

# Identification of a Costimulatory Molecule Rapidly Induced by CD40L as CD44H

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## Summary

The interaction between CD40 ligand and CD40 is critical for activation of T and B cells in vivo. We have recently demonstrated that this interaction rapidly induces a novel costimulatory activity distinct from B7 and independent of CD28. To study the molecular basis of the costimulatory activity, we have produced a novel monoclonal antibody, TM-1, that binds an 85-kilodalton costimulatory molecule rapidly induced by CD40L. Expression cloning reveals that TM-1 binds CD44H. CD44H expressed on Chinese hamster ovary cells has potent costimulatory activity for clonal expansion of T cells isolated from both wild-type mice and those with a targeted mutation of CD28. Thus, CD44H costimulates T cell proliferation by a CD28-independent mechanism. These results revealed that CD44H is a costimulatory molecule rapidly induced by CD40L.

Cognate interactions between T cells and B cells are critical not only for B cell activation but also for T cell responses. The interaction between CD40L and CD40 is one such example. It is now well established that this interaction is essential for proliferation and differentiation of B cells, particularly for the formation of germinal center and memory B cells (1–8). Recent data also illustrate an important role for such interaction in the activation of T cells. Two groups have recently reported that priming of CD4 T cells is defective in mice with a targeted mutation of either CD40 or CD40L (9, 10). While the mechanism for the defect is still unclear, an attractive hypothesis is that this interaction is important because of its induction of costimulatory activity on the APCs (11, 12). This hypothesis would explain the parallel induction of immunological tolerance when the TCR is engaged in the absence of costimulation (13–17) and when CD40L/CD40 interaction is blocked (18). Furthermore, using mice with a targeted mutation of the CD40L gene, we have recently demonstrated that CD40L is critical for optimal induction of costimulatory activity on APCs (19). This hypothesis has not been critically tested largely because the molecular basis of the costimulatory activity induced by CD40L/CD40 interaction is not well understood.

Several groups have presented evidence that CD40L can up-regulate costimulatory molecules such as B7-1 and/or B7-2 (1, 20, 21), and they have suggested the induced-expression of B7 family members as an explanation for the induction of costimulatory activity on B cells by CD40L. However, a careful examination of the kinetics of the induction of the costimulatory activity and that of B7 family members suggests that this is unlikely to be the case. We showed that CD40L induces significant costimulatory activity within 3 h; yet, B7-2 was not induced until 12 h, and B7-1 was not induced in the first 48 h after B cells were stimulated by CD40L (19). In addition, when activated T cells are incubated with B cells, CD40L is essential for optimal induction of costimulatory activity but not for that of B7-2. Furthermore, the CD40L-induced costimulatory activity functions in the absence of CD28, in contrast to B7 family members that induce a poor clonal expansion of CD28(–/–) CD4 T cells. Consistent with this notion, Schultz et al. has recently reported that induction of B7-1 and B7-2 does not explain the CD40L-induced costimulatory activity on a large panel of leukemic cell lines (22). Taken together, these studies demonstrate that CD40L rapidly induces a novel costimulatory activity on APCs.

To identify the costimulatory molecules induced by CD40L, we have generated an mAb, TM-1, that appears to bind a costimulatory molecule induced by CD40L for the following reasons (19). First, TM-1 almost completely inhibits the costimulatory activity induced by CD40L. Sec-

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ond, TM-1 epitope is induced more rapidly than B7-2, and the induction of TM-1 epitope correlates with that of the costimulatory activity. Third, TM-1 binds a molecule of ~85 kD, which is distinct from B7, HSA, and ICAM-1. Here we report that TM-1 and another mAb with similar properties, 9C5, bind CD44H and that CD44H has costimulatory activity for clonal expansion of T cells by a CD28-independent mechanism.

## Materials and Methods

**Experimental Animals, Cell Lines, and mAbs.** CBA/Caj and C57BL/6j mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice with a targeted mutation of CD28 (CD28KO mice) (23) were kindly provided by Dr. T. Mak (University of Toronto, Toronto, Ontario, Canada) and have been backcrossed to C57BL/6j for six generations. Spleens from these three strains of mice were used as the source of T and B cells, which were prepared as has been described (19).

Both COS cells and Chinese hamster ovary (CHO)<sup>1</sup> cells were cultured in DMEM containing 5% of FCS. The transient transfection of COS cells and stable transfection of CHO cells have been described (24).

Generation of hamster mAb TM-1 has been described (19). Another mAb, 9C5, was produced independently by a similar procedure. Fusion protein CTLA4Ig that is comprised of extracellular domain of CTLA4 and Fc portion of murine Ig was produced as described (25). Anti-CD3 mAb 2C11 (26) was used to engage the TCR/CD3 complex. HB224 (27), a hamster IgG mAb to murine CD11c, was used as control.

**Expression Cloning of Costimulatory Molecule Recognized by TM-1.** A cDNA library was prepared from a B leukemic cell line, RAW8.1, that expresses high levels of TM-1 epitope by using a previously described method (28). The cDNA library was used to transfect COS cells by DEAE-dextran method. 3 d after transfection, COS cells were harvested and incubated with TM-1 mAb (5 µg/ml) at 4°C for 1 h. Unbound mAb was removed by washing, and the COS cells were incubated in petri dishes precoated with goat anti-hamster IgG antibodies (Caltag, San Francisco, CA). 2 h later, the unbound cells were washed extensively with PBS; the cells adhering to the plates were lysed, and the episomal DNA was prepared as has been described (28). The cDNA was used to transform *Escherichia coli* MC1061/p3. The antibiotic-resistant colonies were amplified and pooled. Plasmids were prepared from pools of 50 colonies and used to transfect COS cells. Individual colonies from the positive pools were amplified, and their plasmids were tested for their ability to transfer TM-1 epitope into COS cells. The positive clone was sequenced by an automatic DNA sequencer.

**T cell Proliferation.** Given numbers of CD4 T cells isolated from mouse spleens were stimulated with 1:40 dilution of anti-CD3 hybridoma supernatants (2C11; Reference 26) in the presence of accessory cells. Syngeneic B cells cultured with either Ψ-2 or CD40L-transfected Ψ-2 for given periods were fixed with 0.1% paraformaldehyde for 5 min and used as accessory cells. In addition, FcR-transfected CHO cells (CHOFcR), or human B7-1-transfected CHOFCR, or CD44H-transfected CHOFCR were

treated with mitomycin C (50 µg/ml) and used as accessory cells. T cells cultured with accessory cells and anti-CD3 mAb for 42 h were pulsed with 1 µCi/well of <sup>3</sup>H-TdR for an additional 6 h, and the incorporation of <sup>3</sup>H-TdR was used as an indicator for T cell proliferation. Anti-CD44 mAbs or human CD44Ig that was known to block CD44H binding to hyaluronic acid (HA) (29) was added at the beginning of the culture to test the inhibition of T cell proliferation. (Data presented are means of duplicates, with variations always <15% of the means.)

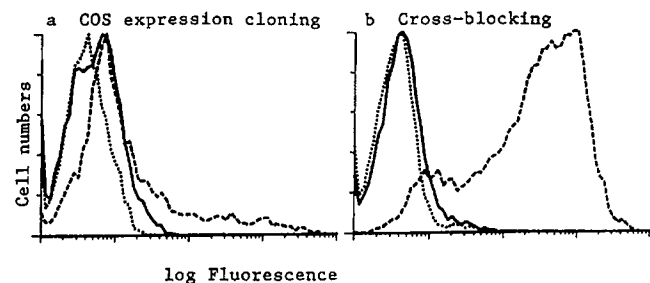
**Cross-blocking of mAb Binding to CD40L-activated B Cells.** To test whether TM-1 and anti-CD44H mAb IM1.7 (30) binds to the same molecules, we preincubated CD40L-activated B cells with unlabeled TM-1 or IM1.7 (100 µg/ml) for 30 min on ice. Biotinylated TM-1 or IM1.7 was then added. After another 30 min of incubation, unbound mAbs were washed away and cell surface binding of the biotinylated mAb was detected by phycoerythrin-streptavidin.

In other experiments, unlabeled rat mAb IM1.7 was used to block the binding of hamster mAb 9C5. The method is essentially identical except that FITC-labeled goat anti-hamster Ig (adsorbed by rat and mouse Ig) was used as the second-step reagent.

**Hyaluronate Binding Assay.** Unstimulated T-depleted spleen cells or those that have been cocultured with either untransfected or CD40L-transfected Ψ-2 cells for 16 h were incubated with FITC-labeled HA (FITC-HA) (31) for 45 min on ice as described (31). The specificity of the staining was verified by blocking with a fivefold excess of unconjugated HA. CHO cells that express endogenous hamster CD44 and bind HA well (29) were used as positive control.

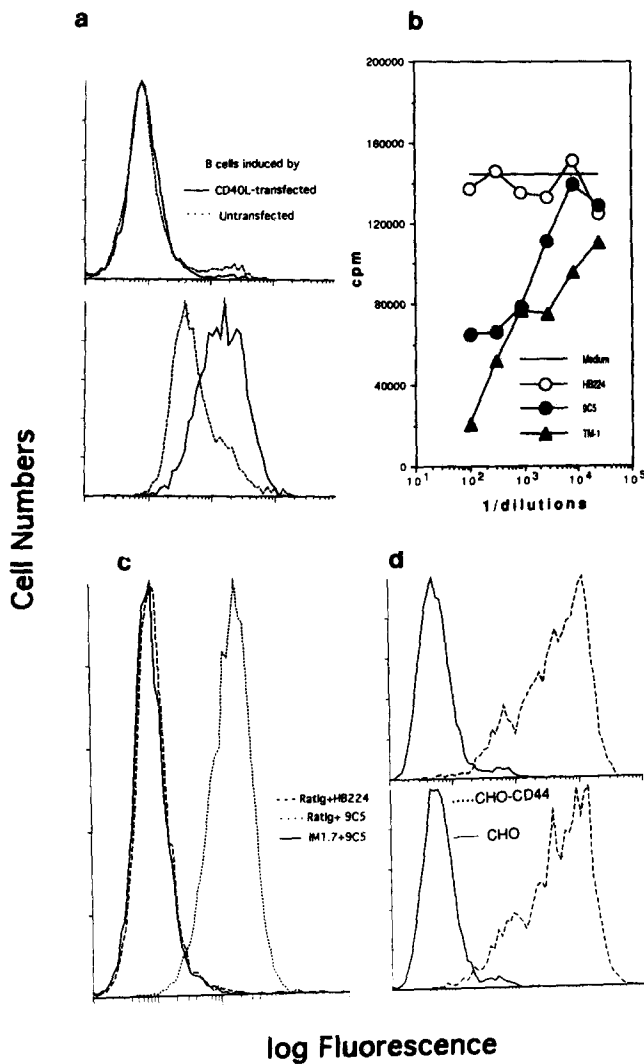
## Results

**Expression Cloning Reveals Two mAbs that Block CD40L Induced Costimulatory Molecules Bind CD44H.** We have prepared cDNA from a B leukemic cell line, RAW8.1, that

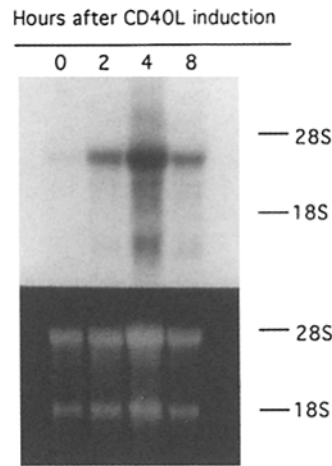


**Figure 1.** Expression cloning reveals that TM-1 binds CD44H. (a) A single clone 3F isolated from the cDNA library prepared from RAW8.1 cells transfer TM-1 epitope into COS cells. Mock-transfected or a single cDNA clone-transfected COS cells were incubated with mAb TM-1. This is followed by incubation with the second-step reagent, the FITC-labeled goat anti-hamster IgG, and analysis of the expression of TM-1 epitope by flow cytometry. ····, second-step reagent; ———, mock-transfected+TM-1; - - -, 3F-transfected+TM-1. (b) TM-1 and anti-CD44H mAb IM1.7 cross-block each other's binding to CD40L-activated B cells. B cells from CBA/Caj mice were stimulated by CD40L-transfected fibroblasts for 48 h. Viable cells isolated by centrifugation through a Ficoll-hypaque medium were incubated with 100 µg/ml of either TM-1, IM1.7, or normal hamster Ig for 30 min at 4°C. Biotinylated mAbs, either TM-1 or IM1.7, were added and incubated with the cells for another 30 min; the binding of biotinylated mAbs was detected by phycoerythrin-labeled streptavidin. ····, second-step control; ——— @CD44+ TM-1-biotin; - - -, medium+TM-1-biotin.

<sup>1</sup>Abbreviations used in this paper: CHO, Chinese hamster ovary; CHOFCR, FcR-transfected CHO cells; FITC-HA, FITC-labeled HA; HA, hyaluronic acid.



**Figure 2.** mAb 9C5 binds to a CD40L-induced costimulatory molecule and recognizes CD44H. (a) 9C5 recognized a CD40L-induced molecule on B cells. B cells from C57BL/6j mice were stimulated with either  $\Psi$ -2 or CD40L-transfected  $\Psi$ -2 for 16 h, and the expression of the 9C5 epitopes was determined by flow cytometry. (b) 9C5 blocks the CD40L-induced costimulatory activity. Normal B cells from C57BL/6j mice were cocultured with CD40L-transfected  $\Psi$ -2 cells for 16 h, fixed with 0.1% paraformaldehyde for 5 min, and used as accessory cells for proliferation of CD4 T cells to anti-CD3 mAb 2C11 (added as 1:40 dilution of hybridomas supernatants). Given dilutions of supernatants from hybridomas (9C5, TM-1, or control hamster mAb HB224 that contain  $\sim 10 \mu\text{g/ml}$  mAb) were added at the beginning of the culture. (c) The binding of 9C5 epitope was blocked by anti-CD44H mAb IM1.7. B cells cocultured with CD40L-transfected  $\Psi$ -2 cells for 16 h were preincubated with either normal rat Ig or anti-CD44H mAb IM1.7 (100  $\mu\text{g/ml}$ ) for 30 min. The 9C5 hybridoma supernatants were added, and its binding was determined by using mouse/rat Ig adsorbed, FITC-labeled goat anti-hamster IgG. (d) mAb 9C5 binds CD44H-transfected, but not untransfected CHO cells. Stable CD44H-transfected or untransfected CHO cells were incubated with either 9C5 (top) or TM-1 (bottom) for 30 min, the binding of mAbs was determined by flow cytometry using FITC-labeled, goat anti-hamster IgG as the second-step reagent.

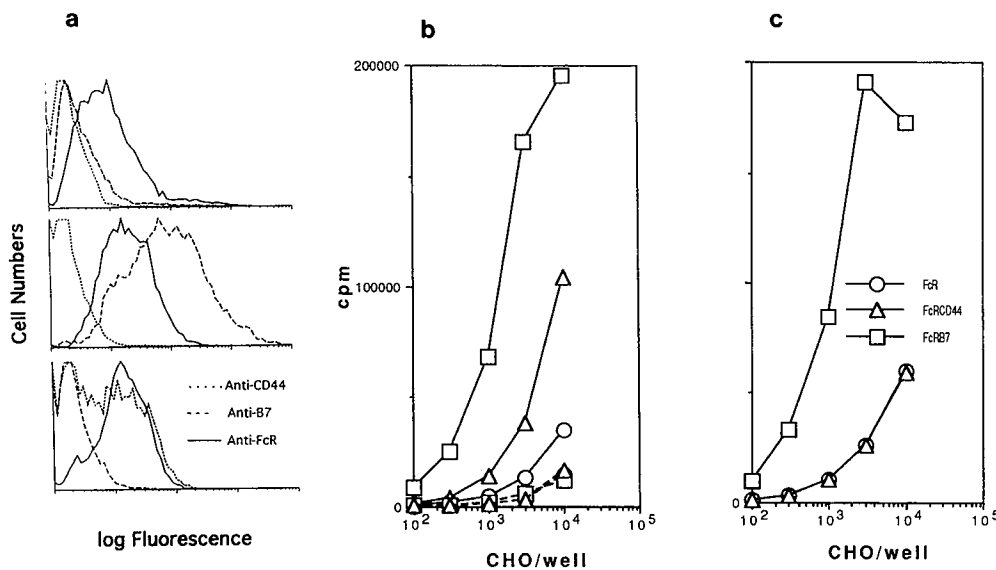


**Figure 3.** Rapid induction of CD44 mRNA by CD40L. B cells from CBA/CaJ mice were stimulated with CD40L-transfected fibroblasts for 0, 2, 4, and 8 h. The total cytoplasmic RNA were isolated, and the amount of the CD44 mRNA was detected by Northern blot using  $^{32}\text{P}$ -labeled CD44H cDNA as probe. The amounts of RNA loaded are shown at the bottom.

has a high level of TM-1 epitopes. The COS cells were transfected with the cDNA library, and the TM-1 epitope-expressing COS cells were enriched by panning using TM-1. The small numbers of cells binding to the TM-1 mAb were lysed, and the episomal DNA were used to transform *E. coli*. 800 colonies were amplified, and cDNA from pools of 50 colonies were used to transfect COS cells. Episomal DNA prepared from one of such pools transferred the TM-1 reactivity to COS cells. A single clone was identified that contains the gene encoding TM-1 (Fig. 1 a). DNA sequencing of the whole insert reveals that the cDNA encodes CD44H (data not shown). Consistent with this, TM-1 and anti-CD44H mAb IM1.7 cross-block each other's binding to CD40L-induced B cells (Fig. 1 b). The extent of blocking suggests that the majority of the molecules recognized by TM-1 is CD44H.

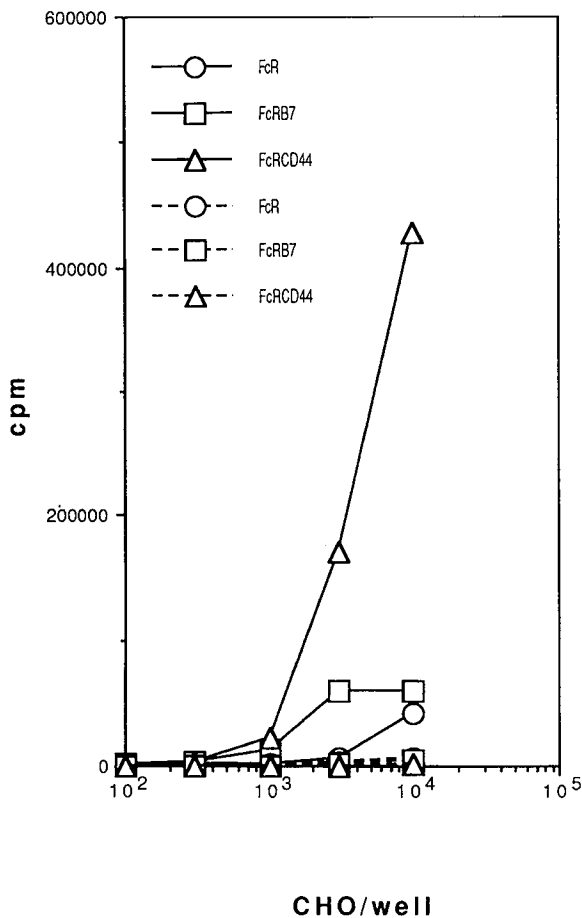
We have recently produced another mAb, 9C5, that has similar properties to TM-1. As shown in Fig. 2 a, 9C5 binds to a molecule induced by CD40L-transfected cells but not untransfected  $\Psi$ -2 cells. In addition, 9C5 is a potent inhibitor of the clonal expansion of CD4 T cells when CD40L-activated T cells were used as accessory cells (Fig. 2 b). To test whether 9C5 also binds CD44H, we tested whether prototypic anti-CD44H mAb IM1.7 inhibits the binding of 9C5 to CD40L-induced B cells. As shown in Fig. 2 c, IM1.7 completely blocks the binding of 9C5, which strongly suggests that 9C5 also binds CD44H. That 9C5 binds CD44H is confirmed by its binding to CD44H-transfected, but not parental, CHO cells (Fig. 2 d). Thus, two independently derived mAbs that recognize CD40L-induced costimulatory activity bind CD44H. Consistent with this, the prototype anti-CD44H mAb IM1.7 also blocks CD44H-induced costimulatory activity (data not shown).

**Rapid Induction of CD44H mRNA by CD40L.** To test whether CD44H is up-regulated by the CD40L at the level of transcription, we isolated total RNA from B cells that have been precultured with CD40L-transfected  $\Psi$ -2 cells. As shown in Fig. 3, CD44 mRNA is rapidly up-regulated by CD40L. After normalizing the RNA loading, we can



**Figure 4.** CD44H expressed on CHO cells has costimulatory activity for the clonal expansion of T cells. (a) Levels of FcR, CD44H, and B7 on CHO cells transfected with FcR (top), FcR + human B7-1 (middle), and FcR + murine CD44H (bottom). The expression of FcR, CD44, and B7 was determined using either mAbs (2.4G2 for FcR; TM-1 for CD44) or fusion protein (CTLA4Ig for B7). (b) Induction of clonal expansion of CD4 T cells. Given numbers of CHO cells transfected with either FcR, FcR + B7, or FcR + CD44H were used as accessory cells after being fixed by 0.15% paraformaldehyde for 5 min. CD4 T cells ( $10^5$ /well) were stimulated with 1:40 dilution of anti-CD3 mAb 2C11 for 42 h, and the proliferation of CD4 T cells was determined by

incorporation of  $^3\text{H}$ -TdR in the subsequent 6 h. Solid lines represent the incorporation of  $^3\text{H}$ -TdR in cultures containing both CD4 T cells and CHO cells; whereas dotted lines represent that of CHO cells alone. (c) Inhibition of CD44H-mediated costimulatory activity by TM-1, as in b, except that TM-1 was added at a final concentration of 1.5  $\mu\text{g}/\text{ml}$ .



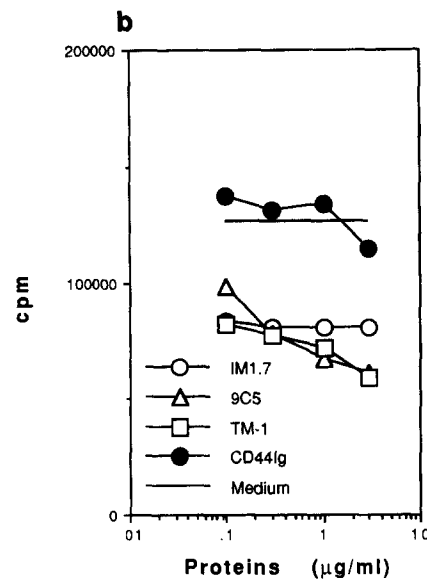
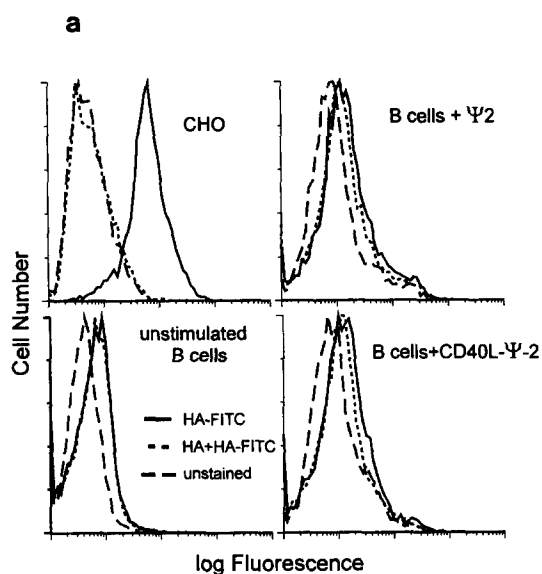
**Figure 5.** CD28 (-/-) CD4 T cells respond to costimulatory activity of CD44H but poorly to that of B7. CD4 T cells ( $1.5 \times 10^5$ /well) isolated from CD28-deficient mice were stimulated with anti-CD3 mAb in

the presence of CHO cells transfected with either FcR or FcR plus either B7 or CD44H, and the proliferation of CD4 T cells was determined by incorporation of  $^3\text{H}$ -TdR, as detailed in Fig. 4 legends. Solid lines represent the incorporation of  $^3\text{H}$ -TdR in cultures containing both CD4 T cells and CHO cells, whereas dotted lines represent that of CHO cells alone.

*CD44H Expressed on CHO Cells Has Costimulatory Activity for the Clonal Expansion of T Cells.* We have transfected CD44H cDNA in conjunction with FcR into CHO cells to test whether CD44H has costimulatory activity for T cells. As shown in Fig. 4, CHO cells cotransfected with CD44H and FcR induce significantly higher proliferation of CD4 T cells, as compared to CHO cells transfected with FcR alone. These results are reproduced by multiple experiments using two independently derived CD44H transfectants (data not shown). Moreover, anti-CD44H mAb TM-1 significantly blocks the costimulatory activity of CD44H-transfected CHO cells, yet it has no effect on the function of CHO cells transfected with B7 + FcR. Thus, CD44H provides sufficient costimulatory activity for clonal expansion of CD4 T cells. Interestingly, we observed a slight enhancement of T cell proliferation by anti-CD44H mAb when CHO/FcR were used as accessory cells.

To test whether the costimulatory activity of CD44H requires CD28, we isolated CD4 T cells from mice with a targeted mutation of CD28. Although CD4 T cells from CD28-deficient mice respond poorly to B7, as has been described (32), they retain their responsiveness to CD44H (Fig. 5).

the presence of CHO cells transfected with either FcR or FcR plus either B7 or CD44H, and the proliferation of CD4 T cells was determined by incorporation of  $^3\text{H}$ -TdR, as detailed in Fig. 4 legends. Solid lines represent the incorporation of  $^3\text{H}$ -TdR in cultures containing both CD4 T cells and CHO cells, whereas dotted lines represent that of CHO cells alone.



**Figure 6.** HA is unlikely to be the receptor that transduces the costimulatory activity of CD44H. (a) CD40L does not induce B cell binding to HA. CHO cells, unstimulated B cells, or B cells cocultured with  $\Psi$ -2 or CD40L-transfected  $\Psi$ -2 cells for 16 h were isolated and assayed for their ability to bind FITC-HA. Solid lines depict the fluorescence profiles of cells incubated with FITC-HA, long dash lines depict those for autofluorescence, whereas the dotted lines depict the fluorescence of cells incubated with unlabeled HA for 20 min before addition of FITC-HA. (b) Human CD44Ig does not block the costimulatory activity of murine CD44-transfected CHO cells. CD28(-/-) CD4 T cells ( $1.5 \times 10^5$ /well) were stimulated with CHO cells

transfected with either FcR or FcR+CD44H, as detailed in the legend for Fig. 5. Anti-CD44H mAbs TM-1, 9C5, and IM1.7 and fusion protein HA-Ig were added at the beginning of the culture. Mitomycin C-treated CHO cells were added at 3,000 cells/well. Mean cpm for CHO/FcR/CD44H cells alone was 2,590. Representatives of two independent experiments.

A critical question is the identity of the receptor on T cells that recognizes the CD44H on CD40L-induced B cells. To determine whether the best characterized CD44H ligand, the HA (29), is involved in costimulation by CD44H, we tested if CD40L enhances B cell binding to HA. As shown in Fig. 6 a, much like resting B cells, CD40L-induced B cells do not bind to HA. CHO cells that express hamster CD44H bind HA significantly. Thus, CD40L-induced CD44H does not bind HA. These results strongly suggest that HA is not the receptor on T cells that receive the costimulatory activity of CD40L-induced CD44. To formally rule out the involvement of HA, we used human CD44Ig, which is known to react with mouse HA, to block T cell proliferation when CHO cells transfected with mouse CD44H were used as accessory cells. As shown in Fig. 6 b, three anti-CD44H mAbs, but not CD44Ig, block T cell proliferation. These results strongly suggest that HA-binding is unlikely to be responsible for the costimulatory activity of CD44H.

## Discussion

CD40L induces costimulatory activity on B cells and perhaps other CD40<sup>+</sup> cells such as dendritic cells, macrophages, and epithelial cells (1, 19–21). Although it is known that CD40L can induce B7 family member B7-1 and B7-2, such induction does not fully explain CD40L-induced costimulatory activity on B cells because the induction of costimulatory activity precedes that of B7-2 (19). This disparity in kinetics of induction reveals that the CD40L-induced costimulatory activity is at least partly distinct from B7 family members. To study the molecular basis of CD40L-induced costimulatory activity, we have recently produced an mAb, TM-1, that blocks such costimulatory activity (19).

We showed by expression cloning that TM-1 and 9C5, another independently derived mAb of similar properties, binds CD44H. In addition, we showed that CD44H expressed on CHO cells has costimulatory activity for clonal expansion of CD4 T cells. These results demonstrate that CD44H is a costimulatory molecule rapidly induced by CD40L. CD44H is expressed at a low level in resting T and B cells (33, 34). It is upregulated and posttranslationally modified after lymphocyte activation (34). However, such rapid induction of CD44H by CD40L, as reported here, has not been documented. Several previous studies have implicated a role of CD44H (on T cells) in T cell costimulation, perhaps as a receptor for costimulatory molecules on APC (35, 36). These studies may explain the augmentation of T cell proliferation by anti-CD44H mAb when the FcR-transfected CHO cells were used as APCs. Nevertheless, our study appears to be the first to directly demonstrate a role of CD44H as a costimulatory molecule on the APCs, and the first direct demonstration that recombinant CD44H is sufficient to costimulate proliferation of T cells by a CD28-independent mechanism.

Corresponding to the heterogeneity of the CD44 molecules, a large array of CD44 ligands have been described. The best characterized ligand for CD44 is HA (29). This binding requires at least two basic amino acids spaced by seven amino acids (37). It also requires clustering of CD44 molecules since mutations affecting this process have been reported to interfere with this interaction (31, 38, 39). The CD44H form (containing no variable exon) is known to bind HA (31). On lymphocytes, such binding can be regulated by cellular activation events (40). Several other molecules, such as fibronectin (41), collagen (42), serglycin (43), the chondroitin sulfate form of invariant chain encoded by MHC region (44), as well as CD44R1 (v8-10 containing

CD44) (45), have also been reported to bind CD44. The receptor on T cells that interacts with the CD40L-induced CD44H to transduce the costimulatory signal remains to be identified. As HA-binding of CD44H is known to be regulated by cellular activation (40), it is of great interest to determine whether HA is such a receptor. Our results presented here demonstrate that CD40L-induced B cells do not bind HA and that human CD44Ig that binds HA does not block T cell proliferation when CD44H-transfected CHO cells were used as accessory cells. Thus, it is very unlikely that HA is the receptor responsible for the costimulatory activity of CD44H expressed on APCs. These results may also explain poor costimulatory activity of CHO cells despite of their strong binding to HA.

Recent studies demonstrate that CD40L is involved in T cell priming *in vivo* (9, 10). Several different hypotheses can be proposed to explain the role of CD40L. First, CD40L may be delivering costimulatory signal to T cells, as has been suggested by Cayabyab et al. (46). However, in

our experience, T cells from CD40L-deficient mice respond to costimulatory activity on the previously activated B cells (47). Thus, CD40L is unlikely to be necessary for T cells to receive costimulatory activity from APCs. Second, CD40L may be involved in inducing costimulatory activity on the APCs. The second hypothesis is attractive because results from numerous experiments show that CD40L/CD40 interaction is both necessary and sufficient for inducing costimulatory activity on B cells (1, 19–21). In addition, blocking CD40L/CD40 interaction facilitates induction of tolerance by B cells (18), consistent with the idea that T cell costimulation was blocked by this treatment (13–17). Our current study shows that CD44H is one such costimulatory molecule that fulfills all known properties of the costimulatory molecules induced by CD40L, namely, rapid induction, and CD28-independence in function. Our study should also facilitate the effort to determine the molecular basis of CD40L function in T cell responses.

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