TCR-Independent CD30 Signaling Selectively Induces IL-13 Production Via a TNF Receptor-Associated Factor/p38 Mitogen-Activated Protein Kinase-Dependent Mechanism

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Initiation of T lymphocyte responses to most Ags requires concurrent stimulation through the TCR and costimulatory receptors such as CD28. Following initial activation, secondary receptors are up-regulated that can costimulate T cells in concert with TCR engagement. One such receptor is the TNFR family member CD30. In this study, we report that unlike CD28, ligation of CD30 on normal effector T cells induces IL-13 production in the absence of concurrent TCR engagement. TCR-independent CD30-mediated IL-13 release correlated with activation of c-Jun N-terminal kinase, p38 mitogen-activated protein kinase (MAPK), and NF-κB, and was completely inhibited by the expression of a TNFR-associated factor 2 (TRAF2) dominant-negative transgene (TRAF2.DN-Tg), but not by that of an I-κBα dominant-negative transgene. In parallel, expression of the TRAF2.DN-Tg selectively prevented the induction of c-Jun N-terminal kinase and p38 MAPK, but not that of NF-κB. Furthermore, IL-13 production was reduced in a dose-dependent manner by the p38 MAPK inhibitor SB203580. Together, these results suggest that TCR-independent CD30-mediated production of IL-13 is triggered by association of CD30 with TRAF family members and subsequent activation of p38 MAPK. Inasmuch as IL-13 can promote airway inflammation and cancer progression, production of IL-13 in a TCR-independent manner has important pathological implications in vivo.

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3 Abbreviations used in this paper: TRAF, TNFR-associated factor; CD30L, CD30 ligand; DN, dominant negative; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/ERK kinase; Tg, transgenic; WT, wild type.
function, CD30 can signal in a completely TCR-independent fashion to induce IL-13 production by effector T cells. This TCR-independent function of CD30 correlates with activation of NF-kB, JNK, and p38 MAPK and is dependent on functional TRAF association. Our findings indicate that in addition to its TCR-dependent costimulatory function, CD30 induces TCR-independent biochemical signals that permit selective activation of a transactivation program in T cells.

Materials and Methods

Mice and reagents

DO11.10 transgenic (DO11.10-Tg) mice expressing a TCR specific for OVA peptide (aa 233–339) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were on the BALB/c background. Transgenic animals expressing a DN TRAF2 protein lacking the N-terminal RING finger under the control of the H-2K promoter and IgH chain enhancer (TRAF2.DN-Tg) (19) and animals expressing a l-Bxo.DN transgene driven by the proximal Lck promoter (20) were intercrossed with DO11.10-Tg mice at Vanderbilt University and shipped to the University of Chicago for acute use. Animals were kept in specific pathogen-free facilities and used in agreement with the Institutional Animal Care and Use Committee according to the National Institutes of Health guidelines for animal use.

The anti-CD30 mAb (mCD30.1, Armenian hamster IgG1, κ-L chain) was produced at the University of Miami (7) or purchased from BD PharMingen (San Diego, CA). Isotype control-matched mAb (A19-3) was purchased from BD PharMingen. Anti-CD28 mAb-producing hybridoma (PV-1, Armenian hamster IgG1, κ-L chain) was generously provided by T. Gajewski (University of Chicago). The hybridoma-producing anti-CD3 mAb (145-2C11) was a kind gift from J. Bluestone (University of California, San Francisco, CA). All mAbs were purified by passage of supernatant over a protein A column (Amersham Pharmacia, Arlington Heights, IL).

Generation and restimulation of CD4<sup>+</sup>, CD30<sup>+</sup> mouse T cells

Spleens were isolated from DO11.10-Tg, TRAF2.DN/DO11.10-Tg, and l-Bxo.DN/DO11.10-Tg mice, and RBCs were lysed using ammonium chloride/potassium buffer. Cells were plated at 10<sup>6</sup> cells/ml in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), HEPES, 2-ME (50 µM), and additional amino acids, in the presence of 1.3 ng/ml murine rIFN-γ and OVA peptide (0.4 ng/ml, synthesized by the University of Chicago Peptide Core Facility). Cells were incubated 72 h, and live cells were collected and depleted of dead cells by density gradient centrifugation through Ficoll-Hypaque (Cedarlane Laboratories, Hornby, Ontario, Canada). Expression of CD4 and CD30 was verified by flow cytometry. T cell purity at this stage was routinely above 95%.

Bead-bound Abs were used to restimulate the CD4<sup>+</sup> CD30<sup>+</sup> T cells. The beads (Dynal, Oslo, Norway) were coated with Abs in PBS supplemented with 0.5% BSA, in a rotator for 1–2 h at room temperature. A maximum of 2 × 10<sup>7</sup> beads/ml was used during the coating procedure. A 1:5 cell to bead ratio was used during the in vitro stimulation.

In some experiments, P815 cells (a mouse mastocytoma cell line) were used in place of beads. P815 cells were transfected with CD30 ligand (CD30L)-expressing construct, a control plasmid, or a B7.1-expressing construct. These cell lines were generously provided by Pfizer Laboratories (Groton, CT). P815 cells and T cells were incubated at a 1:1 ratio.

Cytokine production and proliferation assays

Primed DO11.10-Tg T cells were plated on flat-bottom 96-well plates at 50,000 cells/well in 200 µl complete DMEM in the presence of coated beads or P815 cells. For cytokine assays, supernatants were harvested at 24 h of restimulation, and concentration of cytokines in each sample was detected by ELISA using Ab pairs, as instructed by the manufacturer (BD PharMingen). In some experiments, a p38 inhibitor (SB203580) or a mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor (PD98059) purchased from Calbiochem (San Diego, CA) was added at the indicated concentrations. Absorbance was detected in a 96-well spectrophotometer (Spectra Max 250; Molecular Devices, Sunnyvale, CA), and data were analyzed using Softmax software (Molecular Devices) by comparison with a standard curve generated using recombinant cytokines at known concentrations.

For proliferation assays, 1 µCi [3H]thymidine/well was added for the last 8 h of a 48-h culture. Cells were harvested using a Filtermate harvester, and incorporated [3H]thymidine was detected using a TopCount NXT microplate scintillation and luminescence counter with TopCount NXT software (Packard BioScience, Meriden, CT).

RNase protection assay

Total RNA was purified using TRizol (Life Technologies), as directed by the manufacturer. A total of 5 × 10<sup>6</sup> DO11.10-Tg CD4<sup>+</sup> CD30<sup>+</sup> T cells was used for each sample, and the resulting RNA was dissolved in a final volume of 50 µl diethyl pyrocarboxylic acid-H<sub>2</sub>O. The RNA was equalized, as previously described (21), and the concentration also was determined by spectrophotometric analysis. Approximately 2 µg total RNA was used for each sample. The levels of IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, and IFN-γ mRNA relative to the housekeeping genes L32 and GAPDH were determined using the mCK-1 Mouse Cytokine MultiProbe Template Set (BD PharMingen), according to the directions of the manufacturer.

Immunoprecipitation of CD30-TRAF2 complexes

Primed DO11.10-Tg T cells (2 × 10<sup>7</sup>) were incubated with control or anti-CD30 Ab (5 µg) on ice for 5 min and then placed at 37°C for 15 min in the presence or absence of cross-linking Ab. The cells were pelleted and lysed in 0.5% Nonidet P-40 lysis buffer (10 mM Tris, pH 8, 1 mM MgCl<sub>2</sub>, 50 mM NaCl, 0.5% Nonidet P-40, 2 µg/ml aprotinin, and 1 mM PMSF). Lysate supernatants were added to protein G-Sepharose (Amersham Pharmacia) and incubated at 4°C for a minimum of 2 h or overnight. The Sepharose beads were washed four times with ice-cold lysis buffer, and immunoprecipitated protein was resolved on 10% SDS-polyacrylamide gels. Western blots were performed using anti-CD30 (BD PharMingen) or anti-TRAF2 Abs (Santa Cruz Biotechnology, La Jolla, CA).

Electromobility shift assays

Primed DO11.10-Tg T cells were plated at 0.5–1 × 10<sup>6</sup> cells/ml in 24-well plates in the presence of coated beads, as described above. At the indicated time points, cells were harvested and nuclear lysates were prepared, as previously described (20). Nuclear extracts were kept frozen at −80°C until use.

For the gel shift assays, double-stranded probes containing the NF-κB binding site (TGGAGGAGGGAATTCCCCTCTCTTTT) of the IL-2K promoter or an Oct1 consensus binding site (CAACAATCTAGAA-3) were end labeled using T4 polynucleotide kinase and [γ-<sup>32</sup>P]ATP. Typically, 0.2 ng radiolabeled probe and 4 µg nuclear protein were used for each gel shift reaction. DNA-binding reactions were performed as described (20) and resolved on a 6% native acrylamide gel. Protein-DNA complexes were visualized by autoradiography.

Determination of MAPK activation

JNK kinase assay.

Primed T cells (5 × 10<sup>6</sup>) in 0.5 ml complete DMEM were used for each sample and incubated at 37°C with Ab-coated beads. At the indicated time points, cells were washed by addition of 1 ml PBS at room temperature and pelleted by 10-s centrifugation in a microcentrifuge. The cells were lysed in 300 µl ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, and 1 µg/ml leupeptin), and lysates were incubated with 10 µg GST-c-jun fusion protein coupled to glutathione Sepharose beads on a rotator for 3 h at 4°C. The beads were pelleted, and lysates were kept for Western blotting analysis. The beads were washed four times with 0.75 ml HEPES-binding buffer (20 mM HEPES, pH 7.7, 50 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, pH 8.0, 0.05% Triton X-100, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, and 1 µg/ml leupeptin). A total of 30 µl kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl<sub>2</sub>, 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM DTT, 20 mM ATP, and 5 µCi [γ-<sup>32</sup>P]ATP) was added to each sample, and kinase reactions were performed for 15 min at 30°C. The samples were washed three times with 0.5 ml HEPES-binding buffer, and proteins were resolved by SDS-PAGE. The GST-c-jun fusion protein was visualized by staining with Coomassie blue, and the amount of phospho-GST-c-jun was detected using autodigraphy.
p38 activity. Protein lysates saved from the GST-c-Jun pull-down experiments described above were resolved by SDS-PAGE. Western blots were performed using Abs specific for phospho-p38 and total p38 (New England Biolabs Cell Signaling Technology, Beverly, MA).

Results
CD30 engagement costimulates both type 1 and type 2 effector cytokine production
Splenocytes from DO11.10-Tg animals were cultured with OVA peptide and mouse rIL-4 for 5 days and analyzed by flow cytometry for expression of CD30. As shown in Fig. 1A, DO11.10-Tg T cells uniformly expressed high levels of CD30. To confirm the costimulatory role of CD30 on these CD30-expressing T cells, primed DO11.10-Tg T cells were stimulated with beads previously coated with anti-CD30 mAb or an isotype control mAb and increasing concentrations of anti-CD3 mAb. Both IFN-γ and IL-5 were augmented by CD30 cross-linking at low concentrations of anti-CD3 mAb (Fig. 1B). CD30 cross-linking also augmented TCR-dependent induction of T cell proliferation, as inferred by increased [3H]thymidine incorporation (Fig. 1C). To determine whether the augmentation of cytokine production by CD30 engagement was due to CD30-mediated signals or to increased avidity for the Ab-coated beads, experiments were repeated in the presence of Abs coated on separate beads. CD30 cross-linking augmented cytokine production similarly whether coated on the same beads (in cis) or on separate beads (in trans) as the anti-CD3 mAb (Fig. 1D), indicating that these functional effects are dependent on signaling events mediated by CD30 cross-linking and confirming a costimulatory function for CD30.

Cross-linking of CD30 results in TCR-independent production of IL-13
To determine whether CD30 engagement regulated cytokine gene expression, RNase protection assays were performed using probes for a panel of cytokine genes. In the presence of anti-CD3 mAb stimulation, IL-4, IL-5, and IFN-γ mRNA gene expression was augmented by cross-linking of either CD30 or CD28 mAbs (Fig. 2A), consistent with the increased secretion of these cytokines at the protein level, as determined by ELISA (Fig. 1). Unexpectedly, cross-linking of CD30 for 6 h, in the absence of TCR stimulation, consistently resulted in a specific up-regulation of IL-13 mRNA gene expression when compared with treatment with control mAb (Fig. 2A). This was not observed with cross-linking of CD28 alone. Of the nine cytokines analyzed in this RNase protection assay, IL-13 was the only gene induced by CD30 in a TCR-independent manner. In particular, TCR-independent CD30-mediated induction was not observed on expression of IL-10 mRNA, despite detectable levels of transcripts in unstimulated samples, or on expression of IL-4, IL-5, and IFN-γ mRNA, despite effective CD30-mediated augmentation of TCR-dependent gene induction. Differences in

FIGURE 1. CD30 costimulates cytokine production and proliferation. A, DO11.10-Tg splenocytes were cultured in the presence of OVA peptide and murine rIL-4. On day 5, cells were Ficolled, stained with CD4 and either control mAb (thin line) or anti-CD30 (bold line), and analyzed by flow cytometry. The histogram shows expression of CD30 among the CD4+ cells. B, Primed DO11.10-Tg T cells were stimulated with bead-bound Abs, and supernatants were harvested at 24 h. Cytokine concentration for IFN-γ and IL-5 was analyzed by ELISA. Results are represented as the mean and SD of cytokine concentration detected in triplicate wells. C, Primed DO11.10-Tg T cells were stimulated with bead-bound Abs. [3H]Thymidine (1 μCi/well) was added to cultures at 40 h, and plates were harvested at 48 h. Results are represented as the mean and SD of cpm detected in triplicate wells. D, Primed T cells were stimulated with bead-bound Abs. Anti-CD3 and anti-CD30 or isotype control mAbs were either coated on the same beads or on separate beads. Supernatants were harvested at 24 h and analyzed for cytokine content by ELISA. A–C, Representative of >10 independent experiments, whereas D represents results from three separate experiments.
gene expression were quantitated by densitometry. At 6 h, the mean ratio of IL-13/L32 mRNA expression following CD30 cross-linking was 3.2 ± 0.9-fold higher than that following control IgG treatment in two independent experiments. IL-13 mRNA was also induced at 3 h of CD30 cross-linking alone (data not shown).

To determine whether increased IL-13 mRNA expression downstream of CD30 correlated with detectable secretion of IL-13 at the protein level, ELISAs were performed on supernatants from parallel cultures. The concentrations of IL-13 determined by ELISA 24 h following stimulation with control or anti-CD28 mAbs (Fig. 2B) were at or below the level of detection of the assay. However, IL-13 protein levels were significantly induced following stimulation with anti-CD30 mAb. In fact, the amount of IL-13 induced by anti-CD30 mAb alone was almost comparable with that observed following anti-CD3 stimulation (1.2 ± 0.3 ng/ml at 0.1 µg/ml anti-CD3). Interestingly, engagement of anti-CD30 also further augmented anti-CD3-mediated production of IL-13 (3.1 ± 0.4 ng/ml).

It has been suggested that receptor engagement by mAbs may lead to different signaling effects than binding by natural ligands (22). To verify the physiological relevance of CD30-mediated IL-13 production in the absence of TCR stimulation, primed DO11.10-Tg T cells were incubated in the presence of syngeneic (H-2d) P815 cells transfected with control-, B7-1-, or CD30L-expressing vectors. P815 cells are negative for MHC class II and are therefore not expected to present Ag to DO11.10-Tg T cells. As observed following Ab cross-linking of CD30, engagement of CD30 by CD30L resulted in a significant induction of IL-13 production (Fig. 2C). In contrast, no IL-13 was detected when T cells were incubated with control- or B7-1-expressing P815 cells. Together, these results indicate that CD30 engagement alone on effector T cells can induce the specific release of IL-13, in contrast to the coordinate release of IL-4, IL-5, IL-13, and IFN-γ induced by coligation of CD30 and the TCR.

### Cross-linking of CD30 results in rapid nuclear translocation of NF-κB

The signaling properties of CD30 in normal T cells are not known. To begin to investigate the biochemical pathways by which CD30 engagement alone on T cells could lead to IL-13 production, we studied pathways activated by TNFR family members in transient systems or in TCR-stimulated T cells. CD30 cross-linking of spontaneously proliferating tumor cell lines has been reported to result in nuclear translocation of NF-κB (18, 23). Therefore, primed DO11.10-Tg T cells stimulated with beads coated with combinations of mAbs were lysed at various time points, and EMSAs were performed using radioactively labeled NF-κB and Oct-1-binding probes. Supershift analysis indicated that the upper band revealed by the NF-κB-binding probe was composed of heterodimers of the p50 and p65 members of the NF-κB/Rel family, whereas the lower band mostly comprised p50-p50 homodimers (data not shown). Stimulation with beads coated with low concentrations of anti-CD3 mAb (0.1 µg/ml) induced a weak increase in NF-κB activation at 6 h that was further augmented at the 24-h time point when compared with treatment with control mAb-coated beads (Fig. 3A). Both CD30 and CD28 stimulation marginally enhanced TCR-mediated NF-κB activation at these time points. Interestingly, increased NF-κB activation was observed at much earlier time points when CD30 was cross-linked in the absence of TCR stimulation, when compared with samples incubated with control mAb-coated beads (Fig. 3A). Maximal NF-κB activation in this setting was observed 1 h after CD30 cross-linking, although it was already detectable at the 30-min time point, and was sustained up to 6 h. DNA-binding activity was particularly enhanced for the NF-κB p50/p65 heterodimeric complex, which contains a DNA transactivation domain. In contrast, cross-linking of CD28 had no reproducible effect on NF-κB DNA-binding activity in the absence of TCR ligation, confirming that CD28
engagement alone does not signal NF-κB activation in T cells. Levels of Oct1 complexes were comparable for each set of control/anti-CD30/anti-CD28 mAbs stimulating conditions. These results indicate that CD30 ligation alone delivers a signal in normal T cells independent from the TCR.

To rule out potential artifacts of using Ab cross-linking, experiments were repeated in the presence of P815 transfectants. As a positive control, T cells were treated with control or anti-CD30 mAb-coated beads. As shown above in Fig. 3A, anti-CD30 treatment for 1 h resulted in TCR-independent up-regulation of NF-κB activation when compared with control mAb treatment (Fig. 3B, last two lanes). Basal levels of nuclear NF-κB proteins in P815 cells were very low (Fig. 3B, first three lanes). In contrast, robust activation of NF-κB was observed in nuclear extracts from T cells incubated with CD30L-transfected P815 cells, when compared with T cells cultured with either control- or B7-1-transfected P815 cells. Thus, CD30-mediated TCR-independent signaling can occur not just as a result of Ab cross-linking, but also as a consequence of interacting with the natural ligand for CD30.

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**FIGURE 3.** CD30 cross-linking results in nuclear translocation of NF-κB. A, Primed DO11.10-Tg T cells were stimulated with Ab-coated beads. At the indicated time points, nuclear extracts were prepared and assayed for presence of NF-κB and Oct1 complexes by gel shift analysis. This result is representative of five independent experiments. B, Primed DO11.10-Tg T cells were stimulated with transfected P815 cells, or with Ab-coated beads. P815 cells were also incubated in the absence of T cells. At 1 h of stimulation, nuclear extracts were prepared and assayed for presence of NF-κB and Oct1 complexes by gel shift analysis. This result is representative of three independent experiments.

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**Cross-linking of CD30 results in activation of JNK and p38**

Engagement of other TNFR family members has been shown to augment TCR-mediated MAPK induction in T cells (24). Therefore, we next determined whether ligation of CD30 alone activated MAPKs. Primed DO11.10-Tg T cells were stimulated with Ab-coated beads and lysed at different time points. As shown in Fig. 4A, cross-linking with anti-CD30 (with or without anti-CD3 mAb) resulted in a dramatic induction of JNK kinase activity within 15 min of treatment when compared with control mAb-treated samples. The CD30-mediated augmentation of JNK activation was also significant at 30 min of stimulation, and was in some experiments sustained up to 60 min (data not shown). In contrast, cross-linking of CD28 induced JNK activity only when the TCR was coligated. CD28 ligation has been shown to potently induce JNK activation following stimulation of normal T cells with low concentration of TCR stimuli (25). Therefore, absence of JNK activity following CD28 engagement alone confirmed that JNK induction by anti-CD30 mAb alone was not due to carry-over of Ag from the priming conditions.
Lysates used for JNK kinase assays were subsequently used in a Western blot and probed with phospho-specific anti-p38 mAb to detect the active form of p38 MAPK. Levels of total p38 were also assayed by Western blot to control for sample loading. Phospho-p38 was induced within 30 min of CD30 cross-linking, either in the presence or absence of anti-CD3 stimulation (Fig. 4B). CD28 cross-linking alone had no detectable effect on p38 activation. Thus, in contrast to CD28, CD30 ligation was able to activate both JNK and p38 in a manner independent of TCR coengagement. A proliferation assay performed in parallel revealed similar background levels of \(^{3}H\)thymidine incorporation in T cells stimulated with control, anti-CD30-, and anti-CD28-coated beads (98 ± 33, 153 ± 40, and 351 ± 73 cpm, respectively), whereas T cells stimulated with anti-CD3-coated beads (0.03 \(\mu\)g/ml) exhibited 100-fold more \(^{3}H\)thymidine incorporation (11,782 ± 1,026), further suggesting that the biochemical effects observed following CD30 engagement were not due to Ag carry-over.

Induction of JNK and p38 MAPK activity could not be verified following engagement of CD30 with its natural ligand using CD30L transfectants, as high basal levels of active endogenous JNK and p38 in P815 cells obscured any detectable effect on T cells. In contrast to JNK and p38 MAPK, ERK was not induced following ligation of CD30 alone (data not shown).

Expression of the TRAF2.DN-Tg prevents CD30-mediated JNK and p38 MAPK activation

Some signals mediated by TNFR family members are transmitted through the TRAF family of adapter proteins. In transfected 293 cells, CD30 has been shown to associate with TRAF1 and TRAF2 (18). To determine whether CD30 could associate with TRAF family members in normal T cells, CD30 was immunoprecipitated from primed DO11.10-Tg T cells in the presence or absence of an anti-hamster cross-linking Ab, and Western blot analysis was performed using anti-CD30 or anti-TRAF2 Abs. TRAF2 was identified in anti-CD30- but not control mAb-immunoprecipitated samples, indicating that CD30 and TRAF2 interacted specifically in normal T cells (Fig. 5A). In addition, the association was not enhanced by the presence of the cross-linker.

To determine whether TCR-independent CD30-mediated activation of JNK, p38, and NF-\(\kappa\)B was mediated by TRAF signaling, T cells from TRAF2.DN/DO11.10-Tg mice were used. The truncated DN form of TRAF2 is unable to mediate downstream signaling events, but can bind to TNFR family members and presumably blocks binding of endogenous TRAF proteins to the CD30 cytoplasmic sites that can bind TRAF1, 2, and 3. We verified that primed TRAF2.DN-Tg exhibited similar levels of surface CD30 as control DO11.10-Tg CD4\(^+\) T cells (data not shown). To control for the effect of the TRAF2.DN transgene, CD30-expressing primed T cells were stimulated with TNF for 10 min and JNK activation was examined, as this was reported to be inhibited in TRAF2.DN-Tg lymphocytes (19). As expected, TNF-mediated JNK activation was blocked in TRAF2.DN-Tg when compared with wild-type (WT) T cells, confirming the DN effect of the transgene (data not shown).

When CD30 was cross-linked alone, significant induction of JNK activation was observed in wild-type (WT) DO11.10-Tg T cells. In contrast, no augmentation of JNK activation was detectable in the TRAF2.DN-Tg cells treated with anti-CD30 mAb (Fig. 5B). This was not due to an inability of TRAF2.DN-Tg T cells to up-regulate JNK activity, as CD28 cross-linking effectively resulted in up-regulation of JNK in anti-CD3-treated cells when compared with cells treated with anti-CD3 and control mAb (Fig. 5B).

To determine whether CD30-mediated activation of p38 was TRAF dependent, Western blot assays were performed using the lysates from the JNK kinase assay following the GST-c-jun pull-down. As shown for JNK activation, the presence of the TRAF2.DN transgene completely blocked TCR-independent CD30-mediated activation of p38 (Fig. 5C). In contrast, when CD28 was cross-linked in combination with TCR stimulation, p38 activation was increased, indicating that other conditions could induce p38 activation in TRAF2.DN-Tg T cells. Taken together, these results indicate that association of CD30 with TRAF family members is necessary for CD30-mediated TCR-independent activation of JNK and p38.

To address whether NF-\(\kappa\)B activation downstream of CD30 depended on CD30 association with TRAF family members, nuclear extracts were prepared from bead-stimulated primed DO11.10-Tg T cells. CD30 cross-linking in the absence of TCR stimulation resulted in robust activation of NF-\(\kappa\)B in both the control and the TRAF2.DN-Tg T cells (Fig. 5D). Thus, in contrast to JNK and p38 activation, the presence of the TRAF2.DN transgene did not prevent CD30-mediated activation of NF-\(\kappa\)B, suggesting that NF-\(\kappa\)B activation induced by CD30 engagement can occur in a TRAF-independent manner.

**IL-13 production following CD30 ligation is TRAF and p38 dependent**

IL-13 production upon CD30 engagement correlated with TRAF association and activation of JNK, p38, and NF-\(\kappa\)B. In addition, normal TRAF signaling correlated with activation of JNK and p38,
but not of NF-κB. To determine whether IL-13 production was dependent on TRAF association and/or NF-H9260B activation, experiments were repeated using primed T cells from DO11.10-Tg, TRAF2.DN/DO11.10-Tg, and DO11.10-Tg mice crossed with mice expressing an I-kBα.DN-Tg under the control of a T cell-specific promoter/enhancer pair. T cells from I-kBα.DN-Tg exhibit dramatically reduced NF-κB activation upon T cell stimulation (20). EMSAs confirmed a 70–80% reduction of nuclear NF-κB activity, both in naive and stimulated CD4+ I-kBα.DN/DO11.10-Tg T cells when compared with control DO11.10-Tg T cells (data not shown). Primed I-kBα.DN/DO11.10-Tg CD4+ T cells exhibited similar levels of surface CD30 as control DO11.10-Tg and TRAF2.DN/DO11.10-Tg CD4+ T cells (data not shown). Primed T cells were stimulated with P815 cells transfected

FIGURE 5. CD30-mediated activation of JNK and p38 MAPK, but not that of NF-κB, is TRAF dependent. A, Cellular extracts from primed DO11.10-Tg T cells were immunoprecipitated using anti-CD30 or irrelevant isotype control hamster mAbs in the presence or absence of crosslinking anti-hamster IgG. Immunoprecipitated proteins and whole cell extract (WCE) were resolved by SDS-PAGE and blotted using anti-TRAF2 (N) and anti-CD30 Abs. B, Primed DO11.10-Tg T cells from WT or TRAF2.DN-Tg mice were stimulated with Ab-coated beads. At 30 min, the cells were lysed and a JNK kinase assay was performed. C, After pulling down JNK, supernatants from the lysates were separated by SDS-PAGE, and the resulting blot was probed using Abs directed against active phospho-p38 (p-p38) or total p38. D, Primed DO11.10-Tg T cells from WT or TRAF2.DN-Tg mice were stimulated with beads coated with isotype control, anti-CD30, or anti-CD28 mAbs for 1, 3, or 24 h. Nuclear extracts were prepared and assayed for presence of NF-κB complexes by gel shift analysis. All panels are representative of at least three independent experiments.

FIGURE 6. IL-13 production induced by CD30 engagement is TRAF and p38 dependent. A, Primed DO11.10-Tg WT, I-kBα.DN-Tg, and TRAF2.DN-Tg T cells were stimulated with P815 cells transfected with CD30L or B7.1. Supernatants were collected at 24 h and analyzed by ELISA for IL-13 concentration. The graphs represent the mean and SD of the difference between IL-13 induced by CD30L transfectants and by B7.1 transfectants. B, Primed DO11.10-Tg T cells were incubated with control- or CD30L-transfected P815 cells, in the presence of different concentrations of a p38 or a MEK inhibitor. Supernatants were collected at 24 h and analyzed by ELISA for IL-13 concentration. The graph is representative of three independent experiments.
with CD30L- or B7-1 encoding vectors. As shown in Fig. 6A, similar IL-13 induction was observed in control and NF-κB activation defective T cells following CD30 engagement alone. Importantly, IL-13 was not induced by CD30 engagement in TRAF2.DN-Tg T cells, indicating that TCR-independent IL-13 induction by CD30 is absolutely TRAF dependent, but appears to be NF-κB independent.

Thus, IL-13 production upon CD30 engagement correlated with TRAF association, and TRAF association correlated with activation of JNK and p38 MAPK. To determine whether IL-13 production following CD30 cross-linking was dependent on MAPK activation, primed DO11.10-Tg T cells were stimulated with control or CD30L-transfected P815 cells, in the presence of increasing concentrations of the p38 inhibitor SB203580, or of the MEK inhibitor PD98059. IL-13 production induced by CD30 engagement was reduced in a dose-dependent manner by the p38 inhibitor, whereas ERK inhibition had no detectable effect (Fig. 6B). Taken together, these results indicate that the production of IL-13 induced by CD30 cross-linking alone is dependent upon TRAF association and p38 MAPK kinase activation, but can occur in the absence of complete NF-κB activation.

Discussion

CD30 is a costimulatory receptor of activated T cells, the cross-linking of which augments T cell proliferation and cytokine production at low levels of TCR stimulation in vitro. Using normal effector T cells, we report that, in addition to its costimulatory activity and in striking contrast to CD28, CD30 engagement in the absence of TCR stimulation induces readily detectable levels of IL-13 production. This was the only cytokine from a panel of nine found to be up-regulated by CD30 engagement alone. We have elucidated the biochemical pathways leading to this unanticipated function of CD30 and found that TCR-independent CD30 mediated IL-13 production is TRAF and p38 MAPK dependent, but independent of CD30 induced activation of NF-κB. These findings provide evidence for a mechanism whereby TCR-independent CD30 engagement may function to modulate the profile of cytokines released by T cells at sites of inflammation.

TCR-independent T cell function has recently been described in a few systems. Type I IFN and IL-15 have been shown to induce proliferation of memory CD8+ T cells in vivo, in an NF-κB dependent, TCR-independent manner (26). IL-12 and IL-18 synergize to induce transcription of IFN-γ by CD4+ T cells in a TCR-independent manner (27). Cross-linking of TNFR family members such as Fas and TNFR1 results in complex signaling events in T cells leading to apoptosis (13). Signaling by these proapoptotic receptors appears to require a death domain present in their cytoplasmic tail that is not shared by CD30. In this study, we show that CD30 engagement leads to IL-13 production by T cells in a TCR-independent manner. Thus, CD30 represents a TNFR family member that activates effector T cell function via a death domain-independent pathway.

TCR-independent selective cytokine production has not been described in normal T cells for the prototypic costimulatory receptor CD28. In fact, TCR-independent production of IL-2 following CD28 cross-linking only occurred when normal human T cells were transfected with constructs encoding both VAV and SLP76 adaptors (28). Overall, the effects of CD28 engagement in normal T cells appear to be different from those of CD30: in contrast to CD30, ligation of CD28 did not result in activation of JNK, p38 MAPK, NF-κB, or in cytokine production in primed DO11.10-Tg T cells.

Other TNFR family members such as 4-1BB and Ox40 that are up-regulated following initial T cell activation also augment TCR-mediated biochemical signals and cytokine production. 4-1BB in particular has been shown to increase TCR-mediated activation of JNK and p38 in normal T cells at 24 and 48 h of stimulation (24). Whether Ox40 or 4-1BB would also have TCR-independent effects in normal T cells remains to be demonstrated. In a T cell hybridoma, 4-1BB engagement led to rapid activation of JNK and p38 in the absence of TCR stimulation (24). In Jurkat cells, aggregation of the Ox40 or 4-1BB cytoplasmic tail alone resulted in NF-κB activation (29). However, these signaling events were not reproduced in normal T cells (24), and no correlation with a functional T cell outcome was reported. This may be because experiments were performed using naïve T cells that do not express TNFR family members, and perhaps experiments performed in primed effector T cells might reveal such an effect. Thus, whether similar events as we describe for CD30 would be observed following ligation of other TNFR family members in normal T cells, or whether this is a unique feature of CD30, remains to be established.

Several differences between CD30 and 4-1BB are apparent. First, the TCR-dependent costimulatory function of 4-1BB has been found to require TRAF association, as both p38 activation and the enhancement of cytokine production induced by 4-1BB engagement during TCR stimulation were shown to be inhibited in TRAF2.DN-Tg T cells (24, 30). In our system, although TRAF association appeared necessary for TCR-independent induction of p38 and JNK activation by CD30 ligation, the presence of the TRAF2.DN transgene did not prevent the enhancement by CD30 ligation of TCR-mediated IL-5 or IFN-γ production (data not shown).

Second, coupling of 4-1BB to TRAF1 and TRAF2 has been reported to require cross-linking of 4-1BB (30). In the primed CD30 expressing normal T cells used in our study, TRAF2 association with CD30 was detected in the absence of secondary Ab cross-linking. Although it is conceivable that CD30 molecules were already aggregated from the priming conditions, our results suggest that TRAF2 may be constitutively associated with CD30 in normal T cells.

TCR-independent CD30 mediated activation of JNK and p38 was TRAF dependent. Activation of these MAPKs was specific, as no induction of ERK activation was detected under similar TCR-independent stimulating conditions (data not shown). Our results also indicate that IL-13 production mediated by CD30 engagement is p38 dependent, as production of IL-13 following CD30 ligation was significantly reduced in a dose-dependent manner by SB203580. However, it is known that high concentrations of SB203580 can also inhibit JNK activation in addition to p38 activation. As no specific JNK inhibitor was commercially available at the time of these experiments, it is possible that JNK activation may also be involved in CD30 dependent TCR independent IL-13 production. Interestingly, p38 MAPK was also identified as a mediator of IL-12- and IL-18 induced TCR independent T cell proliferation (27), suggesting that p38 MAPK may be a key regulator of TCR independent T cell functions.

In contrast to activation of JNK and p38, NF-κB activation by CD30 alone was not affected by the presence of the TRAF2.DN transgene. It is possible that residual association of CD30 with endogenous TRAF molecules was sufficient for NF-κB activation, but not for JNK and p38 activation. However, in transfection systems using cell lines, both TRAF-dependent and TRAF independent sites for NF-κB activation have been described in the cytoplasmic tail of CD30 (18). Furthermore, one report has indicated that CD30 could also associate with TRAF5 in addition to TRAF1.
TRA2 and TRAF3, and that TRAF5 may bind to an adjacent, but separate region from the two conventional binding sites (16). If this occurs in normal T cells and TRAF5 can also mediate NF-κB activation, the presence of a TRAF2.DN transgene may not prevent this association. TRAF5-deficient lymphocytes have been reported to have defects in CD27 and CD40 signaling (31), suggesting that TRAF5 may play a role in signaling by TNFR family members in normal T cells. In preliminary experiments, we have examined TRAF5-deficient T cells and found that CD30-mediated induction of JNK, p38, and NF-κB is intact (data not shown).

The finding of a mechanism by which CD30/C3D30L interactions alone, in the absence of TCR stimulation, can selectively promote IL-13 release has important implications for the pathophysiology of several diseases. IL-13 plays an important role in elimination of the parasite Nippostrongylus brasiliensis (32, 33). However, IL-13 also has an unwanted role in promoting allergic asthma, probably as a result of eotaxin induction and recruitment of eosinophils into the lungs (34–36). Interestingly, eosinophils express high levels of CD30L that could engage CD30 on primed T cells, inducing further IL-13 production even in the absence of Ag, and thus possibly amplifying the allergic response. Furthermore, IL-13 has recently been shown to promote cancer progression by two independent mechanisms. First, IL-13 appears to limit immune surveillance by T cells, and blockade of IL-13 has been shown to enhance tumor elimination in vivo (37) by a mechanism dependent on STAT6 (38). Second, IL-13 may act as a growth factor for certain tumors, promoting tumor expansion (39). In Hodgkin’s disease, CD30+ Reed-Sternberg (H/RS) cells are surrounded by nonneoplastic cells, many of which are mast cells expressing CD30L (40). IL-13 production following engagement of CD30 on T cells by CD30L on mast cells could potentially both directly promote growth of certain tumor types and reduce tumor elimination by T cells. Thus, our findings raise the possibility that CD30 on T cells may play a role in down-regulating immune surveillance and promoting tumor expansion, and in exacerbating airway inflammation through the ability of CD30L+ eosinophils or mast cells to evoke TCR-independent IL-13 release. Furthermore, CD30 represents an immune regulator the ligation of which dictates distinct T cell effector responses depending on whether the TCR is concurrently engaged or not.

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