that 9D3 (anti-CD9) induces potent costimulation of virgin T cells in the absence of APC. Anti-CD9 mAb almost equally costimulated T cells from the CD28-deficient as well as wild-type mice. Thus, these results indicate that CD9 on murine T cells delivers a potent costimulatory signal that functions for T cell activation in a CD28-independent way.

### **Materials and Methods**

*Mice.* C57BL/6 and BALB/c mice were purchased from Shizuoka Animal Laboratories (Hamamatsu, Japan). CD28-deficient mice were originally generated as described (10), and a colony of the CD28-deficient (CD28<sup>-/-</sup>) mice and wild-type (CD28<sup>+/+</sup>) control mice (13) were maintained in an animal facility at the Naval Medical Research Institute.

**Antibodies.** A 9D3 hybridoma was generated by immunizing rats with cells of a thymic stromal clone, MRL104.8a (12). 9D3 mAb, anti-CD3 mAb (145-2C11) (14) and anti-CD28 mAb (PV-1) (15) were purified from ascitic fluids of hybridomas producing each mAb. Control rat IgG was purchased from Biomed (Foster City, CA).

**Cell Surface Biotinylation and Immunoprecipitation.** Cell surface biotinylation and immunoprecipitation were carried out as described (16). Briefly, cells ( $5 \times 10^7$ /ml) were suspended in saline containing 100 mM Hepes (pH 8.0). Sulfo-succinimidobiotin (Pierce Chemical Co., Rockford, IL) was added to cell suspension at a concentration of 0.5 mg/ml. After a 40-min incubation, cells were washed and lysed in buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml soybean trypsin inhibitor, 2 mM  $MgCl_2$ , 2 mM  $CaCl_2$ , and 0.1% sodium azide. After a 30-min incubation, the cell lysates were recovered and incubated with antibody-coupled Sepharose 4B for 2 h at 4°C. After washing with lysis buffer, bound proteins were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and visualized with avidin-peroxidase (Bio-Rad Laboratories, Richmond, CA) followed by an appropriate substrate.

**Preparation of a Purified T Cell Population.** Lymph node cells were depleted of B cells by immunomagnetic negative selection as described (17). Briefly, lymph node cells were incubated with Advanced Magnetic Particles bound to goat anti-mouse Ig (Advanced Magnetic, Cambridge, MA). Surface Ig-negative cells were obtained by removing cell-bound magnetic particles with a rare earth magnet (Advanced Magnetic). This was followed by complement-mediated cytotoxic treatment with anti-Ia mAb (34-5-3S). Purity of the resulting population was checked by flow microfluorometry (FMF) with anti-CD3. Purified T cells were consistently >98% CD3-positive.

**Expression Cloning of 9D3 Antigen.** A cDNA expression library from FDCP-1 cells (a myeloid cell line) was electroporated into *Escherichia coli* DH10B and plasmid DNA was prepared from pools of transformants (average insert size  $\sim$ 2 kb) using Wizard Maxiprep resin (Promega, Madison, WI). DNA was transfected into COS-1 cells (clone M6) by lipofectAMINE (GIBCO BRL, Gaithersburg, MD) or DEAE-dextran with chloroquine treatment as described (18). Cells in 100-mm plates were screened by *in situ* staining 48 h after transfection. Monolayers were washed twice in binding buffer (PBS, 1% [wt/vol] bovine serum albumin, 0.1% [wt/vol] sodium azide), and then covered in 3 ml binding buffer at 4°C. Primary monoclonal antibody was added at a final concentration of 2  $\mu$ g/ml for 1 h. Monolayers were washed three times with PBS, and then fixed with methanol for 10 min. After washing again in binding buffer, cells were stained with rabbit F(ab')<sub>2</sub> anti-rat IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) for 1 h at 4°C, then washed three times in binding buffer. Alkaline phosphatase activity was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega, Madison, WI). Positive cells were scored by light microscopy.

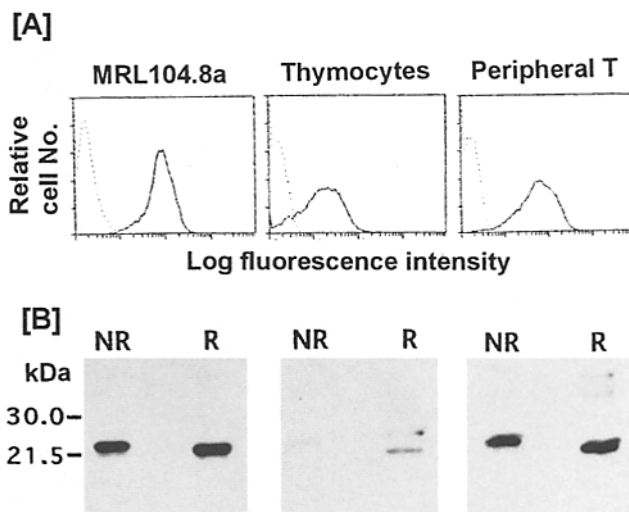
**T Cell Cultures for Proliferation.** mAbs were immobilized to wells of 96-well flat-bottomed microculture plates (Corning 25860, Corning Glass Works, Corning, NY) in a final volume of 0.1 ml. Purified T cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 2-mercaptoethanol at  $1.5$  or  $2.0 \times 10^5$  cells/well at 5% CO<sub>2</sub> at 37°C. The cultures were harvested after an 8-h pulse with 20 kBq/well of [<sup>3</sup>H]TdR. Results were calculated from uptake of [<sup>3</sup>H]TdR and expressed as the mean cpm  $\pm$  SE of triplicate cultures.

## Results

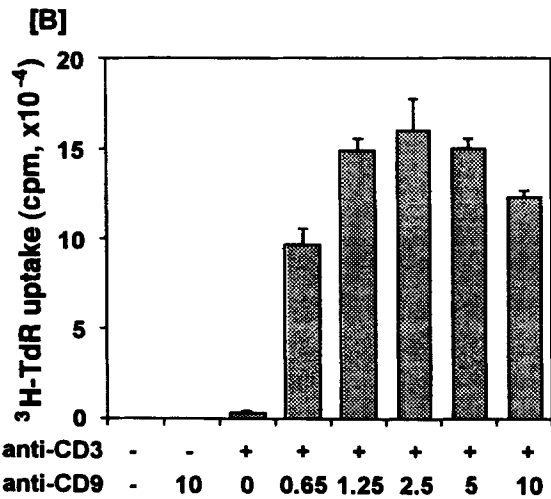
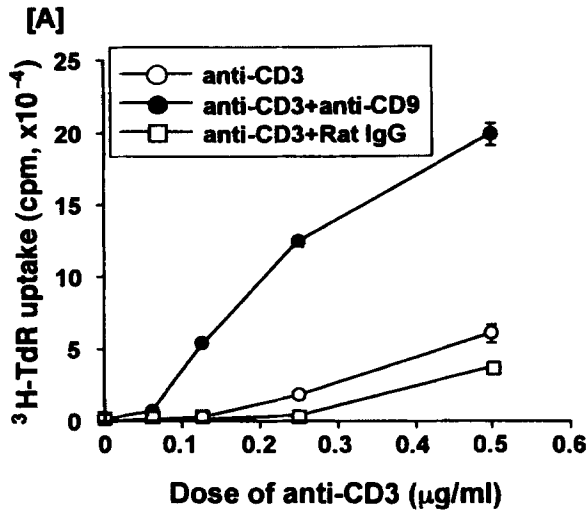
**Characterization of the Antigen Recognized by a Newly Established mAb, 9D3.** Wistar rats were immunized with cells of the MRL104.8a thymic stromal clone (12). A number of hybridomas were generated by fusing splenic B cells from the MRL104.8a-immunized rats with SP2 cells. One of mAbs designated 9D3 (IgG2b) was found to react with thymocytes and peripheral (splenic and lymph node) T cells as well as MRL104.8a stromal cells (Fig. 1 A) and myeloid progenitor cell lines such as FDCP-1 (data not shown).

We determined the sizes of the 9D3 target molecule by immunoprecipitation experiments (Fig. 1 B). MRL104.8a cells, B6 thymocytes and B6 splenic T cells were surface-biotinylated and lysed in detergent-containing lysis buffer. The cell lysates were precipitated with 9D3-bound beads, and bound proteins were subjected to SDS-PAGE. A monomeric protein with almost the same size was immunoprecipitated in three cell lysates and the size was  $\sim$ 24 kD, although it was slightly decreased at a reduced condition. Thus, the results of Fig. 1 show that 9D3 recognizes a protein of  $\sim$ 24 kD molecular mass which is expressed on almost all T cells as well as cells of a thymic stromal cell clone.

**Isolation of 9D3 cDNA Clone.** An FDCP-1 cDNA expression library was screened in transiently transfected COS-1 cells using *in situ* staining with the 9D3 mAb. Cells transfected with one pool of  $\sim$ 11,000 clones and stained with 9D3 mAb showed several hundred positive cells. No signal was found with the same cells stained with a rat isotype control. The positive pool was subdivided into pools



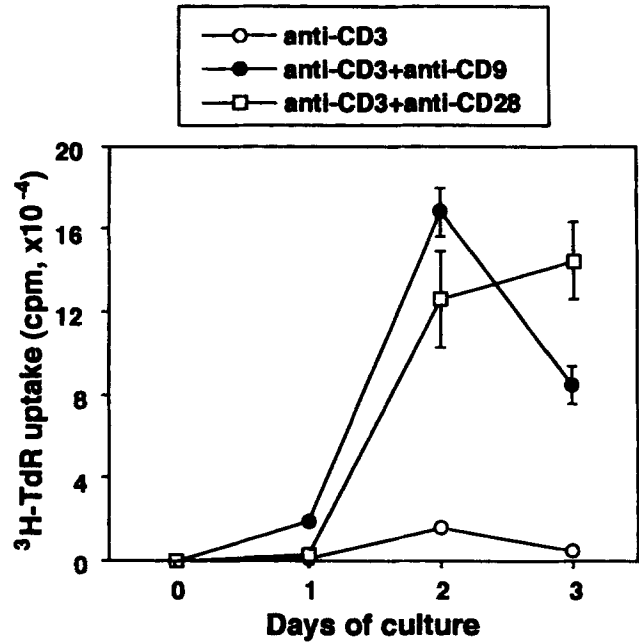
**Figure 1.** 9D3 recognizes  $\sim$ 24 kD molecules expressed on MRL104.8a thymic stromal cells, thymocytes and mature T cells from C57BL/6 mice. (A) MRL104.8a cells, C57BL/6 thymocytes and purified peripheral (splenic and lymph node) T cells were stained with biotinylated 9D3 followed by PE-conjugated streptavidin. Dashed line was the staining with PE-conjugated streptavidin. (B) three types of cells were surface-biotinylated, detergent-extracted and precipitated with 9D3-bound beads. Bound proteins were subjected to SDS-PAGE under non-reduced (NR) or reduced (R) conditions.



**Figure 2.** Anti-CD9 mAb costimulates proliferation of virgin T cells stimulated with suboptimal doses of anti-CD3 mAb. Purified C57BL/6 lymph node T cells ( $2 \times 10^5$ /well) were cultured for 2 d in 96-well flat-bottomed microplates which had been coated with various doses of anti-CD3 mAb plus 10  $\mu$ g/ml anti-CD9 mAb (A) or with various doses of anti-CD9 mAb plus 1  $\mu$ g/ml anti-CD3 mAb (B).

of ~800 clones each for a second round of transfections. A single positive pool from round two was subdivided into pools of 96 clones each. After three rounds, a single plasmid clone (F20) containing a cDNA insert of ~1.2 kb was isolated. Sequencing and analysis of the F20 cDNA revealed complete identity at the nucleotide level to murine CD9 (19). The reactivity of 9D3 to COS-1 cells transfected with F20 cDNA was confirmed by FMF analyses (data not shown).

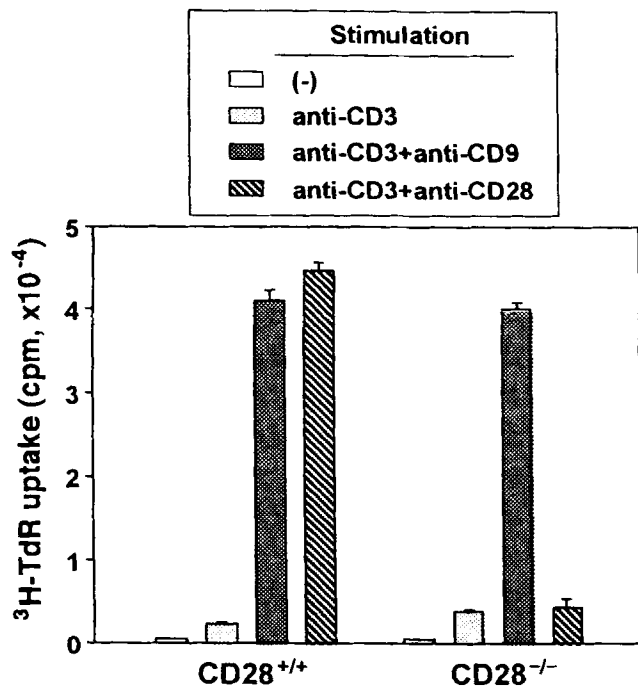
*The 9D3 (anti-CD9) mAb Provides T Cell Costimulation in the Absence of APC.* CD9 is a member of the transmembrane 4 superfamily (TM4SF), together with CD81 and CD82. In considering that CD81 and CD82 have the capacity to modulate the growth of T or B cells (24, 25), we examined the effect of the CD9 costimulation of TCR-stimulated T cells in the absence of APC (Fig. 2). The re-



**Figure 3.** Comparison of T cell costimulation between anti-CD28 and anti-CD9. Anti-CD3 (1  $\mu$ g/ml) alone or together with either anti-CD9 or anti-CD28 mAb (10  $\mu$ g/ml) were co-immobilized in wells of microplates. B6 T cells ( $2 \times 10^5$ /well) were cultured for various days.

sults (a representative of five experiments) show that the 9D3 mAb increased the proliferative response of purified C57BL/6 T cells when coimmobilized with submitogenic doses of anti-CD3 mAb: the 9D3 mAb immobilized at a given concentration (10  $\mu$ g/ml) activated T cells that were stimulated with suboptimal doses of anti-CD3 (Fig. 2 A), and anti-CD3 immobilized at a submitogenic dose (1  $\mu$ g/ml) induced 9D3 dose-dependent T cell proliferation (Fig. 2 B). Anti-CD9 costimulation was also observed using BALB/c purified T cells (data not shown). Neither immobilized 9D3 alone (Fig. 2) nor soluble 9D3 together with immobilized anti-CD3 (data not shown) induced proliferation. Thus, 9D3 cross-linking can provide a costimulatory signal necessary to induce T cell proliferation in response to suboptimal anti-CD3 stimulation.

*Comparison of T Cell Costimulation Provided by Anti-CD28 and Anti-CD9 mAbs.* We compared the costimulatory activity between anti-CD28 and anti-CD9 (9D3) mAbs. Purified C57BL/6 T cells were cultured for 1–3 d in wells coimmobilized with anti-CD28 or anti-CD9 (9D3) plus a suboptimal dose of anti-CD3 mAb. Optimal concentrations of anti-CD28 for costimulation were predetermined as 5–10  $\mu$ g/ml. The results of Fig. 3 (a representative of three performed) show that T cell proliferation by anti-CD9 costimulation is inducible as early as 1 d after culturing, reaching a peak response after 2 d. Anti-CD28 costimulation failed to elicit proliferation on day 1, and induced higher levels of proliferation 3 d rather than 2 d after stimulation. The peak responses induced by anti-CD9 and anti-CD28 were comparable.



**Figure 4.** Anti-CD9 can costimulate proliferation of T cells from CD28-deficient mice. Purified lymph node T cells ( $1.5 \times 10^5$ /well) from CD28<sup>+/+</sup> or CD28<sup>-/-</sup> mice were stimulated with immobilized anti-CD3 (0.5  $\mu$ g/ml) alone or together with 5  $\mu$ g/ml anti-CD9 (coimmobilized) or 2  $\mu$ g/ml anti-CD28 (in a soluble form) mAb.

*Anti-CD9-induced Costimulation Is CD28-independent.* To determine whether signaling through CD9 ligation was linked to CD28-mediated signaling, we examined the ability of anti-CD9 to costimulate T cells from wild-type and CD28-deficient (CD28<sup>-/-</sup>) mice. Purified T cells from either type of mice were stimulated with submitogenic doses of anti-CD3 alone or in the presence of coimmobilized 9D3 mAb. A representative of three experiments performed is shown in Fig. 4. Anti-CD9 cross-linking enhanced the proliferation of the CD28<sup>-/-</sup> T cells equivalently to wild-type T cells. Thus, anti-CD9-mediated costimulatory signals do not require the involvement of CD28 molecules and can function independently of CD28 molecules.

## Discussion

In the present study, we have developed a mAb capable of costimulating fresh T cells for potent proliferation. By expression cloning, the molecule recognized by this mAb (termed 9D3) was identified as CD9. Our results obtained using the 9D3 mAb demonstrated that CD9 is expressed on most mature T cells and thymocytes at high and weak levels, respectively, in addition to the MRL104.8a thymic stromal cells used as immunizing cells for preparation of mAbs. Anti-CD9 costimulation induced T cell proliferation in response to suboptimal anti-CD3 stimulation. The costimulation by anti-CD9 was as potent as that by anti-

CD28 and induced almost equally in T cells from wild-type and CD28<sup>-/-</sup> mice.

CD9 is a member of the recently discovered transmembrane four superfamily (TM4SF) (reviewed in reference 20). The TM4SF comprises a group of cell surface proteins such as CD81 (21–23), CD82 (22, 24), and CD53 (25), which are characterized by the presence of four hydrophobic domains. The precise biochemical function of the TM4SF is unknown, although recent studies largely suggest a role for this superfamily in the regulation of cell development (26, 27), proliferation/activation (26), adhesion (28) and motility (29). Our present results show that CD9, generally as a member of the TM4SF, joins a growing list of molecules responsible for cell activation and specifically in the T cell, delivers a costimulatory signal essential for cell activation (vide infra).

Regarding the detection of the CD9 expression using anti-human (20) or anti-murine CD9 mAb (KMC8) (29), earlier studies have shown that CD9 is not expressed by mature human B and T cells (20, 26) or slightly expressed by only subpopulations of mature murine B and T cells (29). In contrast, anti-CD9 mAb (9D3) established here strongly reacted with all mature murine T cells (Fig. 1) and mature murine B cells (our unpublished results). Thus, our anti-CD9 mAb permitted us to investigate the functional role for the CD9 molecule expressed on mature T cells.

The important finding of this study is that a member of the TM4SF, CD9, functions as a molecule that can deliver a costimulatory signal. Four of the TM4SF members have been suggested to couple to signal transduction pathways. These include platelet activation/aggregation and pre-B cell adhesion of CD9 (28), fresh T cell mitogenic effects of CD53 (25), and anti-proliferative effects of CD81 (21). However, there has been no report which shows the role of CD9 in T cell activation or proliferation, probably due to the lack of an appropriate anti-CD9 mAb. This study demonstrated that anti-CD9 costimulation induced potent proliferation by freshly isolated T cells when combined with suboptimal doses of anti-CD3 stimulation, providing the first evidence for the role of CD9 in T cell activation.

The mechanisms by which the TM4SF molecules including CD9 transduce a signal are not known. In this regard, the observations showing molecular interactions between TM4SF and immunoglobulin superfamily members may be worthwhile. For example, CD81 and CD82 have been shown to associate with the immunoglobulin superfamily molecules CD19 and CD21 (23) and CD4/CD8 molecules (22) on B or T cells. The B cell complex of CD81, CD19 and CD21 is considered to play an important costimulatory role in B cell activation (23). Similarly, the association of CD81 and CD82 with CD4 or CD8 has been observed in T cells (22) although the functional significance of these interactions is unknown. In contrast to CD81 and CD82, CD9 is likely to be associated with the integrin family members such as  $\beta$ 1 in pre-B cells (30) and  $\beta$ 3 integrin in platelets (31). An interesting speculation may be raised that the TM4SF plays a role in anchoring signal transduction complexes. However, it remains to be eluci-

dated whether CD9 on T cells is associated with some members of the immunoglobulin superfamily and/or integrin family and such an association is required for the manifestation of signal transduction.

Our present results illustrate the expression of CD9 molecules on mature T cells as well as its role for T cell activation. Further studies will be required to investigate the ex-

tracellular events leading to CD9 stimulation including the determination of the CD9-associated molecules and to elucidate CD9-mediated signal transduction. Such studies could contribute to a better understanding of differential roles of CD28 vs CD9 molecules in T cell costimulation as well as the biological significance of CD9 molecules in T cell activation.

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