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J Immunol published online 19 October 2011
http://www.jimmunol.org/content/early/2011/10/19/jimmunol.1101804

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/10/19/jimmunol.1101804.4.DC1

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T Cell-Signaling Network Analysis Reveals Distinct Differences between CD28 and CD2 Costimulation Responses in Various Subsets and in the MAPK Pathway between Resting and Activated Regulatory T Cells

Maria Elisabeth Kalland, Nikolaus Günter Oberprieler,1 Torkel Vang, Kjetil Taskén, and Knut Martin Torgersen2

To uncover signaling system differences between T cell stimuli and T cell subsets, phosphorylation status of 18 signaling proteins at six different time points following TCR triggering and CD28/CD2 costimulation was examined in human T cell subsets by phopho-epitope–specific flow cytometry of fluorescent cell barcoded samples, thereby providing a high-resolution signaling map. Compared with effector/memory T cells, naïve T cells displayed stronger activation of proximal signaling molecules after TCR triggering alone. Conversely, distal phosphorylation events, like pErk and pS6-ribosomal protein, were stronger in effector/memory subsets. CD28 costimulation specifically induced signaling necessary for proper NF-κB activation, whereas CD2 signaled more strongly to S6-ribosomal protein. Analysis of resting regulatory T cells (rTregs; CD4+CD45RA−FOXP3+) and activated regulatory T cells (actTregs; CD4+CD45RA−FOXP3+) revealed that, although rTregs had low basal, but inducible, Erk activity, actTregs displayed high basal Erk phosphorylation and little or no Akt activation. Interestingly, the use of Mek inhibitors to block Erk activation inhibited activation-dependent FOXP3 upregulation in rTregs, their transition to actTregs, and the resulting increase in suppressive capacity. In summary, our systems approach unraveled distinct differences in signaling elicited by CD28 and CD2 costimulation and between rTregs and actTregs. Blocking rTreg transition to highly suppressive actTregs by Mek inhibitors might have future therapeutic applications.

The Journal of Immunology, 2011, 187: 000–000.
(14, 15), whereas the second encompasses activation of PI3K, phosphatidylinositol-dependent kinase 1, and Akt. The relatively mild phenotype associated with CD28 deficiency suggests that other costimulatory molecules can compensate for the loss of CD28, and CD2 has been proposed to play such a role (16–18). CD2 can act both as an adhesion molecule and a signaling molecule. In fact, CD2 cross-linking can induce proliferation of T cells, as well as cytokine secretion, both in a ZAP70-dependent manner (19). Independently of TCR stimulation, interactions between CD2 and its ligand CD58 can also induce signaling that leads to formation of CD2 clusters in distinct membrane microdomains (20). Moreover, transgenic expression of CD2 in developing thymocytes results in increased apoptosis at the double-positive stage, suggesting that CD2 generates signals that resemble the ones derived from the TCR (21). Along these lines, both Lck and Fyn have been shown to interact with CD2 and to become activated in response to CD2 cross-linking (22, 23). Similarly to CD28-deficient T cells, CD2-deficient T cells are only mildly affected, indicating some redundancy (24). However, the combined lack of CD28 and CD2 leads to profound defects in activation and proliferation of T cells (16), suggesting the necessity of at least one of these costimulatory molecules for proper T cell function.

Activation of CD4+ T cells is required for a majority of adaptive immune responses, and a balanced reaction is essential to avoid excessive tissue damage and autoimmunity. Consequently, conventional CD4+ T cells are kept in check by regulatory T cells (rTregs; CD45RA+CD25+FOXP3+), and activated Tregs supports this notion (30). These subsets are conventional naive CD4+ T cells in human blood can be divided into five categories based on the expression of CD45RA, CD25, and FOXP3 (30). These subsets are conventional naive CD4+ T cells (CD45RA+CD25+FOXP3+), conventional effector/memory CD4+ T cells (CD45RA−CD25+FOXP3−), CD4+FOXP3+ effector T cells with cytokine-producing capabilities (CD45RA−CD25+FOXP3+), resting Tregs (rTregs; CD45RA−CD25−FOXP3−), and activated Tregs (actTregs; CD45RA−CD25+FOXP3+). Given proper stimulatory conditions, naive Tconv can proliferate and differentiate to become effector/memory Tconv. In a similar manner, rTregs can be considered precursors of actTregs. The observation that, in response to activation, rTregs can proliferate and differentiate into Ki-67+ FOXP3+CD45RO+ cells with suppressive abilities comparable to actTregs supports this notion (30).

Because the abovementioned CD4+FOXP3+ T cell subsets combined constitute <10% of all CD4+ T cells in peripheral blood, traditional biochemical studies of signal transduction in these cells have been challenging. However, recent technical developments, including fluorescent cell barcoding (FCB) (31) and a growing number of phospho-epitope–specific Abs, have made it possible to use phospho-epitope–specific flow (phospho-flow) cytometry-based methods to study signaling processes at single-cell resolution in several phenotypically defined T cell subsets simultaneously (32–34). Furthermore, these developments have enabled an increase in the resolution to a level at which signaling differences can be linked to functional properties in small subsets of cells. To understand systems-level signaling differences between different T cell stimuli and in various T cell subsets, we used this technique to investigate signaling and the role of CD28 and CD2 costimulation in different human CD4+ T cell subsets, including the CD4+FOXP3+ subsets. We were able to reproducibly identify signaling characteristics and define distinct differences between CD28 and CD2 in the activation of NF-kB and the S6-ribosomal protein (S6-Rp) transcriptional program, respectively. In addition, differences in Akt versus Mek1/Erk signaling between rTregs and actTregs, combined with functional studies with signaling inhibitors, suggested that the activity level of Mek1/Erk and Akt pathways is involved in defining a functional switch between these two subsets.

Materials and Methods

Reagents

Cyclosporin A (cat. no. 239835), PI-103 (cat. no. 526100), Akt Inhibitor VIII (cat. no. 124017), rapamycin (cat. no. 553211), and SB 203580 (cat. no. 559395) were purchased from Calbiochem; U0126 (cat. no. 9905), and wortmannin (cat. no. 9951) were purchased from Cell Signaling; and PD0325901 (cat. no. 1408) was purchased from Axon Medchem. Abs used for T cell stimulation included anti-CD2 (clone OKT3) custom produced from the hybridoma by Diatiec, anti-CD28 (cat. no. 13-0289; eBioscience), and anti-CD2 (cat. no. 13-0029; eBioscience). Avdin (cat. no. 43-4401) was purchased from Ouchterlony, and 7-AAD (cat. no. 750065) from Diatec, anti-CD2 (cat. no. 13-0029; eBioscience), and anti-CD28 (cat. no. 13-0289; eBioscience). Vinculin (cat. no. 345766), CD4 (cat. no. 348809 and 557922), CD25 (cat. no. 557741), CD45RA (cat. no. 555489), CD45RO (cat. no. 555493), and FOXP3 (cat. no. 560047 and 560045) were from BD, whereas the Abs used to detect the expression of CD8 (cat. no. 9536-09) was from Southern Biotech.

Flow cytometry

Saffron (cat. no. 130630), and anti-CD28 [5mM] alone or together with anti-CD2 [5mM] or anti-CD28 plus anti-CD2 [5mM] alone or together with 10 ng/ml IL-2. After 5 min, cells were fixed immediately using prewarmed BD Phosflow Fix Buffer I (BD Biosciences) for 10 min at 37˚C, followed by centrifugation (830 × g, 5 min), one wash with flow-washing solution, per-
Abs and fluorescently labeled cell surface-marker Abs (30 min at room temperature), washed twice with flow-washing solution, and made ready for flow cytometric analysis by resuspension in flow-washing solution. FOXP3 staining, when included, was performed on FCB cell stocks prior to permeabilization and storage at −80°C, using a FOXP3 staining kit from BD Pharmingen (cat. no. 560098). Finally, all samples were analyzed using a BD FACSCanto II (4–2–2) cytometer equipped with 405-, 488-, and 633-nm lasers. For each sample, ≥200,000 events were recorded (corresponding to ≥3000 events per square in the heatmaps for prevalent cell populations and >300 events for less prevalent populations). For fluorescence compensation, PerCP-conjugated CD3 Ab, PE-Cy7–conjugated CD4 Ab, and PE-conjugated CD8, CD45RA, or CD45RO Ab staining was used on unstimulated non-FCB cells. Ax647-conjugated CD3 Ab was used to compensate for the phospho-epitope–specific Abs conjugated to Ax647.

The data-analysis program Cytobank (http://cytobank.stanford.edu) and FlowJo 8.8.2 (TreeStar, Ashland, OR) were used for further analysis and visualization of data.

**Sorting of CD4+ T cell subsets**

Purified CD4+ T cells in PBS with 2% FCS were incubated with fluorescently labeled Abs (anti-CD45RA-PE and anti-CD25-PE-Cy7, anti-CD4-Alexa Fluor 700 was also used unless subsequent suppression assays were to be performed) for 30 min on ice. Thereafter, cells were washed once in PBS with 2% FCS, resuspended in PBS with 2% FCS, and sorted on a BD FACSaria Ii cytometer (5–2) equipped with 488- and 633-nm lasers and set up with a sheath pressure of 70-pound-force per square inch and a 70-μm nozzle.

**In vitro stimulation of rTregs**

rTregs, defined as CD4+CD45RA−CD25+ T cells, were sorted as described. Sorted cells were resuspended at 1 × 10^6 cells/ml in complete medium (RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and nonessential amino acids), incubated with or without specific inhibitors of PI3K (0.5 μM PI-103 in combination with 0.1 μM wortmannin), Akt (either 1 μM Akt1/2 inhibitor or 10 nM of the mammalian target of rapamycin (mTOR) inhibitor, rapamycin), Mek (either 1 μM U0126 or 10 μM of the MEK1/2 inhibitor, PD0325901), or NFAT (0.1 μM of the calcineurin inhibitor, cyclosporin A [CsA]) pathways for 20 min at 37°C, followed by stimulation with αCD3/CD28/CD2-coated MicroBeads (Miltenyi Biotec; bead/cell ratio of 1:5), which was used in all bead-based experiments presented in Figs. 5 and 6 for different time periods. The same experimental setup was also used in additional studies, using purified CD4+ T cells to examine all of the inhibitors’ effect on viability and for titration of optimal concentrations of inhibitors on stimulation-induced upregulation of the fractions of rTregs and actTregs. The defined optimal inhibitor concentrations were used in subsequent experiments. We next tested to what extent inhibition of the Mek–Erk– or Akt-signaling pathways affected the stimulation-induced upregulation of FOXP3 in rTregs. One sample was left unstimulated as a reference. Unstimulated, sorted rTregs (defined as CD4+CD45RA−CD25+ T cells) and naive Tconv (defined as CD4+CD45RA−CD25+ T cells) were included as controls. After stimulation for the intended time periods, samples were washed once in flow-washing solution before staining with 7-AAD. Later, cells were fixed with Buffer A from a FOXP3 staining kit (BD Pharmingen), according to the manufacturer’s protocol, and stored at −80°C. Subsequently, all samples were stained for FOXP3 and surface markers and analyzed as described above.

**Suppression assay**

Sorted rTregs were either added directly into a suppression assay or pre-treated (30 min at 37°C) or not with specific inhibitors of PI3K, Akt, Mek, p38, and NFAT pathways, followed by 36 h of culture in complete medium alone or in the presence of αCD3/CD28/CD2-coated MicroBeads. After two rounds of washing, cells were mixed with CFSE-stained, purified CD4+ T cells (called responder cells) at a 1:1 ratio and stimulated with αCD3/CD28/CD2-coated MicroBeads for 84 h. Cells were then stained with antibodies to CD4 and subjected to flow-cytometric analysis using a BD FACSCanto II. The suppressive capacity of different rTreg populations was determined by the level of CFSE dilution in responder cells using FlowJo 8.8.2 software.

**Processing of data and statistical analysis**

Changes in phosphorylation of signaling proteins following activation of T cells were calculated using the inverse hyperbolic sine (arcsinh) of the median fluorescence intensity (MFI) of stimulated versus unstimulated cell populations. The reason for choosing arcsinh for calculating changes was explained by Irish et al. (36). Comparison of the effects of preincubation of rTregs with different specific inhibitors on FOXP3 expression was analyzed using one-way ANOVA, specifying unstimulated rTregs as the control group. Comparisons of the effects of specific inhibitors on the suppressive capacity of rTregs were also analyzed using one-way ANOVA; stimulated rTregs preincubated in the absence of an inhibitor was used as the control group. Differences in mean values were considered statistically significant when p < 0.05. The statistical analyses were conducted using SigmaPlot 11.2 (Systat Software).

**Results**

**High-throughput analysis of T cell-signaling profiles**

Our experimental setup is outlined in Fig. 1A. After subjecting cells to different stimulatory conditions and fixation with formaldehyde, cells from each stimulatory condition were stained with a unique combination of FCB reagents and, therefore, could be tracked from all other sample populations in subsequent assays. Thus, FCB allows for combining all cell samples prior to staining with fluorescently labeled Abs against intracellular phospho-epitopes and cell surface markers, thereby analyzing all samples with the same baseline, minimizing intra-assay variability and allowing for high-throughput analysis (31).

To define the sensitivity of the experimental system, initial assays involved incubation of purified CD3+ human T cells (95±3% purity, data not shown) with different concentrations of anti-CD3 Ab (1 ng/ml–10 μg/ml) on ice, followed by one round of washing, and then cross-linking with avidin at 37°C for up to 60 min. Fig. 1B shows the subsequent analysis of these cells with regard to the phosphorylation status of five selected signaling intermediates downstream of the TCR (see Supplemental Fig. 1 for 13 additional signaling parameters and data from more individuals). TCR-proximal–signaling molecules (such as ζ-chain, ZAP70, LAT, and Slp-76) were considerably activated/phosphorylated only at anti-CD3 concentrations ≥1 μg/ml, and they peaked after 1–3 min (Fig. 1B). In accordance with the principle of signal-cascade amplification, signaling mediators located more downstream (e.g., Erk, p38, NF-κB, and S6-Rp) were activated at lower levels of stimulation and with delayed kinetics compared with TCR-proximal–signaling molecules (Fig. 1B, Supplemental Fig. 1). These observations were apparent for both CD4+ and CD8+ T cells. Moreover, comparison of data from three independent donors revealed a consistent pattern, testifying to the robustness of the method (Fig. 1C).

Alternatively, purified CD3+ T cells were pre-equilibrated at 37°C prior to addition of anti-CD3 Ab, followed 2 min later by cross-linking with avidin and continued incubation for up to 60 min (Supplemental Fig. 2). Distinct differences were observed when analyzing the whole data set with regard to signaling responses and comparing data obtained from cells incubated on ice with cells treated at 37°C (Supplemental Fig. 2). Phosphorylation of TCR-proximal molecules (such as ζ-chain and ZAP70) appeared stronger in cells that had been incubated on ice compared with cells only treated at 37°C. This could be due to a lower level of CD3 cross-linking under the latter condition, slower and/or weaker effects of inhibitory molecules (e.g., protein tyrosine phosphatases) in cells that had been incubated on ice, or a better synchronized effect of the activation giving stronger synergy in the costimulation when allowing Abs to first bind on ice. However, phosphorylation of TCR-distal molecules, such as Mek1, p38, and NF-κB, were elevated at time 0 following incubation on ice, suggesting a direct effect of the temperature changes on several signaling processes. As a result of this, and to optimize signal-to-noise across the full panel of signal markers, all further experiments were conducted on cells that had been pre-equilibrated at 37°C.
Comparison of T cell-signaling profiles for different naive and effector/memory T cell subsets

Having established a well-working phospho-flow cytometry protocol for a set of markers across relevant signal pathways in T cell activation, we next wanted to dissect signaling profiles in different T cell subsets. Three different stimulatory conditions were used: cross-linking of anti-CD3 alone, cross-linking of anti-CD3/anti-CD28, and cross-linking of anti-CD3/anti-CD2/anti-CD2. For all these experiments, a suboptimal concentration of anti-CD3 (1 μg/ml) was used to capture effects of the different costimulatory conditions. In addition to the panel of 18 phospho-specific Abs described above, staining for CD45RO was included so that naive T cells could be discerned from effector/memory T cells (CD45RO- and CD45RO+, respectively). Because of the limited number of channels in the FACS analysis, CD8 staining had to be omitted in this setup. Still, >90% of CD3+CD4- peripheral T cells were CD3+CD8+ T cells (data not shown), indicating that CD4 negativity within the population of peripheral T cells was a good surrogate marker for CD8 positivity. Therefore, CD3+CD4-CD45RO- and CD3+CD4-CD45RO+ T cells are referred to as naive and effector/memory CD8+ T cells, respectively, whereas CD3+CD4+CD45RO- and CD3+CD4+CD45RO+ T cells denote naive and effector/memory CD4+ T cells. The signaling in each subset was initially analyzed relative to the control sample for the same subset (Fig. 2A). Compared with analysis of the entire populations of CD4+ and CD8+ peripheral T cells, the addition of CD45RO-based subgating of CD4+ and CD8+ T cells revealed differences in signaling responses. In general, when costimulation was not present, the amplitude of the signaling responses seemed to be stronger in naive cells compared with effector/memory cells. This was particularly evident when considering Mek1 and Erk, both of which demonstrated a strong signaling response in naive CD4+ T cells but less so in effector/memory cells.
memory cells. The addition of CD28 costimulation only had a modest effect on the signaling amplitudes in all four subsets and then preferentially on downstream mediators, such as S6-Rp. In contrast, combined costimulation of CD28 and CD2 consistently gave high signaling amplitudes for all signaling molecules tested in all of the subsets (Fig. 2A).

**FIGURE 2.** Comparison of T cell-signaling profiles for different naive and effector/memory T cell subsets. A, Primary human T cells were stimulated by cross-linking of the indicated combinations of Abs with avidin and incubated for different time periods. Subsequently, cells were barcoded, stained with fluorescently labeled Abs, and analyzed by FACS. Phospho-signal profiles for the different T cell subsets are presented as heatmaps, as explained in Fig. 1. The panel for each signaling molecule consists of two parts: the upper one distinguishes between CD3+CD4+ and CD3+CD4− T cells, whereas the lower one also includes naive (CD45RO+) and effector/memory (CD45RO−) subsets within these CD4+ and CD4− T cell subsets. Data are representative of experiments for T cells from three individuals (see Supplemental Fig. 2 for all individual data). B, Experiment as in A, but all values are relative to the phospho-signals obtained for the control sample of the naive CD4+ T cell population. Data are representative of experiments with T cells from three individual blood donors. C, Experiment as in B, but data for T cells from three individuals are combined. Data are mean ± SEM (n = 3) of arcsinh median differences of the phospho-epitope–specific fluorescence intensity signals.
FIGURE 3. Analysis of signaling in conventional and regulatory CD4+ T cell subsets reveals elevated Erk activation and decreased Akt activation in actTregs. A, Gating strategy for distinguishing between CD4+ effector and regulatory T cell subsets using CD45RA and FOXP3 Ab staining. B, Primary human T cells were stimulated by cross-linking of the indicated combinations of Abs with avidin and incubated for different time periods. Cells were then barcoded, stained with fluorescently labeled Abs, and analyzed by FACS. Upper part of each panel, Phospho-epitope–specific (Figure legend continues)
Next, signaling responses in all subsets where analyzed using unstimulated naive CD4+ T cells as a reference (Fig. 2B, 2C). With such an approach, several interesting features were observed. First, compared with CD3 stimulation alone, the addition of CD28 costimulation resulted in higher signaling responses in naive CD4+ T cells, even at the level of TCR-proximal molecules, such as ζ-chain and ZAP70. Second, all four subsets displayed higher signaling responses with two rather than one costimulatory factor present and with an especially strong effect of CD2 costimulation. Third, although phosphorylation of TCR-proximal–signaling molecules (such as ζ-chain, ZAP70, and Slp-76) generally was stronger in naive T cells than in effector/memory T cells (both CD4+ and CD8+ cells), the opposite was the case for more downstream mediators (e.g., Erk and S6-Rp). Fourth, in naive T cells (both CD4+ and CD8+), phosphorylation of Mek1 peaked after 1 min of stimulation and subsequently displayed a second wave of activation in the presence of CD2 costimulation. This suggested involvement of a positive-feedback loop as a result of coreceptor signaling. Somewhat surprisingly, the signaling patterns of Erk did not relate directly to their upstream activator Mek1. Finally, the relatively high basal phosphorylation of S6-Rp in effector/memory subsets, and especially for CD8+ T cells, indicated an activated transcription and translation program, as expected for effector cell populations (Fig. 2C). We observed that addition of CD2 costimulation produced a reproducible increase in S6-Rp phosphorylation in all of the subsets, irrespective of basal status, supporting a role for CD2-induced signals in the regulation of translation at the level of S6-Rp (37). Although the CD2-regulated S6-Rp phosphorylation was in agreement with the kinetics of Akt activation in naive T cells, albeit with a different amplitude consistent with downstream amplification at the S6-Rp level, this did not appear to be the case in effector/memory subsets, indicating the influence of other components of a CD2-regulated signal network in effector/memory T cells (Fig. 2B, 2C, bottom panels), as further addressed in Figs. 3 and 4 below.

Analysis of signaling in conventional and regulatory CD4+ T cell subsets reveals elevated Erk activation and decreased Akt activation in actTregs

As previously described for humans (30, 38), CD4+ Tregs can be divided into two functionally distinct subsets based on CD45RA and FOXP3 expression: CD4+CD45RA+FOXP3+ rTregs and CD4+CD45RA-FOXP3++ actTregs (Fig. 3A). We hypothesized that this functional delineation would be reflected in signal-transduction processes and used the established phospho-flow cytometry protocol to investigate signaling in these subsets. For global overview purposes, the analyses also included naive (CD45RA-FOXP3-) and effector/memory (CD45RA-FOXP3-) CD4+ Tconvs, as well as the CD4+CD45RA-FOXP3+ effector T cell subset with cytokine-secretory ability (30).

As shown in Fig. 3B and 3D, phosphorylation levels of ζ-chain (as well as other TCR-proximal–signaling molecules; Supplemental Fig. 3) were comparable among actTregs, rTregs, and naive CD4+ Tconvs, both in response to CD3 stimulation and when different types of costimulation were added. The same observation was made for rTregs and naive CD4+ Tconvs with regard to Mek1 phosphorylation, whereas the signals for actTregs were weaker and comparable to the ones seen for effector/memory CD4+ Tconvs. Interestingly, levels of Erk activation in actTregs were very high, both in unstimulated cells and after CD3 stimulation with or without costimulation. For all other CD4+ T cell subsets tested, robust Erk activation was observed in response to stimulation, but the signals were always significantly weaker than those in actTregs. ActTregs also differed from most other subsets with regard to Akt signaling (Fig. 3C, 3D), which was nearly absent in actTregs, even in the presence of the strongest stimulus tested (combined cross-linking of CD3/CD28/CD2). In contrast, CD4+ Tconvs, as well as rTregs, displayed potent Akt activation, especially in the presence of costimulation. Again, all subsets significantly increased the levels of S6-Rp phosphorylation when CD2 costimulation was added, even when the basal activity was elevated, as seen for CD4+ effector/memory T cells, which also differed from the kinetics of Akt activation. The basal phosphorylation levels and the patterns of induced responses were reproducible in all three donors tested (Fig. 3D, 3E, Supplemental Fig. 3).

CD28 and CD2 costimulation trigger overlapping but distinct signaling pathways

To delineate differences between CD28 and CD2 in costimulation, we examined more carefully the relative contribution of CD2 and CD28 by cross-linking, either alone or combined, with CD3. Costimulation with CD28 or CD2 separately increased the amplitude of proximal TCR-signaling events at the level of ζ-chain and Slp-76 phosphorylation compared with CD3 stimulation alone (Fig. 4). Similar responses were seen for phosphorylation of ZAP70, Mek1, and histone 3 (Supplemental Fig. 4). Additive effects of CD28 and CD2 were generally observed (as also seen in Fig. 3B–D). However, at the level of Erk activation, the addition of CD28 and/or CD2, compared with CD3 alone, had only a modest effect on the signaling amplitudes in the different subsets tested, indicating that Erk phosphorylation depends mainly on the TCR signal (Fig. 4). Interestingly, the phosphorylation responses of Akt in naive CD4+ T cells and rTregs and in all subsets tested for NF-κB phosphorylation depended mainly on CD28, indicating that the CD28 signal may be essential for proper activation of an Akt–NF-κB pathway in naive CD4+ T cells and rTregs. In contrast, the CD28-dependent activation of NF-κB in other subsets did not seem to be mediated by Akt (Fig. 4B, Supplemental Fig. 4). Stimulation through the CD2 receptor appeared to result in stronger activation of S6-Rp compared with CD28 in the effector T cell subsets and in actTregs. A clear additive effect of the activation of S6-Rp was observed when both costimulators were present.

Mek-Erk–dependent upregulation of FOXP3 in rTregs

rTregs most likely represent a thymus-derived population that, upon stimulation, can expand and mature both in vitro and in vivo to become actTregs, which are characterized by enhanced suppressive capabilities (30). Key events in this maturational process are increased FOXP3 expression and proliferation. Given the significant differences between rTregs and actTregs with respect to activation of Akt and Erk (as described in Figs. 3, 4), we aimed to address
FIGURE 4. Costimulation with CD28 is essential for proper activation of NF-κB–related signaling. A, Primary human CD4+ T cells were stimulated by cross-linking of the indicated combinations of Abs with avidin and incubated for different time periods. Cells were then barcoded, stained with fluorescently labeled Abs, and analyzed by FACS. The data are presented as in Fig. 3 and are representative of experiments from three separate blood donors (see Supplemental Fig. 4 for all individual data). B, Amalgamated data with normalization to the CD4+CD45RA−FOXP3+ subset, as in the lower panels in A. Data are mean ± SEM (n = 3) of arcsinh median differences of the phospho-epitope–specific fluorescence intensity signals.
the importance of different signaling pathways in the transition of rTregs to actTregs. To first assess the induction of FOXP3 protein in rTregs upon activation, CD4+CD25+CD45RA+ T cells were sorted and stimulated with αCD3/CD28/CD2-coated Microbeads in vitro for up to 92 h, followed by flow cytometry analysis of FOXP3 levels. As seen in Fig. 5A and 5B, FOXP3 expression in rTregs increased markedly in a time-dependent manner in response to stimulation. Peak levels, which were reached after 36–44 h, even exceeded the FOXP3 levels observed for actTregs isolated directly from blood (Fig. 5A). At later time points, FOXP3 expression in stimulated rTregs decreased, suggesting that transiently high expression of FOXP3 was necessary to drive the transcriptional program necessary for the maturation of these cells. No significant stimulation-induced upregulation of FOXP3 was observed in conventional naive and effector/memory CD4+ T cell subsets under the same conditions (data not shown).

We next tested to what extent inhibition of the Mek–Erk- or Akt-signaling pathways affected the stimulation-induced upregulation of FOXP3 in rTregs. To do so, all inhibitors were first tested for toxicity, and their effect on upregulation of rTreg and actTreg subsets was assessed over a range of concentrations (see Fig. 5C for titration of the Mek inhibitor PD 0325901). Subsequently, sorted rTregs (purity >98%, data not shown) were incubated with the panel of inhibitors at defined concentrations prior to stimulation for 36 h, as shown in Fig. 5D. Interestingly, Mek inhibitors (PD 0325901 and U0126) that would prevent activation of Erk potently inhibited the stimulation-induced upregulation of FOXP3 (>90%) without any effect on viability (data not shown), whereas pretreatment with inhibitors against PI3K (PI-103 in combination with wortmannin), mTOR (rapamycin), Akt (Akt1/2-inhibitor), or calcineurin (CsA) reduced the FOXP3 induction by ~50%. These effects were consistent between several donors (Fig. 5E), indicating a crucial role for Mek-related signaling in activation-induced upregulation of FOXP3 in rTregs. In comparison, inhibition of p38 (SB 203580) had no significant effect.

**Induction of rTreg-suppressive capacity is Mek dependent**

We next tested the functional consequence of blocking the activation-induced upregulation of FOXP3 in rTregs. In these assays, Treg function was defined as the ability to suppress the proliferation of CFSE-labeled purified CD4+ T cells. Notably, suppressive function could not be demonstrated for sorted rTregs

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**FIGURE 5.** Mek-dependent upregulation of FOXP3 in rTregs. A, Sorted rTregs were stimulated or not with αCD3/CD28/CD2-coated MicroBeads for the indicated time periods, followed by FOXP3 staining and FACS analysis. Sorted actTregs were included as controls. Data are representative of experiments with T cells from three separate blood donors. B, Experiments as in A were performed with T cells from three individuals. The bars represent relative increase in FOXP3 expression after stimulation for the indicated time periods. Relative increase in FOXP3 expression was calculated using MFI and the following formula: \[ \Delta \text{MFI} = \frac{\text{MFI (stimulated)} - \text{MFI (unstimulated)}}{\text{MFI (unstimulated)}} \]. Data are given as mean ± SEM (n = 3).

C, Effects of various concentrations of Mek inhibitor on stimulation-induced increase in rTreg (left panel) and actTreg (right panel) subsets. Purified CD4+ T cells were incubated with indicated concentrations of Mek inhibitor (PD0325901, 20 min) and then stimulated (αCD3/CD28/CD2-coated MicroBeads) for 36 h. One sample was kept unstimulated and used as a reference. Thereafter, FOXP3 staining and FACS analysis were conducted. The bars (mean ± SEM; n = 3) represent increases in rTreg and actTreg subsets after stimulation relative to the unstimulated reference sample. Data are representative of three individual blood donors analyzed in duplicate. D, Experiment as in A, but sorted rTregs were incubated with the indicated inhibitors of Mek (either PD0325901 or U0126), p38 (SB 203580), PI3K (PI-103 in combination with wortmannin), Akt (Akt1/2-inhibitor), mTOR (rapamycin), or calcineurin (CsA) for 20 min, followed by stimulation with αCD3/CD28/CD2-coated MicroBeads for 36 h. E, Experiments as in D were performed with T cells from three individuals and otherwise analyzed as outlined in B. Data are presented as mean ± SEM (n = 3). **p < 0.01, ***p < 0.001.
added back on the day of sorting (Fig. 6; purity >98%, data not shown). Further studies of suppressive capacity of rTregs from six individual blood donors compared with that of actTregs revealed little or no suppression by rTregs without prior stimulation (Table I). However, when rTregs were stimulated for 36 h (to assure proper FOXP3 upregulation) before initiation of the CFSE assay, significant suppression was observed. Finally, incubation of rTregs with a Mek inhibitor prior to stimulation and subsequent initiation of the CFSE assay completely blocked the ability of these cells to become suppressive. In comparison, inhibition of Akt resulted in 50% reduction in suppressive capacity. These results were consistent among all donors tested (Fig. 6B) and suggest that the degree to which these inhibitors prevent FOXP3 upregulation defines their potency to restrain induction of suppressive function in rTregs.

**Discussion**

T cell signaling has been extensively studied for decades, and the various signaling pathways involved have been well characterized. However, there is a need for an overview of how distinct signal pathways integrate and cross-talk to form signaling networks. Furthermore, with the increasing number of different T cell subsets being defined, especially among the group of CD4+ T cells, and the possibility of signaling differences between various subsets, there is a need for more subset-specific analyses to link signaling activity to functional properties. Because several CD4+ T cell subsets are relatively low in abundance (e.g., rTregs and actTregs), such analyses are challenging using traditional biochemical methods. Recent technical advances have made it possible to obtain cell-signaling data even from a relatively low number of cells. One such approach is phospho-flow cytometry in combination with FCB. In this report, we described how this technique can be exploited to assess the phosphorylation status of 18 important signaling intermediates downstream of the TCR and co-stimulatory molecules in various CD4+ and CD8+ T cell subsets. Of note, this assay system captured clear signaling differences between CD28 and CD2 with respect to activation of NF-κB and the S6-Rp program, as well as between rTregs and actTregs where our results indicated that a Mek–Erk-signaling pathway is necessary for the maturation and functional control of human act-Tregs.

The comprehensive analysis of T cell signaling described in this report revealed time-dependent activation/inactivation of the signaling intermediates tested. Generally, TCR-proximal signaling proteins were transiently activated within 1–3 min after TCR cross-linking; the time kinetics for intermediate signaling molecules were slower; and, finally, the most TCR-distal molecules analyzed (e.g., histone 3) displayed the slowest activation kinetics, peaking after 10–30 min and with signals persisting throughout the duration of the assay (60 min). These findings are in agreement with data obtained with conventional methods (e.g., immunoblotting) in the past, indicating that the phospho-flow approach is sufficiently sensitive to capture relevant and established alterations in the phosphorylation status of various signaling molecules.

**Table I.** Proliferation of Tconvs is strongly suppressed by actTregs but not equally well by rTregs

<table>
<thead>
<tr>
<th>Donor</th>
<th>actTregs</th>
<th>rTregs</th>
<th>Tconvs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.0</td>
<td>78.0</td>
<td>78.9</td>
</tr>
<tr>
<td>2</td>
<td>35.9</td>
<td>77.8</td>
<td>86.7</td>
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<td>3</td>
<td>53.8</td>
<td>94.3</td>
<td>94.2</td>
</tr>
<tr>
<td>4</td>
<td>12.8</td>
<td>76.5</td>
<td>80.6</td>
</tr>
<tr>
<td>5</td>
<td>24.7</td>
<td>73.1</td>
<td>79.0</td>
</tr>
<tr>
<td>6</td>
<td>79.1</td>
<td>94.6</td>
<td>93.8</td>
</tr>
</tbody>
</table>

Sorted CD4+ Tconvs (CD45RA+CD25- and CD45RA-CD25+) were CFSE stained and mixed at 1:1 ratio with non-CFSE–stained add back cells (sorted actTregs, sorted rTregs, or sorted CD4+ Tconvs). Thereafter, cells were stimulated with αCD3/CD28-coated T cell expander beads (bead/cell ratio 1:10) for 90 h, followed by assessment of CFSE dilution in cells. Numbers indicate the percentage of proliferation = 0.001, add back of actTregs versus Tconvs; p = 0.099, add back of rTregs versus Tconvs; p = 0.001, add back of actTregs versus rTregs; paired two-tailed t test.

FIGURE 6. Induction of rTreg suppressive capacity is Mek dependent. A. Sorted rTregs were either directly added to CFSE-labeled CD4+ T cells or pretreated with indicated inhibitor and then stimulated with αCD3/CD28-coated MicroBeads for 36 h before being added to CFSE-labeled CD4+ T cells. As a control sample, purified CD4+ T cells were mixed with CFSE-labeled CD4+ T cells (marked 0:1 in the panels). As soon as rTregs (or purified CD4+ T cells) had been mixed with CFSE-labeled CD4+ T cells (1:1 ratio), αCD3/CD28/CD2-coated MicroBeads were added, and incubations continued for 84 h, followed by assessment of CFSE dilution in viable cells. Data are representative of experiments conducted on T cells from three different donors. B. Data from three experiments as in A were combined. The suppressive capacity was calculated as Δproliferation = % proliferation (responder cells alone, 0:1) − % proliferation (responder cells in presence of Tregs, 1:1). Data are presented as mean ± SEM (n = 3). **p < 0.01, ***p < 0.001. Day 0 rTreg, sorted rTregs directly mixed with responder cells; 36 h srTreg, sorted rTregs pretreated with inhibitor or not and then stimulated with αCD3/CD28/CD2-coated MicroBeads for 36 h before being added to responder cells.
For all T cell subsets tested (both among CD4+ and CD8+), our experiments revealed that signaling was clearly augmented in the presence of CD28 costimulation, in agreement with previous observations. As expected, we also found that signaling was even stronger with the additional presence of CD2 costimulation. Based on studies of mice deficient in CD28, CD2, or both (16–18), it is clear that some level of costimulation is necessary for proper T cell activation and commitment; but, these studies also indicated that the loss of CD28 can be compensated for by CD2, and vice versa. However, we also unraveled clear signaling differences in this regard. Indeed, CD28 was necessary and sufficient to trigger activation of NF-κB, indicating that although there may be redundancy in activation of PLCγ pathways, activation of NF-κB may be more exclusively controlled by CD28. Furthermore, it is interesting to note that augmented phosphorylation of many signal molecules is observed when both costimulatory pathways are triggered simultaneously. One possible explanation for this may be that the combined action of both costimulatory molecules is necessary for fine-tuning of some T cell responses. The costimulatory effects of either CD28 or CD2 are believed to be mediated by signaling molecules that are also activated downstream of the TCR. One such candidate is PLCγ1 (39, 40), which was previously suggested to be the most TCR-proximal molecule activated by CD28. However, another upstream candidate is Lck. It is noteworthy that the SH3 domain of Lck can bind proline-rich regions in the cytoplasmic parts of both CD28 and CD2 (41, 42). Because only ~50% of Lck molecules in a CD4+ T cell are bound to CD4 and, hence, are directly involved in TCR signaling, the remaining 50% of Lck molecules are available for interactions with other proteins (43). Mobilization and activation of some of these latter Lck molecules may significantly boost T cell activation. This notion is supported by our data showing that TCR-proximal signaling events, such as c-chain phosphorylation and ZAP70 activation, both Lck-dependent processes, are much stronger when CD28, CD2, or combined CD28/CD2 stimulation is added to TCR stimulation. In addition to the stronger TCR-proximal signals seen when CD28 costimulation was added to TCR/CD28 stimulation, we noticed a robust increase in S6-Rp phosphorylation by CD2 alone, especially in effector/memory subsets. This observation supports a specific role for CD2-induced signals in S6-Rp–mediated regulation of translation, which appears independent of the activation of Akt, a well-known upstream activator of S6-Rp.

Our analyses indicated clear differences in signaling patterns between naive and effector/memory cells. This was seen for both CD4+ and CD8+ Tconvs. Generally, for a given stimulus, naive cells displayed stronger TCR-proximal responses than did effector/memory cells. The opposite was the case when TCR-distal processes were analyzed. The most obvious explanation for this observation is altered expression and/or regulation of certain signaling molecules in differentiated cells compared with naive cells, thereby shifting the activation threshold. Such differences may also provide a molecular explanation for why effector/memory cells, compared with naive cells, generally respond more rapidly and strongly to rechallenge with a previously encountered Ag. Because effector/memory responses are potent and potentially harmful to the host, the activation of these cells is tightly controlled, both by cell-intrinsic mechanisms (e.g., controlled by other cells, such as Tregs) and mechanisms that are intrinsic to the effector/memory T cells. In this respect, we recently observed that effector/memory cells have a constitutively active protein kinase A-signaling node (34), which could be important to prevent aberrant activation of CD8+ effector/memory T cells, possibly through protein kinase A–mediated phosphorylation and activation of C-terminal Src kinase.

A similar, but weaker, trend was seen for CD4+ effector/memory T cells, indicating that additional mechanisms are also operative. Because our starting material was buffy coats, the Treg populations that we studied were mainly thymus-derived Tregs, also known as natural Tregs. A recent publication (44) demonstrated that these cells are remarkably stable and continue to express the Treg-defining transcription factor FOXP3, despite being subjected to an inflammatory environment. Still, differences in FOXP3 expression, as well as differential expression of CD25 and CD45RA, have led to the division of natural Tregs into two groups, rTregs (FOXP3+) and suppressive actTregs (FOXP3+); the former is considered a precursor of the latter (30). Our phospho-flow analyses of rTregs and actTregs revealed clear signaling differences between these subsets. The most striking feature was that activation-induced Akt signaling was present in rTregs and diminished in actTregs. In contrast, actTregs displayed high basal Erk activation, indicating an active Mek. Previous reports suggested an inverse relationship between Akt signaling and the suppressive function of Tregs (45, 46). Furthermore, it was recently shown that the Fxo proteins Fxo1 and Fxo3A, both of which are inactivated by Akt, drive FOXP3 expression (47–49). Combined with our data, this suggested that a well-functioning Akt-signaling pathway in rTregs keeps FOXP3 expression in check and, hence, controls the function of rTregs until proper activation and Mek-Erk–dependent maturation into actTregs.

Transcription of the FOXP3 gene is controlled by a plethora of transcription factors acting on the promoter region (e.g., AP-1, SP1, NF-κB, NFAT, Fxo1, Fxo3a, STAT5) and Runx) and conserved noncoding sequence (CNS)1 (NFAT, Smad 2/3), CNS2 (Fxo1, Fxo3a, NF-κB, CREB, ATF, Runx, STAT5, and FOXP3 itself), and CNS3 (NF-κB) (50). The transition of rTregs into highly suppressive actTregs involves upregulation of FOXP3, as well as several other proteins. We observed that TCR/CD28/CD2–mediated upregulation of FOXP3 in rTregs peaked after 36 h in a Mek-dependent manner. Importantly, at this time, these “activated” rTregs had acquired a suppressive capability comparable to that of actTregs. In contrast, sorted rTregs that were added back without prior stimulation were only mildly suppressive. Hence, our data indicated that potent FOXP3 upregulation may be necessary for these cells to acquire strong suppressive capacity. In a recent publication, Miyara et al. (30) demonstrated that rTregs also exert suppression prior to their transition to actTregs. This suppression was substantially weaker than that of actTregs, which is in line with our observations. Although we used anti-CD3/CD28/CD2– or anti-CD3/CD28–coated beads for stimulation of responder cells in our suppression assays, Miyara et al. used plate-bound anti-CD3 in the presence of irradiated autologous accessory cells. Because the level of Treg-mediated suppression depends on the strength and quality of the stimulus provided, these differences in experimental conditions may explain the observed variation in the suppressive capacity of rTregs.

Because actTregs in the phospho-flow analysis displayed elevated basal Erk activation, we speculated that the differentiation of rTregs into actTregs involves a shift in Mek-Erk–mediated signaling necessary to keep FOXP3 levels high, perhaps through AP-1. However, with the experimental evidence presented in this article, it is difficult to define cause and action. Based on the aforementioned inhibitory effects of Akt signaling on Treg function, it was somewhat surprising that Akt inhibitors did not augment TCR/CD28/CD2–mediated upregulation of FOXP3 in rTregs. In fact, the presence of Akt inhibitors resulted in lower TCR/CD28/CD2–mediated FOXP3 upregulation and subsequent lower suppressive capacity. Combined with the data obtained with Mek inhibitors, this suggested that the differentiation of rTregs into actTregs is a complex process involving several pathways and
possibly distinct stages where the Akt and Mek/Erk pathways play important roles.

The functional interplay between Tconvvs and Tregs is important for balancing immune activation and immune control. Disturbance of this balance can contribute to autoimmunity, inappropriate inflammation, and cancer. Hence, a more detailed view of the signaling processes in different functional T cell subsets might increase our understanding of the complex molecular mechanisms forming the basis for specific subset functions and reveal disease adaptability and potential escape mechanisms that prevent an efficient immune response. Overall, the data from this report indicated that inhibitors of the Mek/Erk-signaling pathway might be used therapeutically to control the function of Tregs and peripheral tolerance. Further studies are needed to clearly define the precise roles of Mek and Erk in immune suppression by Tregs.

Acknowledgments
We thank Drs. Jonathan Irish and Garry Nolan for access to the Cytobank flow cytometry analysis software at the Stanford server and Dr. Eirik A. Torheim for technical advice on suppression assays.

Disclosures
The authors have no financial conflicts of interest. The University of Oslo Technology Transfer Office, Inven2 AS, has filed a pending patent application on methods to inhibit Treg activation.

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