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CD30 Expression Identifies the Predominant Proliferating T Lymphocyte Population in Human Alloimmune Responses

Keith W. Chan,* Corwyn D. Hopke,† Sheri M. Krams,*† and Olivia M. Martinez2*†

CD30 is an inducible member of the TNFR superfamily that is expressed on activated T and B cells and some lymphoid malignancies. We have previously shown that human CD30+ T cells elicited with allogeneic APC are a major source of IFN-γ and IL-5 production. In the present study we have used alloantigen, as well as anti-CD3 plus anti-CD28 mAb stimulation, to further characterize human CD30+ T cells with respect to function and the expression of other activation-dependent cell surface molecules, including the related TNFR family members OX-40 and 4-1BB (CD137). Our results indicate that human CD30+ T cells are a subset of activated T cells that also express CD25 and CD45RO. Moreover, we observed that allogeneic APC consistently induced a greater proportion of CD30+ cells within the activated T cell population than did stimulation with plate-bound anti-CD3 plus anti-CD28 mAb or stimulation with soluble anti-CD3 plus anti-CD28 and autologous APC. The enhanced induction of CD30 expression by alloantigen was not common to other inducible TNFR family members because anti-CD3 plus anti-CD28 mAbs were far more effective in inducing expression of 4-1BB and ON-40. Furthermore, CD30 expression marked the predominant proliferating T cell population induced by alloantigen as determined by CFSE staining and flow cytometry. These results indicate that CD30, but not 4-1BB or ON-40, is preferentially induced by alloantigen, suggesting that CD30 may be important in human alloimmune responses. The Journal of Immunology, 2002, 169: 1784–1791.

CD30 is a 120-kDa type 1 transmembrane glycoprotein and a member of the TNFR superfamily (1). CD30 was originally identified as a molecule expressed on Hodgkin and Reed Sternberg cells in Hodgkin’s disease but has subsequently been detected on certain non-Hodgkin’s malignancies as well as some virally transformed cells (2). CD30 is also expressed on a subset of normal human T lymphocytes following activation with Ag or mitogen (3, 4). Expression of CD30 has been proposed to preferentially mark Th2 cells (5), although CD30 has also been detected on CD4+ Th0 and Th1 cells, indicating that CD30 is not restricted to Th2 cells (6, 7). We (8) and others (9) have demonstrated that primary stimulation of human T cells with alloantigen or anti-CD3 mAbs and IL-2 elicits a population of CD30+ T cells that is a major source of IFN-γ and IL-5 production.

Several lines of evidence indicate that CD30 acts as a costimulatory molecule in T cell responses. The human CD30 ligand (CD30L; CD153) is expressed in the periphery on activated T cells, monocytes, B cells, neutrophils, and eosinophils. Engagement of CD30 by CD30L+ cells or agonist anti-CD30 mAbs induces IFN-γ production (9) and proliferation (10) in anti-CD3-stimulated peripheral blood T cells, NF-kB activation, and viral expression in an HIV-infected T cell line (11), and Ca2+ flux in Jurkat cells (3). Signaling through CD30 has also been suggested to induce apoptosis during negative selection in the thymus (12, 13), although some studies fail to support a role for CD30 in this process (14).

Other inducible members of the TNFR family expressed on T cells, including 4-1BB and ON-40, have also been shown to have costimulatory function. The interaction between 4-1BB (CD137) and 4-1BB ligand (4-1BBL), expressed on activated B cells, macrophages, and dendritic cells, is particularly important in CD8-mediated responses to alloantigen (15–17), viruses (18, 19), and tumors (19), although recent studies also suggest a role for 4-1BB/4-1BBL interactions in CD4+ T cell function (20). ON-40, in contrast, primarily promotes cytokine production by CD4+ T cells (21–23) and has been implicated in T cell function in animal models of allogeneic graft-vs-host disease (GVHD) (24), experimental allergic encephalomyelitis (25), leishmaniasis (26), inflammatory bowel disease (27), and tumor immunity (28). 4-1BB and ON-40, like CD30, tend to be expressed relatively late following activation, suggesting these molecules may be important in sustaining T cell responses after the initial CD28/B7-1 and B7-2 interactions. Despite the observed similarities in expression and function of CD30, ON-40, and 4-1BB in murine systems, relatively little is known about the expression patterns of these inducible TNFR family members on human T lymphocytes. The purpose of the current study was to examine the relationship of CD30 expression to the expression of other inducible TNFR family members on human T cells and to further characterize the phenotype and function of CD30+ T cells in alloimmune responses.

Materials and Methods

Abs and reagents

The following Abs were purchased from DAKO (Carpinteria, CA): anti-CD3-FITC, anti-CD3-PECy5; anti-CD4-PE, anti-CD4-PECy5, anti-CD8-PECy5, anti-CD30-FTC, anti-CD25-PE, IgG1-FITC, IgG1-PE, and PE-conjugated rabbit anti-mouse Ig. PE-conjugated mAbs specific for CD25, CD4, CD19, CD27, and CD45RO, as well as IgG1-PECy5, were obtained from BD PharMingen (San Diego, CA). Anti-CD28 and anti-CD30 mAbs were purchased from BD PharMingen and anti-CD3 mAb was purchased from Coulter (Miami, FL). Human rIL-2 was obtained from the Department of Surgery and † Program in Immunology, Stanford University School of Medicine, Stanford, CA 94305-5492. E-mail address: omm@stanford.edu

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3 Abbreviations used in this paper: CD30L, CD30 ligand; 4-1BBL, 4-1BB ligand; NF-κB, nuclear factor-κB; IFN-γ, interferon-γ; IL-5, interleukin-5; T cells, CD4+ T cells, CD8+ T cells, alloantigen, alloreactive T cells, allogeneic T cells, APC, antigen-presenting cells, OX-40, CD95 ligand; 4-1BB, CD137 ligand; CD27, CD28, CD30, OX-40, 4-1BB, CD27, and CD45RO, as well as IgG1-PECy5, were obtained from BD PharMingen (San Diego, CA). Anti-CD28 and anti-CD30 mAbs were purchased from BD PharMingen and anti-CD3 mAb was purchased from Coulter (Miami, FL). Human rIL-2 was obtained from the Department of Surgery and † Program in Immunology, Stanford University School of Medicine, Stanford, CA 94305-5492. E-mail address: omm@stanford.edu

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Lymphoblastoid B cell lines (LCL) were maintained in RPMI 1640 with 10% FCS and 1% penicillin-streptomycin (PS) in a 5% CO₂ atmosphere at 37°C. Blood was collected from healthy donors by venipuncture in heparinized tubes. PBMC were isolated from blood samples by Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) density centrifugation. PBMC were washed, counted, and cultured in RPMI 1640 with 10% normal human serum (NHS; Sigma-Aldrich) and 1% PS at 37°C in a humidified incubator with 5% CO₂. Cells were isolated by magnetic cell selection using the MiniMACS (Miltenyi Biotec, Auburn, CA) separation protocol. To obtain purified T cells, PBMC were washed and resuspended at a concentration of 1.25 × 10⁶/ml. Twenty microliters of Hapten-Ab Cocktail (Miltenyi Biotec), containing hapten-conjugated-anti-CD11b, anti-CD16, anti-CD19, anti-CD36, and anti-CD356 mAbs, was added per 10⁵ cells and the cells were incubated for 10 min at 4°C. The cells were then washed and resuspended at a concentration of 1.25 × 10⁶/ml in PBS containing 1% FCS and 2 mM EDTA (PBS/BS/EDTA). Twenty microliters of MACS AntiHapten Microbeads (Miltenyi Biotec) was added per 10⁵ cells and the cell suspension was incubated for 15 min at 4°C. The cells were washed and resuspended in 500 μl of PBS/BS/EDTA, loaded onto a MACS Column Type LS/VS² (Miltenyi Biotec), and allowed to filter through the column. The column was flushed four times with 3 ml of PBS/BS/EDTA and the unbound cells were eluted. This negative selection enrichment routinely resulted in a cell population consisting of >98% T cells as determined by immunofluorescent staining and flow cytometry.

**Mixed lymphocyte reaction**

Stimulator-responder cell pairs in MLR were selected for HLA disparity based on previously performed HLA typing. Mismatches were confirmed in preliminary experiments using one-way MLR and [³H]thymidine incorporation on day 6 as a measure of cell proliferation. MLR were done as previously described (8). Briefly, PBMC were suspended at 1 × 10⁶ cells/ml in RPMI 1640 with 10% heat-inactivated NHS and 1% PS and plated in 24-well cell culture plates (Costar, Cambridge, MA) at 1 × 10⁶ cells/well. One million irradiated (5000 rad) HLA-incompatible LCL as allogeneic APC were added to each well to yield a final volume of 2 ml. MLR were incubated at 37°C at 5% CO₂ for 1–8 days depending upon the speed of the immune response. The wells were washed twice with PBS/BS and supernatants were collected for flow cytometry and cytokine assays, respectively. A total of four different stimulator-responder pairs were used in subsequent experiments on CD30 expression and function and each yielded similar findings.

**Primary and secondary in vitro T cell activation**

Purified T cells were washed and cultured at 1 × 10⁶/ml in RPMI 1640 with 10% heat-inactivated NHS and 1% PS in 24-well plates (Costar) that had been precoated with anti-CD3 mAbs (10 μg/ml) plus anti-CD28 mAbs (10 μg/ml). For stimulation with soluble mAbs, PBMC (1 × 10⁷/ml) were cultured with anti-CD3 alone (10 ng/ml) or anti-CD3 (10 ng/ml) plus anti-CD28 (5 μg/ml). Parallel cultures were established containing 1 × 10⁶ T cells and 1 × 10⁶ irradiated, allogeneic APC. In some experiments cells were harvested on days 3–8 for phenotypic analysis by immunofluorescent staining and flow cytometry. In other experiments, after 4 days the cultures were supplemented with IL-2 (100 U/ml) and maintained for an additional 3 days. On day 7, all cultures were washed four times with PBS and resuspended at 1 × 10⁶/ml. Twenty microliters of Hapten-Ab Cocktail was added per 10⁵ cells and the cells were incubated for 10 min at 4°C. The cells were then washed and resuspended in 500 μl of PBS/BS/EDTA. Twenty microliters of MACS AntiHapten Microbeads (Miltenyi Biotec) was added per 10⁵ cells and the cell suspension was incubated for 15 min at 4°C. The cells were washed and resuspended in 500 μl of PBS/BS/EDTA, loaded onto a MACS Column Type LS/VS² (Miltenyi Biotec), and allowed to filter through the column. The column was flushed four times with 3 ml of PBS/BS/EDTA and the unbound cells were eluted. This negative selection enrichment routinely resulted in a cell population consisting of >98% T cells as determined by immunofluorescent staining and flow cytometry.

**Immunofluorescent staining and flow cytometry**

Freshly isolated PBMC, anti-CD3 plus anti-CD28 mAb-stimulated, and alloactivated mononuclear cells were analyzed for cell surface Ag expression by two- and three-color immunofluorescence and flow cytometry. Cells were incubated with FITC-, PE-, and PE-Cy5-conjugated mAbs specific for CD3, CD4, CD8, CD25, CD45RO, OX-40, 4-1BB, and CD27, or fluorescein-conjugated isotype-matched control mAbs for 30 min at 4°C. The cells were then washed and resuspended in PBS/BS/EDTA and acquired on a FACScan (BD Biosciences, Mountain View, CA) with CellQuest software. Cells were gated on forward and side scatter and a minimum of 20,000 cells were collected for each sample. When cells were stained with anti-CD3-PECy5, anti-CD4-PECy5, or anti-CD8-PECy5 in addition to FITC- and PE-conjugated mAbs, the cells were further gated on the CD3-, CD4-, or CD8-positive populations to analyze T cell subsets.

**CFSE labeling and analysis of cell division by flow cytometry**

Isolated PBMC were washed, counted, and resuspended at 5 × 10⁶/ml in RPMI 1640 with 1% PS. A stock solution of CFSE (5 mM) was diluted 1/10 with RPMI 1640 and 15 μl of the CFSE was added per milliliter of cells. The cell suspension was incubated for 10 min at 37°C. The cells were then washed and cultured in RPMI 1640 supplemented with 10% NHS and 1% PS on plates precoated with anti-CD3 plus anti-CD28 mAbs or with allogeneic APC in MLR as described above. Cells were collected on days 3–6, washed with PBS/FCS, and stained with unconjugated mouse anti-human CD30 mAbs for 20 min at 4°C. Cells were washed twice and labeled with PE-conjugated rabbit anti-mouse Ig for 20 min at 4°C. Cells were again washed and labeled with PE-Cy5-conjugated anti-CD3 mAb for 20 min at 4°C. After a final wash, the cells were acquired and analyzed on a FACScan. Twenty thousand cells were collected and gating was set by forward and side scatter.

**Measurement of cytokine production**

Quantification of IFN-γ, IL-2, IL-5, and TNF-α was performed as previously described (29). Briefly, on day 1, a 96-well microtiter plate (Costar) was coated with 50 μl capture Ab (2 μg/ml; BD PharMingen) specific for the cytokine of interest and incubated overnight at 4°C. On day 2, the plate was washed twice with PBS/0.05% Tween and blocked with PBS containing 3% BSA for 2 h at room temperature. The wells were washed twice with PBS/Tween and sample supernatants were loaded onto the plate in duplicate. A standard curve (0–1000 pg/ml) was generated by diluting recombinant cytokine (BD PharMingen) with PBS containing 1% BSA. The plate was covered and incubated at 4°C overnight. On day 3, the plate was washed four times with PBS/Tween, biotinylated anti-human cytokine Ab (1 μg/ml) was added, and the plate was incubated for 45 min at room temperature. The plate was then washed six times with PBS/Tween and was incubated with avidin-HRP (5 μg/ml; Sigma-Aldrich) for 30 min at room temperature. The plate was washed six times with PBS/Tween before developing with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) in phosphate-citrate buffer. The reaction was stopped with 2 N H₂SO₄ and the plate was read at 450 nm on an ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

**Results**

CD30 is expressed on a subset of activated human T cells that also express CD25 and CD45RO.

We previously demonstrated that CD30 is induced on both CD4 and CD8 human T cells following stimulation with alloantigen (8). Alzona et al. (9) reported that CD30 expression and function and each yielded similar findings. A total of four different stimulator-responder pairs were used in subsequent experiments on CD30 expression and function and each yielded similar findings. In preliminary experiments using one-way MLR and [³H]thymidine incorporation on day 6 as a measure of cell proliferation, MLR were done as previously described (8). Briefly, PBMC were suspended at 1 × 10⁶ cells/ml in RPMI 1640 with 10% heat-inactivated NHS and 1% PS and plated in 24-well cell culture plates (Costar, Cambridge, MA) at 1 × 10⁶ cells/well. One million irradiated (5000 rad) HLA-incompatible LCL as allogeneic APC were added to each well to yield a final volume of 2 ml. MLR were incubated at 37°C at 5% CO₂ for 1–8 days depending upon the speed of the immune response. The wells were washed twice with PBS/BS and supernatants were collected for flow cytometry and cytokine assays, respectively. A total of four different stimulator-responder pairs were used in subsequent experiments on CD30 expression and function and each yielded similar findings. In preliminary experiments using one-way MLR and [³H]thymidine incorporation on day 6 as a measure of cell proliferation, MLR were done as previously described (8). Briefly, PBMC were suspended at 1 × 10⁶ cells/ml in RPMI 1640 with 10% heat-inactivated NHS and 1% PS and plated in 24-well cell culture plates (Costar, Cambridge, MA) at 1 × 10⁶ cells/well. One million irradiated (5000 rad) HLA-incompatible LCL as allogeneic APC were added to each well to yield a final volume of 2 ml. MLR were incubated at 37°C at 5% CO₂ for 1–8 days depending upon the speed of the immune response. The wells were washed twice with PBS/BS and supernatants were collected for flow cytometry and cytokine assays, respectively. A total of four different stimulator-responder pairs were used in subsequent experiments on CD30 expression and function and each yielded similar findings.
FIGURE 1. CD30⁺ T cells also express CD25 and CD45RO following primary stimulation with allogeneic APC (A) or anti-CD3 plus anti-CD28 mAbs (B). For CD25 analysis, PBMC were cultured with allogeneic APC for 8 days or plate-bound anti-CD3 and anti-CD28 mAbs for 4 days and then labeled with fluorochrome-conjugated isotype controls or the following mAbs: CD3-Cy5PE, CD25-PE, and CD30-FITC. For CD45RO analysis, PBMC were cultured with allogeneic APC for 7 days or plate-bound anti-CD3 plus anti-CD28 mAbs for 3 days, and then labeled with fluorochrome-conjugated isotype controls or the following mAbs: CD3-Cy5PE, CD30-FITC, and CD45RO-PE. The data were analyzed by three-color immunofluorescence with gates established on CD3⁺ cells. The percentage of cells found in each quadrant is indicated. Similar results were obtained from different experiments and data shown are from one representative experiment.

Effect of APC on the induction of CD30 expression on T lymphocytes

In the previous experiments we observed that allogeneic APC were more effective than plate-bound anti-CD3 plus anti-CD28 mAbs in inducing the expression of CD30 on T cells. One possible explanation for the difference in the extent of CD30 expression is that the plate-bound anti-CD3 plus anti-CD28 system lacks APC, whereas in the MLR allogeneic APC are present. Alternately, there may be intrinsic differences in T cell activation by alloantigen as compared with anti-CD3 plus anti-CD28 mAbs that explain the enhanced CD30 expression in MLR. To distinguish between these possibilities we compared CD30 expression on T cells activated by soluble anti-CD3 plus anti-CD28 mAbs and autologous APC with CD30 expression on T cells activated by plate-bound anti-CD3 plus anti-CD28 mAbs in the absence of APC (Fig. 2). Stimulation with soluble anti-CD3 plus anti-CD28 mAbs and autologous APC with CD30 expression on T cells activated by plate-bound anti-CD3 plus anti-CD28 mAbs led to distinct expression patterns of the TNFR family members CD30, 4-1BB, and OX-40.

To determine the effect of allostimulation on the expression of other TNFR family members we analyzed the coexpression of CD30 with the activation-dependent molecules 4-1BB and OX-40 and the constitutively expressed molecule CD27. In these experiments T cells were enriched by negative selection which routinely yielded a population of >98% CD3⁺ cells as determined by immunofluorescence and flow cytometry (data not shown). Purified T cells were stimulated for 4 days with alloantigen or plate-bound anti-CD3 plus anti-CD28 mAbs and then analyzed for expression of CD30, CD25, 4-1BB, CD27, and OX-40. As shown in a representative experiment in Fig. 3A, >50% of T cells expressed CD30 following primary alloactivation and, as shown previously, the CD30⁺ T cells were uniformly CD25⁺ (Fig. 3, middle left panels). In contrast, only 16% of T cells expressed CD30 in response to anti-CD3 plus anti-CD28 mAbs despite the fact that, on the basis of CD25 expression, 98% of the T cells display an activated phenotype. Moreover, the level of CD30 expression was lower on T
cells activated with anti-CD3 plus anti-CD28 mAbs as indicated by the reduced fluorescence intensity of CD30 staining as compared to CD30+ T cells elicited by allogeneic APC.

Analysis of 4-1BB expression after allostimulation revealed two populations of CD30+ T cells (Fig. 3A, middle panel). The predominant population of CD30+ T cells was 4-1BB+/CD30+ (36% of all T cells), while approximately one-third of the CD30+ T cells coexpressed 4-1BB+CD30+ (17% of all T cells). Conversely, the major population elicited by anti-CD3 plus anti-CD28 mAbs stimulation was 4-1BB-/CD30+ (30% of all T cells), while a minor population of T cells coexpressed 4-1BB and low levels of CD30 (Fig. 3B, middle panel). Overall, 19% of T cells activated with alloantigen expressed 4-1BB, whereas 36% of T cells expressed 4-1BB when activated with anti-CD3 plus anti-CD28 mAbs. Taken together, these results indicate that 4-1BB, like CD30, is expressed on a subset of activated T cells and that 4-1BB and CD30 are expressed independently.

CD27 is known to be constitutively expressed on T cells. We observed that activation with either alloantigen or anti-CD3 plus anti-CD28 mAbs did not effect expression of CD27 (Fig. 3, middle right panels). However, in the case of allostimulation, the CD27+ cells were evenly divided between CD30+ and CD30- T cells (Fig. 3A, middle right panel). In contrast, 89% of T cells activated with anti-CD3 plus anti-CD28 mAbs were CD27+CD30-, while 10% were CD27+CD30low (Fig. 3B, middle right panel), reflecting the modest induction of CD30 by anti-CD3 plus anti-CD28 mAbs.

Unlike CD27, there were marked differences in OX-40 expression depending on the mode of T cell stimulation. Primary alloactivation resulted in low levels of OX-40 expression (23% of all T cells), but most of these OX-40+ T cells were found in the CD30- population (Fig. 3A, right panel). Stimulation with anti-CD3 plus anti-CD28 mAbs induced OX-40 expression on >80% of T cells, and these cells were primarily CD30- (Fig. 3B, right panel).

Taken together, these results suggest that the method of stimulation affects the extent of CD30 expression. Allostimulation yields a greater proportion of CD30+ T cells than does activation with anti-CD3 plus anti-CD28 mAbs. In contrast, stimulation with anti-CD3 plus anti-CD28 mAbs leads to a more pronounced induction of OX-40 and, to a lesser extent, 4-1BB than alloantigen. It is important to note that OX-40 and 4-1BB, like CD30, are maximally expressed relatively late after activation. However, the predominance of 4-1BB and OX-40 induction in response to anti-CD3 plus anti-CD28 mAbs stimulation vs alloantigen was consistently observed over the course of 8 days of culture (data not shown). Thus, the preferential induction of the TNFR family members according to the mode of stimulation we observed could not be explained by differences in the kinetics of expression of these molecules.

Effect of secondary stimulation on CD30, 4-1BB, CD27, and OX-40 expression on T cells

To determine whether secondary stimulation affected the pattern of CD30, 4-1BB, CD27, and OX-40 expression, purified T cells were activated with allogeneic APC or anti-CD3 plus anti-CD28 mAbs for 4 days, rested in IL-2 for 7 days, and then restimulated with either allogeneic APC or anti-CD3 plus anti-CD28 mAbs for an additional 3 days before immunofluorescent staining and flow cytometry. Fig. 4 shows the results of a representative experiment. After two rounds of stimulation with alloantigen or anti-CD3 plus anti-CD28 mAbs, as would be expected, essentially all the T cells displayed an activated phenotype on the basis of CD25 expression (Fig. 4, middle left panels). Whereas <10% of the CD3-CD25+ T cells expressed CD30 in the anti-CD3 plus anti-CD28 mAb-treated cultures, approximately one-half (45%) of the CD3-CD25+ T cells in the alloactivated cultures expressed CD30.

With respect to 4-1BB, the general pattern of expression in both alloactivated T cells and anti-CD3 plus anti-CD28 activated T cells was similar to that found after primary stimulation. Alloantigen induced minimal 4-1BB while anti-CD3 plus anti-CD28 mAbs yielded a discrete population of 4-1BB+CD30+ cells (Fig. 4, A, middle panel, vs B, middle panel). Similarly, restimulation with alloantigen did not significantly alter the relatively low levels of expression of OX-40 that were observed after primary allogeneic stimulation (compare Fig. 4A, right panel, and Fig. 3A, right panel). However, T cells restimulated with anti-CD3 plus anti-CD28 mAbs had notably lower levels of OX-40 expression than did T cells following primary anti-CD3 plus anti-CD28 mAbs stimulation.

The most significant difference in primary and secondary stimulation was observed in the expression of CD27. Secondary stimulation with alloantigen induced a more polarized pattern of CD30 and CD27 expression as compared with primary allogeneic activation, in that the major T cell populations expressed either CD30 or CD27 but not both molecules (Fig. 4A, middle right panel). The CD30+CD27+ population, which comprised 47% of all T cells after primary alloactivation, consisted of only 14% of T cells after secondary stimulation. These results indicate that CD30+ T cells...
also comprise a significant component of the T cell response during secondary stimulation with alloantigen. However, restimulation with anti-CD3 plus anti-CD28 mAbs does not induce significant CD30 expression. Furthermore, secondary stimulation with alloantigen and anti-CD3 plus anti-CD28 mAbs results in downregulation of CD27 and OX-40, respectively.

**Differential cytokine production in T cell lines generated by alloantigen or anti-CD3 plus anti-CD28 mAbs**

We have previously demonstrated that CD30+ T cells generated in response to primary alloantigenic stimulation produce abundant IL-5 and IFN-γ (8). To begin to address whether the differential phenotypes induced by stimulation with alloantigen or anti-CD3 plus anti-CD28 mAbs were associated with distinct cytokine profiles, purified T cells were stimulated with two rounds of alloantigen or anti-CD3 plus anti-CD28 mAbs as described previously. On day 3 following the second stimulation the T cells were recovered and cultured in plates containing immobilized anti-CD3 mAbs. Supernatants were recovered after 48 h and analyzed for cytokine production by specific ELISA (Fig. 5). T cells stimulated with two rounds of alloantigen produced significant levels of IL-2, IL-5, and IFN-γ and modest levels of TNF-α. Secondary stimulation with immobilized anti-CD3 plus anti-CD28 mAbs yielded T cells that produced comparable levels of IL-2 but minimal IL-5, IFN-γ, and TNF-α. IL-4 could not be detected in either type of culture (data not shown). Thus, differences in cytokine profile accompany the distinct phenotypes induced by alloantigen and anti-CD3 plus anti-CD28 mAbs. Although we did not fractionate CD30+ and CD30− T cells in these experiments, previous studies from our laboratory and others using primary allogeneic stimulation (8) or anti-CD3 plus IL-2 (9) suggest that the IL-5 and IFN-γ detected in supernatants following secondary allogeneic stimulation are likely derived from CD30+ T cells. The lack of CD30+ T cells, IL-5, and IFN-γ following secondary stimulation with anti-CD3 plus anti-CD28 mAbs is also consistent with this observation. Whether or not differential expression of other TNFR family members such as 4-1BB, CD27, or OX-40 can be used to further distinguish functional subsets of T cells activated with alloantigen remains to be determined.

**CD30+ T cells are the predominant proliferating cell population in response to alloantigen**

One possible explanation for the relatively large proportion of CD30+ T cells in alloactivated cultures is that these cells are expanding through cellular proliferation. Therefore, we analyzed the proliferative capacity of CD30+ and CD30− T cells. PBMC were labeled with CFSE and stimulated with either irradiated, allogeneic APC or plate-bound anti-CD3 and anti-CD28 mAbs. Normal peripheral blood T cells do not express CD30 and minimal cell division could be detected before day 3 in alloactivated cultures (data not shown). Therefore, cells were harvested on days 3–6 and labeled with anti-CD3-PE mAbs to allow gating on T lymphocytes. Nonresponding cells can be observed as a population of CD30− CFSEhigh cells (Fig. 6A). On day 3 of alloactivation CD30+ T cells are evident and a significant proportion of the CD30+ T cells are CFSElow, indicating that they have undergone multiple cell divisions. It is worth noting that CD30+ CFSEhigh cells are also detectable, indicating that cell division is not required for CD30 expression. Overall, CD30+ T cells constitute the predominant proliferating cell population throughout the time course of the experiment, although some alloactivated CD30+ CFSElow T cells are also present. In contrast, anti-CD3 plus anti-CD28 mAb stimulation elicits a dividing T cell population composed of both CD30+ and CD30− cells (Fig. 6B).

Quantitative analysis indicates that the proportion of dividing T cells that express CD30 remains relatively constant from day 3 to day 6 of the MLR (range = 65–78%) (Fig. 6C). Moreover, the persistence of CD30 expression on proliferating T cells in the alloactivated cultures has been observed through day 11 (data not shown). When cells are stimulated with immobilized anti-CD3 plus anti-CD28 mAbs, the CD30+ T cells and CD30− T cells

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**Figure 4.** Phenotypic analysis of CD30+ T cells generated by secondary stimulation with allogeneic APC (A) or anti-CD3 plus anti-CD28 mAbs (B). PBMC were cultured with allogeneic APC or anti-CD3 plus anti-CD28 mAbs for 4 days, maintained in IL-2 (100 U/ml) for 7 days, then restimulated with allogeneic APC or anti-CD3 plus anti-CD28 mAbs for 3 days. The cells were labeled with fluorochrome-conjugated isotype controls or the following mAbs: CD3-Cy5PE, CD30-FITC, and CD25-PE, 4-1BB-PE, CD27-PE, or OX-40-PE and analyzed by three-color immunofluorescence with gates established on CD3+ cells. The percentage of cells in each quadrant is indicated. One representative experiment from three experiments with similar results is shown.

**Figure 5.** Cytokine production by T cells following secondary stimulation with allogeneic APC or anti-CD3 plus anti-CD28 mAbs. PBMC were stimulated with allogeneic APC in MLR or plate-bound anti-CD3 plus anti-CD28 mAbs for 4 days, rested for 7 days in IL-2 (100 U/ml), then restimulated in MLR (filled bars) or with immobilized anti-CD3 plus anti-CD28 (open bars) for 3 days as described in Fig. 3. Cells were then harvested and cultured in microtiter plates coated with anti-CD3 mAbs (10 µg/ml). Supernatants were harvested after 48 h and analyzed for cytokine production by specific ELISA. Shown is one representative experiment of three experiments that yielded similar results. Data represent the mean ± SEM of three determinations.
constitute similar proportions of the total cell population that has undergone cell division on days 3 and 4 (Fig. 6D). However, by day 5 the majority (60.5%) of dividing cells induced by anti-CD3 plus anti-CD28 mAbs are CD30\textsuperscript{+}H11002\textsuperscript{+} and on day 6 CD30\textsuperscript{+}H11002\textsuperscript{+} T cells represent 72.5% of the cells that have undergone cell division. These results indicate that CD30\textsuperscript{+}H11001\textsuperscript{+} T cells constitute the primary proliferating cell population elicited by alloantigen, whereas plate-bound anti-CD3 plus anti-CD28 mAbs stimulate proliferation of both CD30\textsuperscript{+} and CD30\textsuperscript{−} T cells.

**Discussion**

Numerous studies have demonstrated the important role of the T cell costimulatory molecules CD28 and CD154 (CD40L) in alloimmune responses and graft rejection (30–32). Recently, however, several members of the TNFR family have also been implicated as T cell costimulatory molecules that participate in graft rejection. In particular, 4-1BB costimulation has been shown to augment proliferation of alloreactive CD8 T cells in vitro (17), enhance the generation of allospecific CTL in a murine model of acute GVHD, and accelerate the rejection of cardiac and skin grafts (16). Mice deficient in both 4-1BBL and CD28 exhibit a delay in skin allograft rejection (15). In addition, in a rat model of GVHD, OX-40\textsuperscript{+} donor-derived T cells were shown to be alloreactive and may constitute effector cells (24) while CD27-CD27L interactions promote proliferation and generation of allospecific CTL (33).

Another member of the TNFR family, CD30, also has costimulatory function and has been characterized as a signaling molecule that can promote proliferation, cytokine production, and apoptosis depending on the cell type and its state of differentiation and whether other cell surface receptors are concomitantly engaged. Furthermore, CD30\textsuperscript{+} T cells have been proposed to function as regulatory cells through their ability to produce cytokines. However, little is known about the potential role of CD30\textsuperscript{+} T cells in alloimmune responses and, more generally, about the relative expression of inducible TNFR family members on human T cells. We have taken the approach of characterizing the phenotype and function of the human CD30 cells elicited by alloantigen as a means to begin to understand their contribution to alloreactivity.

We previously reported that alloantigen induces the robust expression of CD30 on human T cells and that these cells are the primary source of IL-5 and IFN-\gamma (8). We now demonstrate that CD30\textsuperscript{−} T cells generated by allostimulation express high levels of CD25 and are found within the CD45RO population. A similar pattern of CD30, CD25, and CD45RO coexpression was also found in T cells stimulated with plate-bound anti-CD3 plus anti-CD28 mAbs in the current study and with anti-CD3 plus IL-2 in previous studies by others (3).

Moreover, using CFSE labeling to identify dividing cells, we found that CD30\textsuperscript{−} T cells generated by allostimulation express high levels of CD25 and are found within the CD45RO population. A similar pattern of CD30, CD25, and CD45RO coexpression was also found in T cells stimulated with plate-bound anti-CD3 plus anti-CD28 mAbs in the current study and with anti-CD3 plus IL-2 in previous studies by others (3).

**FIGURE 6.** Cell division of CD30\textsuperscript{−} T cells and CD30\textsuperscript{−} T cells activated by alloantigen or anti-CD3 plus anti-CD28 mAbs. PBMC were labeled with CFSE, then cultured with allogeneic APC (A) or anti-CD3 plus anti-CD28 mAbs (B). Cells were recovered on days 3–6 and labeled with anti-CD30 FITC and anti-CD3-Cy5 PE mAbs. Gating was established on CD3\textsuperscript{+} cells for analysis. Data are representative of three independent experiments. Quantitative analysis of T cell proliferation in response to allogeneic APC (C) or anti-CD3 plus anti-CD28 mAb (D) was also performed. Data shown are the proportion of total dividing T cells that are CD30\textsuperscript{−} (filled bars) or CD30\textsuperscript{−} (open bars). Data represent the mean ± SEM of three experiments.
cells as compared with stimulation with anti-CD3 plus anti-CD28 mAbs, despite the fact that only a subset of naive T cells (estimated to be 1–10%) are capable of responding to alloantigen, whereas essentially all T cells should be activated by anti-CD3 plus anti-CD28 mAbs. In the initial phase of the response to anti-CD3 plus anti-CD28 mAbs, similar proportions of CD30+ and CD30− T cells underwent cell division, whereas in the latter phase of the response the predominant proliferating T cell population was CD30−. This shift is likely attributable to loss of CD30 expression on CD30+ T cells, rather than death of CD30+ T cells, because we were unable to detect significant apoptosis in the CD30+ fraction (K. W. Chan and O. M. Martinez, unpublished data).

We also observed marked differences with respect to the expression of other TNFR family members on T cells, depending on the mode of cell activation. Primary and secondary stimulation with anti-CD3 plus anti-CD28 mAbs led to induction of a discrete population of activated CD30+ T cells that express moderate to high levels of 4-1BB. In contrast, only low levels of 4-1BB expression were induced by alloantigen. Kim et al. (34) also analyzed expression of CD30 and 4-1BB on human T cells after stimulation with anti-CD3 plus anti-CD28 mAbs. These authors concluded that 4-1BB and CD30 were expressed on mutually exclusive T cell subsets. However, their analysis was restricted to CD4 T cells that had undergone secondary stimulation with anti-CD3 plus anti-CD28 mAbs. Indeed, when we analyzed CD3+ T cells after secondary stimulation with anti-CD3 plus anti-CD28 mAbs we found only a small population (5%) of cells that coexpress CD30 and 4-1BB. In contrast, our data indicate that primary allologenic stimulation can induce a CD30+/4-1BB+ T cell population.

We also found that primary stimulation with anti-CD3 plus anti-CD28 mAbs induced OX-40 on the majority of T cells, whereas <25% of activated T cells stimulated with alloantigen expressed OX-40. Primary stimulation with either alloantigen or anti-CD3 plus anti-CD28 mAbs resulted in CD27 on >90% of activated T cells. However, secondary stimulation with alloantigen resulted in down-regulation of CD27 on the CD30+ subset. Interestingly, there was a slightly higher frequency of CD8+CD30+ cells and CD8−4-1BB+ cells compared with CD4+CD30+ and CD4−4-1BB+ cells, whereas there was no difference in the expression of OX-40 and CD27 in T cell subsets (K. W. Chan and O. M. Martinez, unpublished data). Wen et al. (35) also found that a greater proportion of CD8 cells express 4-1BB compared with CD4 cells when human T cells were stimulated with immobilized anti-CD3. Taken together our data suggest that the mode of stimulation can influence the expression of the TNFR family members CD30, 4-1BB, CD27, and OX-40.

Although numerous studies in murine models support a role for 4-1BB and OX-40 in allogeneic responses (15–17, 24) our studies indicate that a relatively small subset of alloreactive human T cells express these molecules. Whether there are differences in the role of these molecules in allogeneic responses in human and mouse systems or whether the small subset of alloactivated human T cells which express 4-1BB or OX-40 contributes to alloimmunity remains to be determined.

Recent studies in murine systems using DO11.10 transgenic T cells as well as transgenic T cells specific for an LCMV-derived peptide indicate that individual T cells can simultaneously express CD30, OX-40, and 4-1BB (20). Our results indicate a more restricted, less overlapping pattern of expression on human T cells among these TNFR family members. Several factors may account for the observed discrepancy, including differences in murine and human systems, the use of transgenic T cells vs normal T cells, or the use of peptide Ag vs alloantigen and anti-CD3 plus anti-CD28 stimulation.

In any case, the factors that account for the differential induction and regulation of these TNFR family members on human T cells are unknown. An obvious difference in the two activation systems used in the current study is that the MLR contains purified responder T cells and allogeneic APC, whereas the anti-CD3 plus anti-CD28 mAbs system contains only purified T cells and no APC. The LCL used as allogeneic APC in the present study are known to express a variety of molecules including B7-1, B7-2, ICAM-1, CD30L, and CD40, which could influence the subsequent expression of inducible molecules on T cells. It should be noted that the use of allogeneic PBMC as APC in place of allogeneic LCL yielded similar results with respect to CD30 expression (K. W. Chan and O. M. Martinez, unpublished data). Furthermore, our experiments comparing CD30 induction by plate-bound anti-CD3 plus anti-CD28 mAbs with CD30 induction by soluble anti-CD3 plus anti-CD28 mAbs and autologous APC indicate that it is the mode of T cell activation, rather than the presence or absence of APC, that dictates the extent of CD30 expression.

CD30 expression has also been reported to be influenced by cytokines and CD28. Some studies have suggested that IL-4 is essential for CD30 expression on human T cells (36), but other studies found that IL-4 inhibits expression of CD30 on human T cells (3). Experiments in murine models indicate that IL-4 is important in regulation of CD30 expression (37). However, IL-4 does not appear to play a role in our studies because we have been unable to detect IL-4 in supernatants from MLR or anti-CD3 plus anti-CD28 mAb-stimulated T cell cultures. CD28 costimulation has also been implicated as a requirement for CD30 expression (38). In the current study CD28 signaling was provided by anti-CD28 mAbs in the plate-bound Ab system or by B7-1 and B7-2 on LCL stimulators in the MLR system. Because CD28 engagement occurs in both systems it is not likely that CD28 signaling accounts for the difference in expression of CD30; however, we cannot rule out the possibility that the nature of CD28 signaling differs when agonist anti-CD28 mAbs are used instead of the natural ligands B7-1 and B7-2.

Another obvious difference in the two modes of stimulation is the strength of the TCR signal. Anti-CD3 mAbs have a higher affinity for CD3 than MHC does for the TCR. However, we have seen a similar pattern of CD30 expression on T cells in the plate-bound anti-CD3 plus anti-CD28 system even when suboptimal doses of anti-CD3 mAbs are used (C. D. Hopke and O. M. Martinez, unpublished data). These results suggest that the strength of the TCR signal alone does not determine the pattern of CD30 expression.

The function of the CD30 molecule on alloactivated T cells remains unclear. Several reports have suggested that CD30 may play a role in delivering an apoptotic signal to CD4+CD8+ thymocytes in negative selection (12) and in elimination of activated, peripheral T cells (13, 39). However, we did not detect enhanced apoptosis in the CD30+ activated T cell population generated by either alloantigen or anti-CD3 plus anti-CD28 mAbs (K. W. Chan and O. M. Martinez, unpublished data). Alternately, it is plausible that CD30 costimulation in alloactivated T cells influences cellular survival, proliferation, or cytokine production. Experiments are under way to test these possibilities.

In summary, we have determined that alloimmune stimulation is characterized by induction of CD30 in a discrete subset of activated T cells. Importantly, while CD30 expression is limited to a subset of activated cells, its expression marks the predominant proliferating cell population in response to alloantigen. Thus, it will be important to address the function of the CD30 molecule on alloactivated T cells and the role of CD30+ T cells in transplantation.
References