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To delineate the molecular mechanisms regulating Th2 cell differentiation, CD28-mediated generation of Th2 effectors was analyzed. In the absence of TCR ligation CD28 stimulation induced Th2 differentiation of memory but not of naive CD4+ T cells, whereas costimulation via CD28 and the TCR enhanced Th2 differentiation from naive T cells but suppressed it from memory T cells. Stimulation of T cells via the CD28 pathway, therefore, provided critical signals facilitating Th2 cell differentiation. By comparing the responses to CD28 stimulation in memory and naive T cells and by using specific inhibitors, signaling pathways were defined that contributed to Th2 differentiation. CD28-induced Th2 differentiation required IL-4 stimulation and the activation of the mitogen-activated protein kinases p38 and extracellular signal-regulated kinases 1/2. CD28 engagement directly initiated IL-4 gene transcription in memory T cells and induced activation of phosphatidylinositol 3-kinase, p38, and c-Jun NH2-terminal kinase/stress-activated protein kinase pathways. Extracellular signal-regulated kinase phosphorylation that was necessary for Th2 differentiation, however, required stimulation by IL-2. These results indicate that optimal TCR-independent generation of Th2 effectors requires coordinate signaling via the CD28 and IL-2 pathways. TCR-independent generation of Th2 effectors might provide a mechanism to control Th1-dominated cellular inflammation.

demonstrated that the extent of stimulation through CD28 regulates the extent of Th2 effector generation (24, 26). For example, as a result of unopposed signaling through CD28, there is a massive polyclonal expansion of Th2 cells in CTLA-4 knockout mice (27). Taken together, the data indicate that CD28 is a key regulatory molecule in T cell differentiation. However, despite the significance of CD28-mediated signals for T cell activation and differentiation, the relevant signaling events initiated by CD28 ligation are just beginning to be delineated (28).

CD28 engagement has been shown to induce phosphorylation of phosphatidylinositol 3-kinase (PI3-kinase) and activation of the mitogen-activated protein (MAP) kinase cascades (28). However, the role of PI3-kinase and MAP kinase activation in transducing signals following CD28 stimulation and the functional consequences of such activation in normal human T cells has not been completely delineated. Given the significance of CD28 in T cell activation and differentiation, analysis of the signaling pathways involved in transducing CD28-generated signals might provide detailed insights into the understanding of molecular mechanisms involved in T cell activation and, importantly, in regulating Th2 cell differentiation.

In this report, we investigated CD28-mediated, Ag-independent Th2 cell differentiation in isolated naive and memory CD4+ human T cells and analyzed the functional contribution of individual signals downstream of CD28 in the generation of Th2 effectors. We demonstrate that TCR-independent Th2 differentiation from resting human memory but not from naive T cells could be induced by CD28 engagement and that CD28-mediated Th2 differentiation was dependent on IL-4 and the activation of the MAP kinases, p38, and extracellular signal-regulated kinase (ERK). CD28-mediated, Ag-independent T cell differentiation might be an important mechanism to control inflammatory responses initiated by Ag-specific Th1 cells.

Materials and Methods

Cell purification

Resting naive and memory CD4+ T cells were isolated from heparinized cord blood or from healthy adult peripheral blood, respectively, by negative selection using mAbs to CD8 (OKT8), CD19 (HD37), CD16 (B73.1), HLA-DR (L243), and CD45RA (111-1C5, a generous gift from R. Vilella, Barcelona, Spain) (21). Homogeneity and purity of the recovered cells were assessed by flow cytometry. Typically, >95% of the cells were positive for CD3 and CD4 and >95% of the isolated naive T cells were positive for CD45RA. The cells were negative for the activation markers CD25, CD30, CD69, and HLA-DR.

Generation and analysis of effector T cells

All cell cultures were carried out in RPMI 1640 medium supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml), l-glutamine (2 mM), 10% normal human serum, and recombinant human IL-2 (10 U/ml). T cell differentiation was studied using a previously described multistep ex vivo cell culture system (21). In brief, differentiation was induced by priming of purified resting naive or memory T cells with 1 µg/ml anti-CD28 mAb (28.2; BD PharMingen, San Diego, CA) in the presence of IL-2. After 5 days, cells were harvested, rested for 60 h, and their phenotype was analyzed. Where indicated, cells were primed in the presence of IL-4 (31.25 ng/ml), anti-IL-4 mAb (25D2, 10 µg/ml; both Endogen, Woburn, MA), immobilized anti-CD3 (OKT3, 1 µg/ml), or inhibitors to PI3-kinase (wortmannin, 20 nM), to MAP kinase kinase 1 (MAPKK1), an upstream activator of ERK1 and ERK2 (PD98059, 10 µM), to p38 MAP kinase (SB203580, 400 nM), or in the presence of a negative control for the MAP kinase inhibitors (SB202474, 10 µM; all Calbiochem, La Jolla, CA).

3 Abbreviations used in this paper; PI3-kinase, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK/SAPK, c-Jun NH2-terminal kinase/stress-activated protein kinase; MAPKK1, MAP kinase 1.

Preparation of mRNA and amplification of cDNA

Purified naive or memory CD4+ T cells were stimulated with anti-CD28 (10 µg/ml) in the presence of IL-2 (10 U/ml). Actinomycin D (10 µg/ml) was added to some cultures to prevent gene transcription. Total cellular RNA was extracted with TriReagent, (Sigma, St. Louis, MO), and mRNA was transcribed to cDNA followed by amplification of target sequences for a number of cycles that was within the linear range of the exponential amplification (27). Primers for IL-4 were purchased from Clontech Laboratories (Palo Alto, CA). The primers specified for the constitutively expressed endogenous mRNA, cyclophilin, and the transcription factors GATA-3 and c-maf were deduced from the published sequences (30–32) (cyclophilin: 5'-CCGTGTTCTTCGACAT TG3-3', sense, nt 28–46; 5'-TCAGTGTGTCACAGTTCGC-3', antisense, nt 489–508; GATA-3: 5'-ACTGTCAGACCCACACACCC-3', sense, nt 1078–1097; 5'-AGGACAGTTGTCAGGAGG-3', antisense, nt 1307–1326; c-maf: 5'-AGAAATGGCATCAGAACTTGGC-3', sense, nt 805–824; 5'-CAGTGTAGTCCTCCAGGTGCG-3', antisense, nt 1064–1085).

Western blot analysis

Purified CD4+ T cells subsets (memory or naive, 4 × 10⁶/ml) or Jurkat T cells (American Type Culture Collection, Manassas, VA) were deprived from serum overnight and incubated with anti-CD28 (10 µg/ml), anti-CD3 (10 µg/ml), IL-2 (100 U/ml), or a combination thereof for 10 min on ice. Where indicated, cells were pretreated with specific kinase inhibitors or, for control, with the solvent of the inhibitors (DMSO) for 1 h (45 min at 37°C and 15 min on ice). Subsequently, cross-linking was accomplished with goat anti-mouse IgG for 10 min at 37°C. Activation of the kinases was assayed using PhosphoPlus protein kinase assay kits (New England Biolabs, Beverly, MA). Positive and negative control cell lysates (New England Biolabs) were included in all samples for the calibration of the assays (data not shown). The activity of ERK and p38 MAP kinase was assayed with nonradioactive kinase assays (New England Biolabs) according to manufacturer’s instructions.

Assay for PI3-kinase activity

Jurkat T cells or purified CD4+ memory T cells (10 x 10⁶/ml) were treated with increasing concentrations of wortmannin or, for control, with DMSO for 1 h (45 min at 37°C and 15 min on ice) and incubated with anti-CD28 (10 µg/ml) for 10 min on ice and subsequently with goat anti-mouse IgG for 10 min at 37°C. The cells were lysed in lysis buffer (0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 10 mM NaF, 2 mM EDTA, 1 mM Na3 VO4, and 100 µg/ml PMSF in 50 mM Tris-HCl, pH 8.0), and the lysates were cleared by centrifugation. PI3-kinase was immunoprecipitated with an anti-PI3-kinase mAb (p85a; BD PharMingen) and recombinant protein G-agarose (Pharmacia, Uppsala, Sweden) for 1 h at 4°C. The immunoprecipitates were washed twice with 1% Nonidet P-40, 10% glycerol, 450 mM NaCl, 10 mM NaF, 2 mM EDTA, 1 mM Na3 VO4, and 100 µg/ml PMSF in 50 mM Tris-HCl (pH 8.0), twice with 500 mM LiCl and 1 mM Na3 VO4 in 100 mM Tris-HCl (pH 8.0), and once with water, and finally resuspended in 10 µl of 20 mM HEPES (pH 7.4) containing 10 µg of phosphatidylinositol (Sigma). Kinase activity was initiated by the addition of 400 µCi of PI3-kinase (10 nM MgCl2, 200 µCi of [γ-32P]ATP, and 200 µl of 20 mM HEPES, pH 7.4) containing 10 µM ATP and 10 µCi of [γ-32P]ATP. After 10 min at room temperature, the reaction was stopped with 100 µl of 1 M HCl. The phosphatidylinositol lipids were extracted with 200 µl of chloroform:methanol (1:1) and resolved by TLC. Radiolabeled lipids were visualized by autoradiography.

Results

Signal transduction through CD28 is sufficient to induce Th2 cell differentiation from memory but not from naive CD4+ T cells

To investigate the capacity of CD28 to induce Th2 cell differentiation independent of TCR-mediated signals, isolated naive and memory CD4+ T cells were primed with anti-CD28 and IL-2 in

some experiments, T cells were primed with human myeloma cells expressing human CD80, CD86, or CD80 and CD86 after transfection with a recombinant adeno-associated virus vector containing the full-length cDNA for CD80 and/or CD86 (29) (a kind gift from M. Hallek, Munich, Germany).

To determine the phenotype of the cells as defined by their capability to produce cytokines, cells were stimulated with ionomycin (1 µM) and PMA (20 ng/ml) in the presence of 2 µM monensin, fixed, stained with saturating amounts of directly labeled mAbs, and analyzed by flow cytometry for the production of cytoplasmic cytokines (6).
the absence or presence of TCR ligation, and the phenotype of the cells generated was determined by cytometric analysis of intracellular cytokines. In agreement with previous reports (23, 24), Th2 cells could be generated from resting naive CD4$^+$ T cells by priming with anti-CD3 and anti-CD28 (Table I and Fig. 1A). Exogenous IL-4 was not required for Th2 cell differentiation from naive T cells and only marginally increased Th2 cell frequencies (Fig. 1B). By contrast, in naive T cells, priming with anti-CD28 in the absence of TCR engagement did not result in Th2 effectors capable of producing IFN-γ in Th2 cell generation. After culture of naive CD4$^+$ T cells with anti-CD3 and anti-CD28 in the absence of TCR-mediated signals (Table I and Fig. 1A). Low concentrations of IL-2 (10 U/ml) were required for the differentiation (data not shown) but did not impose a functional bias (Fig. 1A). By contrast, in naive T cells, priming with anti-CD28 in the absence of TCR engagement did not result in Th2 cell generation. After culture of naive CD4$^+$ T cells in IL-2 alone, small numbers of effectors capable of producing IFN-γ could be detected, indicative of a Th1 bias in naive T cells (23, 24). The generation of these cells was not affected by CD28 but could be inhibited by IL-4 (Fig. 1B). Thus, Th2 differentiation could be induced in a TCR-independent manner in memory but not in naive CD4$^+$ T cells. Moreover, CD28 in combination with IL-2 was sufficient for the induction of Th2 differentiation from memory CD4$^+$ T cells. Signaling via the TCR, on the other hand, was necessary for Th2 differentiation of naive T cells but was suppressive of Th2 differentiation from memory T cells.

**Engagement of CD28 by its natural ligands, CD80, and/or CD86 induces Th2 cell differentiation from CD4$^+$ memory T cells**

To exclude the possibility that CD28-mediated Th2 cell differentiation from memory T cells could be induced by engaging CD28 with a mAb to CD28 but not its natural ligands, Th2 cell differentiation was assessed after priming of purified CD4$^+$ memory T cells with transfectants expressing human CD80 and/or CD86 (29). For control, memory T cells were cultured with mock-transfected cells. Whereas the mock control did not induce Th2 cell differentiation, priming in the presence of myeloma cells expressing CD80, CD86, or CD80 and CD86 significantly increased Th2 cell frequencies (Fig. 2). These data indicate that the initiation of CD28-mediated Th2 cell differentiation from memory T cells is not a characteristic of the anti-CD28 mAb used but can be induced by engaging CD28 with its natural ligands, CD80, and/or CD86.

**CD28-induced Th2 cell differentiation of memory T cells is dependent on IL-4**

Th2 cell differentiation induced by Ag-specific stimulation is IL-4 dependent (33, 34). To assess whether Ag-independent generation of Th2 cells required IL-4, CD4$^+$ memory T cells were stimulated with anti-CD28 in the presence or absence of a blocking mAb to

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**Table I. Frequency of Th2 cells in resting and primed effector populations**

<table>
<thead>
<tr>
<th>Effector Cells Primed with</th>
<th>n</th>
<th>Resting Cells</th>
<th>Anti-CD28 anti-CD3</th>
<th>Anti-CD28 recombinant IL-4</th>
<th>Anti-CD28</th>
<th>Anti-CD28 recombinant IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive CD4$^+$ T cells</td>
<td>11</td>
<td>0.2 ± 0.1</td>
<td>5.3 ± 4.1$^a$</td>
<td>5.6 ± 3.0$^c$</td>
<td>0.4 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Memory CD4$^+$ T cells</td>
<td>16</td>
<td>3.1 ± 1.6</td>
<td>3.2 ± 1.7</td>
<td>4.8 ± 2.5$^d$</td>
<td>6.1 ± 2.8</td>
<td>10.1 ± 3.6$^e$</td>
</tr>
</tbody>
</table>

$^a$ Intracellular cytokines were detected in freshly isolated and primed naive and memory T cells by flow cytometry.

$^c$ Statistically significant differences compared to the starting population (p < 0.002 paired two-tailed Student’s t test).

$^d$ Not significantly different from the starting population or the medium control.

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**FIGURE 1.** Signaling through CD28 is sufficient to induce Th2 cell differentiation from memory but not from naive CD4$^+$ T cells. CD4$^+$ naive (upper panel) and memory T cells (lower panel) were primed with anti-CD28 and the presence or absence of anti-CD3 (A) or the presence of IL-4 (B). For control, cells were cultured in IL-2 and control Ig (medium). Intracellular cytokines were detected by flow cytometry as described in Materials and Methods. Data from one representative experiment of at least four independent experiments carried out are shown.
were as low as in unstimulated controls (Fig. 4D), indicating that the increase in IL-4 mRNA after CD28 stimulation is caused to a significant extent by the induction of IL-4 gene transcription.

The transcription factors GATA-3 and c-maf have recently been implicated in controlling Th2 cell differentiation (35, 36). To determine whether CD28 ligation induced the transcription of GATA-3 or c-maf, CD4+ memory T cells were stimulated with anti-CD28 and the mRNA levels for GATA-3 and c-maf were assessed. Whereas low mRNA levels for both of the transcription factors could be detected in freshly isolated CD4+ memory T cells, they did not increase after engagement of CD28 (Fig. 4E).

**CD28 and IL-4 are synergistic in generating Th2 cells from memory T cells but are not sufficient to induce Th2 cell differentiation from naive T cells**

The induction of IL-4 gene transcription in memory but not in naive T cells by anti-CD28 was a major difference between the T cell subsets. A simple explanation for the inability of CD28 to induce Th2 cell differentiation in naive T cells, therefore, would be the lack of IL-4 production in response to CD28 engagement. To determine whether IL-4 could complement CD28-mediated signals for the initiation of Th2 differentiation in naive cells, IL-4 was added into cultures of naive T cells that were primed with anti-CD28. Whereas IL-4 in combination with IL-2 alone did not induce T cell differentiation in either memory or naive T cells (data not shown), IL-4 had a synergistic effect with anti-CD28 on the generation of Th2 cells in memory T cells (Fig. 1B). By contrast, no Th2 cells could be generated from naive T cells even when IL-4 was added during priming (Fig. 1B). Thus, the differences in induction of IL-4 transcription are not sufficient to explain the different responses of memory and naive T cells to CD28 with respect to Th2 cell differentiation.

**CD28 stimulation induces tyrosine phosphorylation of MAP kinases and Akt in CD4+ T cells**

The MAP kinase pathways transduce multiple signals involved in cell proliferation, differentiation, or death and might also be linked to CD28 stimulation through the sequential activation of GRB2/SOS/p21ras/Raf-1 (37, 38). To delineate their role in CD28-mediated Th2 differentiation, phosphoactivation of the MAP kinases ERK1 and ERK2, p38, and c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) in response to CD28 engagement and their individual contribution to Th2 cell differentiation were studied.

Low levels of phosphorylated ERK2 could be detected in freshly isolated memory but not in naive T cells (Fig. 5A). Stimulation with anti-CD3 induced a marked increase of activated ERK1 and ERK2 to a comparable extent in both T cell subsets. Interestingly, IL-2 alone induced activation of both ERK isoforms. The response to IL-2, however, was lower in naive T cells compared to memory T cells. Of note, CD28 was without any obvious

**FIGURE 2.** Engagement of CD28 by its natural ligands, CD80, and/or CD86 is sufficient to induce Th2 cell differentiation from CD4+ memory T cells. CD4+ memory T cells were primed with human myeloma cells expressing CD80, CD86, or CD80 and CD86 after transfection employing a recombinant adeno-associated virus vector. For control, memory cells were cultured with mock-transfected myeloma cells. Intracellular cytokines produced by CD4+ memory T cells were detected by flow cytometry as described in Materials and Methods. Data from one of two independent experiments carried out are shown.

**FIGURE 3.** CD28-mediated Th2 cell differentiation is dependent on IL-4. Th2 cell differentiation was induced from CD4+ memory T cells by priming with anti-CD28 in the presence or absence of a neutralizing mAb to IL-4. Intracellular cytokines were detected by flow cytometry as described in Materials and Methods. Data from one representative experiment of three independent experiments carried out are shown.
FIGURE 4. CD28 induces IL-4 gene transcription in memory but not in naive CD4+ T cells. CD4+ memory (A) and naive (B) T cells were stimulated with anti-CD28 in the absence of TCR ligation. Cells were harvested at various times after stimulation as indicated, and IL-4 mRNA levels were determined by RT-PCR, employing 32 (A) or 35 (B) amplification cycles, respectively. Cyclophilin mRNA was used as control. (C and D) Actinomycin D inhibits CD28-induced elevation of IL-4 mRNA. C, CD4+ memory T cells were stimulated with anti-CD28 in the absence or presence of actinomycin D, and IL-4 mRNA levels were assessed by RT-PCR at the time indicated. D, CD4+ memory T cells were stimulated by anti-CD28 in the absence of TCR ligation for 5 h. Then actinomycin D was added where indicated and IL-4 mRNA levels were determined at different times by RT-PCR. E, CD4+ memory T cells were stimulated with anti-CD28 and mRNA levels for the transcription factors GATA-3 and c-maf were assessed by RT-PCR at the times indicated. IgG, Culture with isotype-matched control Ig. Data from one of at least two independent experiments are shown.

Effect on steady-state, IL-2-induced, or anti-CD3-mediated phosphorylation of ERK1 and ERK2 in either T cell subset (Fig. 5A).

In contrast, engagement of CD28 induced a marked degree of phosphorylation of p38 MAP kinase in memory T cells but not in naive T cells (Fig. 5B). An additive effect on p38 MAP kinase phosphorylation was observed after costimulation of CD3 and CD28. In naive T cells, phosphorylation of p38 MAP kinase was dependent on stimulation of the TCR. Anti-CD28 alone (Fig. 5B) or in combination with IL-2 (data not shown) was without effect on p38 MAP kinase activation in naive T cells. It should be noted that in some naive T cell samples, a small increase in p38 MAP kinase phosphorylation was observed after stimulation with anti-CD28 in the absence of TCR ligation. However, the response to anti-CD28 in naive cells was significantly weaker compared to CD3-induced phosphorylation and was minimal compared with the extent of the anti-CD28-induced phosphorylation of p38 MAP kinase in memory T cells.

Phosphorylated JNK/SAPK could be detected in primary CD4+ naive and memory T cells (Fig. 5C). It should be noted, however, that JNK/SAPK levels appeared to be lower in naive T cells compared to memory T cells as the detection of JNK/SAPK required a much more prolonged exposure of the Western blots compared to memory T cells. Despite this, JNK/SAPK phosphorylation was induced by stimulation with anti-CD28 in naive T cells (Fig. 5C) and costimulation with anti-CD3 and anti-CD28 further increased the levels of activated JNK/SAPK. In contrast, an isotype-matched control Ig did not activate JNK/SAPK. In resting memory T cells, activation of JNK/SAPK was not induced to a significant extent by anti-CD28 alone but was induced modestly by costimulation via CD3 and CD28 (Fig. 5C).

As previous studies have suggested that, in contrast to the findings reported here for primary naive T cells, activation of JNK/SAPK in Jurkat T cells depended on costimulation of CD3 and CD28 (39, 40), JNK/SAPK phosphorylation via CD3 and/or CD28 was also assessed in Jurkat T cells. Activated JNK/SAPK could hardly be detected in resting Jurkat T cells (Fig. 5E). Furthermore, activation of JNK/SAPK was only noted when these cells were strictly costimulated with anti-CD3 and anti-CD28, whereas no activity was induced with either signal alone (Fig. 5E).

MAP kinase activation is necessary for Th2 cell differentiation

The previous experiments revealed quantitative and qualitative differences in signal kinase phosphorylation between naive and memory T cells in response to CD28 ligation. In contrast to p38, ERK1/2 and PI3-kinase pathways, activation of JNK/SAPK was more prominent in naive T cells and was therefore thought to be unlikely to contribute to CD28-mediated Th2 cell differentiation. Consequently, the role of PI3-kinase and the ERK1/2 and p38 MAP kinase pathways in CD28-mediated Th2 differentiation was assessed by using highly specific inhibitors for the signaling molecules during the priming cultures.

PI3-kinase activity was not required for Th2 cell differentiation, as its inhibition by wortmannin did not reduce the frequencies of Th2 cells below the level of the vehicle control (Fig. 6A). Importantly, wortmannin completely inhibited the anti-CD28-induced PI3-kinase activity in memory T cells (Fig. 6D). By contrast, inhibition of p38 MAP kinase or MAPKK1, an upstream activator of ERK1/2 completely prevented Th2 cell differentiation (Fig. 6B and C). A chemically related control for the MAP kinase inhibitors was without effect on Th2 differentiation (data not shown). Of note, whereas the kinase inhibitors inhibited the kinase activity of the respective targets (Fig. 6 D–F), they did not inhibit proliferation of the cells, and the viability of the cells was not affected by the presence of the inhibitors, excluding nonspecific toxicity.
The dominant role of p38 MAP kinase activation in CD28-induced Th2 cell differentiation is not restricted to controlling IL-4 production

To delineate whether p38 activation exerts its effect on Th2 cell differentiation predominantly by inducing IL-4 production (20), Th2 cells were generated from resting memory T cells in the presence of the p38 MAP kinase pathway inhibitor SB203580 and in the presence of exogenous IL-4. As shown in Fig. 7, priming with anti-CD28 induced Th2 cell differentiation that was enhanced by exogenous IL-4. Notably, however, IL-4 could not compensate for the inhibitory effects of SB203580, which prevented the generation of Th2 effectors even in the presence of IL-4.

Discussion

Th2 effector cells exhibit a number of functions that might down-regulate Th1-mediated cellular immunity, both by counteracting Th1 effector functions such as repressing macrophage activation and by directly inhibiting the development and/or perpetuation of Th1-driven inflammatory responses. Since CD28 is a major regulatory molecule in the differentiation of Th2 effectors from uncommitted resting precursor CD4+ T cells and has recently been implicated in driving Th2 cell differentiation from memory T cells in the absence of TCR ligation, we investigated the signaling pathways elicited by CD28 in isolated human memory and naive CD4+ T cells and the contribution of the individual signaling molecules to CD28-mediated Th2 cell differentiation. In memory but not in naive T cells, engagement of CD28 was sufficient to induce Th2 cell differentiation in the presence of low concentrations of IL-2 by initiating transcription of IL-4 and phosphoactivation of MAP kinases.

Although it is generally agreed that engagement of cell surface receptors such as CD28 with mAbs mimics the interaction of the surface receptor with its natural ligands, some concerns remain as to whether activation of a surface molecule with a mAb in vitro fully recapitulates the physiological contact between the receptor/counterreceptor in an in vivo situation. Here, we demonstrate that Th2 cell differentiation from memory T cells could not only be induced by priming with a mAb to CD28 but also by priming with transfectants expressing the physiological ligands for CD28, human CD80, and/or CD86. The data, therefore, demonstrate that CD28-mediated Th2 cell differentiation is not limited to stimulation by anti-CD28 mAb but rather indicate that TCR-independent Th2 cell differentiation can be induced by engagement of CD28 with its natural ligands.

We have previously shown that the increase of cells capable of producing IL-4 after priming of memory T cells with anti-CD28 is not simply caused by an expansion of cells that have already been committed to the Th2 lineage and is not related to a preferential survival of an already existing T cell subset but is rather clearly caused by differentiation of uncommitted early CD27+ memory T cells into Th2 effectors (21). In those experiments, early CD27+ memory T cells that cannot produce IL-4 could be induced to differentiate into IL-4-secreting Th2 effectors after priming with anti-CD28 in the presence of IL-2 and exogenous IL-4. Therefore, the ability to secrete IL-4 was acquired during differentiation of the early CD27+ memory T cells. Thus, it appears that in humans a subset of early memory T cells has sustained the flexibility to differentiate into either Th cell subset and that the pool of memory T cells is not solely comprised of precommitted, already differentiated Th cells. In this regard, it is of interest to note that the homing receptor CCR7 has recently been described to characterize uncommitted memory T cells that could not produce IL-4 or IFN-γ but could acquire the ability to secrete those cytokines during differentiation into the respective Th cell subsets (42).

Recognition of a specific peptide ligand in the context of the appropriate MHC by the TCR triggers Ag-specific T cell activation. For optimal T cell activation, however, a second signal deriving from an Ag-independent ligation of a costimulatory molecule, such as CD28, is mandatory. In fact, ligation of the TCR in the absence of sufficient costimulation results in Ag-specific anergy of T cells (43). By contrast, ligation of costimulatory molecules in the absence of TCR stimulation has long been regarded to be without cellular responses in normal T cells. Not surprisingly, therefore, a large body of evidence has suggested that signaling through the TCR-CD3 complex is an indispensable requirement for T cell activation. It has only recently begun to be appreciated that T cells might be activated in vitro and in vivo through conventional costimulatory ligands, such as CD28, in a TCR-independent, hence, Ag-independent manner (14, 19, 21, 44, 45). Thus, T cell effector functions might be provoked independent of the TCR and become involved in controlling immune responses. As demonstrated in the current study, ligation of CD28 in the absence of TCR engagement may mediate the initiation of Th2 cell differentiation in memory T cells that might provide a mechanism to generate IL-4-producing effector cells potentially contributing to the regulation of Th1-driven cellular immunity. However, it should be
emphasized that the generation of Th2 effectors in response to anti-CD28 stimulation was dependent on the stage of T cell maturation as it was restricted to the memory T cell population and did not occur in naive cells.

In contrast to memory T cells, Th2 cell differentiation in naive T cells required additional signals provided by the TCR. Analysis of freshly isolated resting naive and memory T cells with flow cytometry after staining with mAbs to CD28 revealed no differences in the surface density of CD28 (data not shown). Moreover, naive T cells have been shown to be extremely sensitive to co-stimulatory signals by anti-CD28, which are highly effective in potentiating IL-2 production and proliferation (46). As demonstrated here, CD28 engagement activated signaling pathways in naive T cells, as evidenced by the induction of Akt and JNK phosphoactivation. Despite similar densities of surface CD28, the responsiveness of naive T cells to CD28-mediated co-stimulatory signals in Ag-dependent T cell activation and the transduction of activation signals, however, anti-CD28 failed to stimulate T cell differentiation of naive T cells. Thus, the differences between memory and naive T cells with regard to CD28-triggered Th2 cell differentiation cannot simply be explained by impaired CD28 signaling in naive T cells but provide an ideal opportunity to define CD28-mediated signals involved in and required for Th2 cell differentiation.

T cell differentiation has been correlated with cell cycle progression and it has been demonstrated that proliferation is required for CD28-induced Th2 cell differentiation. As demonstrated here, CD28 engagement activated signaling pathways in naive T cells, as evidenced by the induction of Akt and JNK phosphoactivation. Despite similar densities of surface CD28, the responsiveness of naive T cells to CD28-mediated co-stimulatory signals in Ag-dependent T cell activation and the transduction of activation signals, however, anti-CD28 failed to stimulate T cell differentiation of naive T cells. Thus, the differences between memory and naive T cells with regard to CD28-triggered Th2 cell differentiation cannot simply be explained by impaired CD28 signaling in naive T cells but provide an ideal opportunity to define CD28-mediated signals involved in and required for Th2 cell differentiation.

FIGURE 6. CD28-induced Th2 cell differentiation in memory T cells is dependent on p38 and ERK1/2 but independent of PI3-kinase. Th2 cell differentiation was induced from freshly isolated CD4⁺ memory T cells by priming with anti-CD28 in the presence or absence of inhibitors specific for PI3-kinase (wortmannin, A), MAPKK1 (PD98059, B), or p38 MAP kinase (SB203580, C). Controls contained IL-2 and control Ig (medium). Ethanol (solvent for the inhibitors) was added in equal concentrations to all cultures. Frequencies of Th2 cells were determined by flow cytometry for cytoplasmic IL-4 as described in Materials and Methods. D, CD28 ligation induces PI3-kinase activity in Jurkat and memory CD4⁺ T cells that can be blocked by wortmannin. Jurkat (upper panel) and memory CD4⁺ (lower panel) T cells were pretreated for 1 h with increasing concentrations of wortmannin and stimulated with anti-CD28 or control Ig. PI3-kinase was immunoprecipitated and PI3-kinase activity was assessed as described in Materials and Methods. E and F, PD98095 and SB202580 inhibit the activity of ERK and p38, respectively. CD4⁺ memory T cells were pretreated for 1 h with increasing concentrations of the inhibitors, stimulated with IL-2 (E) or with anti-CD28 or a control Ig (F). The activities of the immunoprecipitated active kinases were determined in kinase assays using specific substrates of the kinases, as described in Materials and Methods. PI3P, phosphatidylinositol 3-phosphate; pElk1, phosphorylated Elk1; pATF2, phosphorylated activating transcription factor 2. Data from one representative experiment of at least three independent experiments are shown.

FIGURE 7. The role of p38 MAP kinase in CD28-induced Th2 cell differentiation is not restricted to controlling IL-4. Th2 cell differentiation was induced from freshly isolated CD4⁺ memory T cells by priming with anti-CD28 in the presence or absence of recombinant IL-4 and the presence or absence of a specific inhibitor of p38 MAP kinase (SB202580). Frequencies of Th2 cells were determined by flow cytometry for cytoplasmic IL-4 as described in Materials and Methods. Data from one of two independent experiments are shown.
for resting T cells to gain the ability to express IL-4 and IFN-γ (47). In the absence of IL-2, the mAb to CD28 used in the current study did not induce T cell proliferation or activation and, consequently, did not initiate Th2 cell differentiation (data not shown). However, when low concentrations of IL-2 were added along with the anti-CD28 mAb into the priming cultures, the T cells were activated and T cell proliferation occurred in both subsets, indicative of cell cycle progression. Whereas IL-2 itself did not impose a functional bias for memory T cells, small amounts of IFN-γ-producing Th1 effectors were generated in naïve T cells by IL-2 alone. These observations indicate that adequate T cell activation had been provided to allow memory and naive T cells to initiate differentiation. The findings from the current study are supportive of recent reports that naïve T cells possess a default differentiation pathway into Th1 effectors, unless Th2 cell differentiation is enforced by regulatory stimuli such as IL-4 and/or costimulation through CD28 (23, 24). In the experiments carried here, however, IL-4 could inhibit the generation of the IL-2-induced IFN-γ producers but was not sufficient to supplement IL-2-mediated signals to induce Th2 cell differentiation. Of importance, whereas costimulation through CD28 complemented TCR stimulation to generate Th2 effectors from naïve T cells, CD28-mediated signals had no obvious regulatory potential when the TCR was not engaged. Together, the data can be interpreted to suggest that in the absence of TCR ligation, CD28 did not provide the signals required for Th2 cell differentiation in naïve T cells despite the fact that the cells were sufficiently activated by IL-2 to progress into the cell cycle and induce cell differentiation.

Similar to Ag-specific Th2 cell differentiation, CD28-mediated Ag-independent Th2 cell differentiation from memory T cells was dependent upon IL-4. With no exogenous IL-4 added, therefore, CD28 stimulation must have been sufficient to induce IL-4 production in memory T cells. Stimulation of CD28 alone has been shown previously to activate the IL-4 promoter (14) and to increase mRNA levels for IL-4 in human T cells (19) and in an IL-4-producing Jurkat T cell line (14). The data presented here suggest that the CD28-mediated increase in IL-4 mRNA levels in human memory T cells is caused to a significant extent by gene transcription rather than by the mRNA-stabilizing effects of CD28 (12, 13). By contrast, the activation of CD28 did not induce a significant expression of transcription factors that have previously been implicated in controlling Th2 cell development (35, 36). It is interesting to note that in the rat, a mitogenic anti-CD28 mAb was able to increase GATA-3 activation (48). The molecular mechanisms, therefore, of CD28-induced IL-4 gene transcription in humans remain to be shown. Of note, although IL-4 was induced by anti-CD28 and was required for Th2 cell differentiation, it did not induce Th2 cells from memory T cells in the absence of CD28 engagement (data not shown). Thus, the induction of IL-4 gene transcription by CD28 in memory T cells was a requirement but was not sufficient for the development of Th2 effectors.

In contrast to the different effects on transcription of the IL-4 gene, other signals associated with cellular growth and/or differentiation were activated in a comparable manner in naïve and memory T cells by CD28 ligation. For example, phosphoactivation of Akt was induced by CD28 ligation in the absence of other stimuli in both naïve and memory T cells. However, although quantitative differences in the extent of Akt phosphoactivation in response to CD28 might have occurred, the contribution of the PI3-kinase pathway to Th2 cell differentiation became less likely when the specific inhibitor of PI3-kinase, wortmannin, was unable to inhibit Th2 cell differentiation.

The MAP kinase pathways which are thought to provide a mechanism for cross-talk between CD3- and CD28-mediated signals during T cell costimulation (38) are involved in the transduction of a variety of growth and differentiation signals. Although MAP kinase activation has been documented in different experimental systems of T cell activation (49–51), the contribution of MAP kinases to CD28-induced T cell activation in the absence of TCR ligation, in particular in resting peripheral blood T cells, was less clear. In this study, we show that CD28 alone can activate p38 and JNK/SAPK but does not induce ERK phosphorylation in CD4+ T cells.

It has recently been reported that full activation of JNK/SAPK in T cells occurred only after costimulation of the TCR and CD28 (39, 40). In support of this conclusion, costimulation of CD3 and CD28 was required to activate JNK in Jurkat T cells, as demonstrated here (Fig. 5E). Moreover, the modest activation of JNK in memory T cells was only noted when they were costimulated via CD3 and CD28. The conclusion that JNK/SAPK activation in T cells requires costimulation through the TCR and CD28 has recently been challenged when it was demonstrated that in primary resting T cells CD28 engagement is fully capable of triggering the JNK/SAPK cascade without converging TCR-derived signals (52). Likewise, in the experiments described here, CD28 ligation activated JNK/SAPK in primary human naive T cells independent of TCR-mediated signals. It should be noted, however, that the levels of JNK protein appeared to be lower in naive cells compared to memory T cells. Nevertheless, an increase in JNK activation was consistently observed in naïve T cells after stimulation with anti-CD28 that was even more pronounced after costimulation via CD3 and CD28. The molecular mechanisms of CD28-mediated JNK/SAPK activation in human naive T cells remains to be shown.

Of note and in contrast to naïve T cells, CD28 ligation did not induce significant JNK/SAPK activation in human memory T cells. Moreover, costimulation via CD3 and CD28 also had little effect on JNK/SAPK phosphoactivation. The purification of the cells included negative selection against HLA-DR-positive cells. Consequently, the resulting population of memory T cells did not express the activation markers CD25, CD30, CD69, CD96, and HLA-DR (data not shown) and, therefore, was a population of resting memory T cells. Thus, it is possible that coligation via CD3 and CD28 might induce JNK activation in preactivated cells, such as Jurkat T cells (39, 40), but might not be sufficient for phosphoactivation of JNK/SAPK in resting memory T cells. Consistent with this possibility is the recent finding that coligation of CD28 and CD3 failed to induce JNK activation in primary murine splenic T cells and in murine thymocytes (53). The significance of the differences in the capacity of ligation of CD28 to induce JNK activation in human naive and memory T cells as demonstrated here remains to be elucidated.

Whereas JNK/SAPK activation was most prominently observed in naïve T cells, p38 activation appeared to be confined to the CD45RO+ memory T cell subset. This suggested that in contrast to JNK/SAPK, p38 MAP kinase might play a role in CD28-mediated Th2 cell differentiation in human memory T cells. In fact, induction of Th2 cell differentiation in the presence of the highly specific inhibitor of p38 MAP kinase, SB203580, failed, indicating that activation of the p38 MAP kinase pathway was an essential requirement for CD28-induced Th2 cell differentiation. Similar results were obtained with a different inhibitor of the p38 MAP kinase pathway, SB202190 (data not shown). p38 MAP kinase has recently been implicated in regulating IL-4 production by memory T cells after costimulation of CD3 and CD28 (20). To exclude that blocking of p38 MAP kinase with SB203580 inhibited CD28-induced Th2 cell differentiation directly by preventing the production of IL-4, CD28-mediated Th2 cell differentiation was examined in the presence of SB203580 and exogenous IL-4. In these
experiments, IL-4 could not compensate for the inhibitory effects of SB203580 (Fig. 7), emphasizing the dominant role of p38 MAP kinase in CD28-induced Th2 differentiation that was not restricted to controlling IL-4 production. As p38 activation was also induced by simultaneous ligation of CD3 and CD28 in memory T cells, the unique features of CD28-mediated signals in Th2 cell differentiation warrant further elucidation. However, the data presented here strongly suggest a critical role for p38 in CD28-mediated Th2 cell differentiation.

Despite the lack of ERK1/2 phosphorylation in response to CD28, activation of the ERK1/2 pathway was also essential for Th2 cell differentiation, as determined by the use of the MAPK1 inhibitor PD 98059. The apparent discrepancy might be best explained by the fact that IL-2 alone was able to phosphoactivate ERK1/2 in memory T cells and, to a lesser extent, in naive T cells. As IL-2 was required for anti-CD28-induced Th2 cell differentiation (data not shown), it can be surmised that in the presence of IL-2 and anti-CD28, ERK1/2 activation occurred in the priming cultures and that blocking of ERK1/2 activation might therefore interfere with some initial signaling events. Moreover, the data are consistent with the conclusion that IL-2 induced ERK phosphorylation, which complemented CD28-mediated signals required for Th2 cell differentiation. Nevertheless, it also appears that phosphoactivation of ERK1/2, although important for differentiation, was not induced upon CD28-mediated signals.

In summary, we have provided evidence that Th2 cell differentiation can be induced by signaling through CD28 in the absence of TCR ligation, and hence in an Ag-independent manner, in human peripheral blood memory but not in naive T cells. Engagement of CD28 initiated IL-4 gene transcription in memory but not in naive T cells and activated the PI3-kinase, the JNK/SAPK, and the p38 MAP kinase pathways. CD28-induced Th2 cell differentiation could be triggered by interaction of CD28 with its natural ligands, CD80, and/or CD86, and was as dependent on IL-4 and activation of the MAP kinases p38 and ERK. Since CD28 along with IL-2 was sufficient to provide all of the signals required for Ag-independent Th2 cell differentiation, CD28-mediated Th2 cell generation might provide a mechanism to regulate Th1-dominated cellular immunity by generating activated Th2 effectors with the potential to down-modulate Th1-driven effector functions.

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