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Kinetics and Activation Requirements of Contact-Dependent Immune Suppression by Human Regulatory T Cells

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Naturally occurring regulatory T cells (Tregs) maintain self tolerance by dominant suppression of potentially self-reactive T cells in peripheral tissues. However, the activation requirements, the temporal aspects of the suppressive activity, and mode of action of human Tregs are subjects of controversy. In this study, we show that Tregs display significant variability in the suppressive activity ex vivo as 54% of healthy blood donors examined had fully suppressive Tregs spontaneously, whereas in the remaining donors, anti-CD3/CD2/CD28 stimulation was required for Treg suppressive activity. Furthermore, anti-CD3/CD2/CD28 stimulation for 6 h and subsequent fixation in paraformaldehyde rendered the Tregs fully suppressive in all donors. The fixation-resistant suppressive activity of Tregs operated in a contact-dependent manner that was not dependent on APCs, but could be fully obliterated by trypsin treatment, indicating that a cell surface protein is directly involved. By add-back of active, fixed Tregs at different time points after activation of responding T cells, the responder cells were susceptible to Treg-mediated immune suppression up to 24 h after stimulation. This defines a time window in which effector T cells are susceptible to Treg-mediated immune suppression. Lastly, we examined the effect of a set of signaling inhibitors that perturb effector T cell activation and found that none of the examined inhibitors affected Treg activation, indicating pathway redundancy or that Treg activation proceeds by signaling mechanisms distinct from those of effector T cells. The Journal of Immunology, 2012, 188: 5459–5466.

Human CD4+CD25+FOXP3+ regulatory T cells (Tregs) represent a T cell subset that is absolutely crucial for maintaining peripheral tolerance and thereby preventing autoimmune disease. In animal models, depletion of Tregs precipitates both widespread organ-specific and organ-unspecific autoimmune disease, whereas adoptive transfer of Tregs resolves autoimmunity and may foster immune tolerance after solid organ transplantation (1–3). Both in mice and humans, mutations in the FOXP3 gene that cause dysfunctional Tregs invariably lead to widespread autoimmunity and lymphoproliferative disease (4–7).

In humans, several T cell subsets within both the CD4+ T cell and CD8+ T cell compartments have immunosuppressive properties (8). Thymic derived naturally occurring CD4+CD25+FOXP3+ Tregs (nTregs) may represent the most stable and long-lived Treg subset. Other CD4+ T cell and CD8+ T cell subsets with immuno-negulatory properties may develop peripherally as conventional T cells acquire inhibitory activity following chronic activation or interaction with and influence from the microenvironment (inducible Tregs), and we and others have defined experimental conditions for their generation in vitro (9–12). Although the regulatory properties of nTregs are a key phenotypic feature of these cells, their in vitro suppressive activity varies widely. Ex vivo experiments have demonstrated that CD4+ Tregs from mice needed to be triggered through the TCR/CD3-Ag receptor complex (TCR/CD3) to acquire immunosuppressive capacity (13, 14). Human Tregs are also generally considered to be suppressive only postactivation, although the precise activation requirements have not been clearly defined. Once activated, Tregs may suppress in both a contact-independent and contact-dependent manner (15), but the mechanisms remain to be fully elucidated. IL-10 and TGF-β have been suggested to be implicated in the suppressive function of Tregs, but may play a more important role in nTreg maturation, differentiation, and lineage commitment (8). Furthermore, it is not clear whether the contact-dependent immunosuppressive activity of human nTregs requires the presence of dendritic cells or other APCs. nTregs constitutively express CTLA-4, and a CTLA-4–dependent inhibition of dendritic cell maturation, cytokine secretion, and Ag presentation may indirectly prevent adequate T cell activation and thereby suppress T cell effector immune responses (16, 17). Although CTLA-4–dependent inhibition of dendritic cells may be one of several core mechanisms of nTreg-mediated immune suppression that acts through APCs, other contact-dependent mechanisms may also contribute to the inhibitory activity. The presence of APCs does not appear to be required for suppressive activity in vitro experiments, as nTregs are able to suppress effector T cell responses in cocultures with purified T cells (18).

In this study, we demonstrate that the immunosuppressive capacity of human nTregs isolated from healthy blood donors varies widely. We discovered that the contact-dependent immunosuppressive capacity of human nTregs is preserved following fixation in paraformaldehyde. This method, which prevents dynamic changes in cell surface protein exposure and excludes the contribution of cytokine secretion, allowed us to separately examine the

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kinetics and the activation requirements of the contact-dependent immune suppression. The procedure also allowed us to examine the susceptibility of conventional T cells to inhibition by Tregs prior to and following activation because we avoided concomitant activation of the Tregs. Normal blood donors often had nonsuppressive Tregs that became fully suppressive after stimulation. However, more than half the donors had Tregs that were fully immunosuppressive without the need of activation. This demonstrates heterogeneity in humans that may have bearing on the disposition to immune-related disease such as autoimmunity, allergy, infectious diseases, and cancer. Surprisingly, inhibitors of conventional T cell activation targeting downstream pathways of the TCR did not prevent activation of Tregs, whereas trypsin treatment of the Tregs completely perturbed the suppressive function, indicating that a cell surface protein mediates the immunosuppressive mechanism.

**Materials and Methods**

**Reagents and Abs**

Isopaque-Ficoll density medium (Lymphoprep) was purchased from Nycomed Pharma AS (Oslo, Norway). Regulatory T cell isolation kit II and anti-CD3/CD2/CD28–coated MicroBeads were purchased from Miltenyi Biotec (Auburn, CA). RosetteSep human CD4+ T cell enrichment mixture was purchased from STEMCELL Technologies SARL (Grenoble, France). Iatinib mesylate, CP 690550, and PD 032901 were purchased from Axon Medchem (Groningen, The Netherlands). Cyclosporin A, Akt inhibitor VIII, SCR-I, PI 103, rapamycin, and SB2035 were purchased from Calbiochem (San Diego, CA). Rolipram, brefeldin A, paraformaldehyde (PFA), A2A antagonist SCH 58261, and CFSE were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 and 25% trypsin-EDTA were obtained from Life Technologies Invitrogen (Paisley, U.K.). IL-2 was provided by Fluka Analytical (Buchs, Switzerland). IL-10, anti–IL-10, TGF-β, anti–TGF-β, anti–Fas-L, and isotype controls mouse IgG1 Ab (TGF-β) and mouse IgG2B Ab (anti-Fas-L) were purchased from BioLog Life Science Institute (Bremen, Germany). Anti-human CD152 (CTLA-4), anti-human B7-1 (PDL-1, CD274), anti-human B7-DC (PDL-2, CD273), anti-human PD1 (CD279), carboxylate control mouse IgG1, x (PD1), and mouse IgG2a (CTLA-4) were purchased from eBioscience (San Diego, CA). Ab for flow cytometry toward CD3, CD4, CD8, IFN-γ, TNF-α, CD45RA, CTLA-4, and FOXP3 together with propidium iodide, FACS permeabilizing solution, and human FOXP3 buffer set were all purchased from BD PharMingen. Abs for flow cytometry toward Helios were purchased from BioLegend (San Diego, CA).

**Isolation of cells**

PBMC were isolated by Isopaque-Ficoll gradient centrifugation of buffycoats from the Oslo University Hospital Blood Center (study approved by the regional Ethics Review Board). In most experiments, Tregs were isolated as CD4+CD25+CD127+ T cells using a Treg isolation kit II, according to the manufacturer’s instructions, and the Treg-depleted PBMC were used as responder cells in the subsequent add-back and coculture experiments. Isolated T cells were analyzed by flow cytometry, and the purity of the CD4+CD25+CD127+ population was routinely >85%, whereas the Treg-depleted PBMC routinely had <5% Tregs. In some of the experiments, Tregs and CD4+ effector T cells were purified by cell sorting. The purification of T cells was first performed by incubation of buffycoats with RosetteSep human CD4+ T cell enrichment mixture for 20 min prior to isolation by Isopaque-Ficoll gradient centrifugation. The cells were then stained with CD25-PE, CD3-PerCP, and CD127-Alexa Fluor 647 and sorted on a FACSaria CellSorter (BD Biosciences, San Jose, CA). The CD3+CD4+CD25+CD127+ T cell population was isolated as Tregs, whereas the CD3+CD4+CD25- T cells were used as effector cells in subsequent assays. When analyzed by flow cytometry, the purity of the CD3+CD4+CD25-CD127+ Treg population and the CD3+CD4+CD25+ T cells used as effector cells were both >95%.

**Prestimulation of cells**

Isolated CD4+CD25+CD127+ Tregs or CD3+CD4+CD25- T cells used as controls were either used directly, fixed in 4% PFA, and washed twice in PBS, or fixed and washed in PBS after stimulation with anti-CD3/CD2/CD28–coated MicroBeads (bead-to-cell ratio 1:2) for different time periods in culture in RPMI 1640 supplemented with 10% heat-treated FCS, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 1:100 nonessential amino acids (complete medium).

**Coculture experiments and suppression assays**

Purified T cells or PBMC depleted of CD4+CD25+CD127+ Tregs were used as effector cells. The cells were resuspended in RPMI 1640 and labeled with 4 µM CFSE for 10 min at 37°C. Labeling was quenched with complete medium, and the cells were washed twice. Isolated CD4+CD25+CD127+ Tregs were added back at a ratio of 1:3 at indicated time points, and either used unfixed or fixed in paraformaldehyde, as described, prior to add-back. Stimulation was performed with anti-CD3/CD2/CD28–coated MicroBeads in a mixture or each Ab independently (head-to-cell ratio 1:5). For proliferation assays, the cell cultures were incubated in complete medium for 4 d. The cells were then stained for cell surface markers for CD4 (allophycocyanin), CD8 (allophycocyanin), and CD3 (PerCP); subjected to analysis by flow cytometry (FACSCalibur; BD Biosciences); and analyzed using FlowJo software (Tree Star, San Carlos, CA). Assessment of effector function was performed by propidium iodide staining together with cell surface markers.

For the analysis of intracellular cytokine expression, the cell cultures were stimulated with anti-CD3/CD2/CD28–coated MicroBeads (bead-to-cell ratio 1:5) for 24 h during which brefeldin A was added to a final concentration of 5 µM for the last 18 h of the incubation period. In these experiments, the CD4+CD25+CD127+ Tregs were prestained with 2 µM CFSE to separate the Tregs from the responding effector T cells. The cells were fixed in 4% PFA, permeabilized in FACS permeabilizing solution, and stained for intracellular IFN-γ and TNF-α in addition to cell surface markers prior to analysis by flow cytometry.

In some experiments, the CD4+CD25+CD127+ Tregs were preincubated for 30 min with anti-IL-10, anti–TGF-β, anti–PD1, anti–Fas-L, corresponding isotype controls, the protein kinase A type I antagonist Rp-8-Br-cAMPS, the protein kinase A type I agonist Sp-8-Br-cAMPS, SRC-1, PI 103, rapamycin, SB2035, PD2, cyclosporin A, Akt inhibitor VIII, imatinib mesylate, CP 690550, or rolipram prior to anti-CD3/CD2/CD28 stimulation for 6 h and fixation. Anti-human CD152 (CTLA-4) and its isotype control were added 30 min before fixation. IL-10, TGF-β, and PGE2 were added to the CD4+CD25+CD127+ Tregs without any further stimulation and fixed after 6 h of incubation. When used, brefeldin A was added to the CD4+CD25+CD127+ Treg population to a final concentration of 5 µM at the same time of stimulation with anti-CD3/CD2/CD28, and the cells were fixed after 6 h of incubation. In some experiments (Fig. 6B), the CD4+CD25+CD127+ Tregs were treated with trypsin for 5 min after fixation.

In some experiments, PBMC depleted of CD4+CD25+CD127+ Tregs were preincubated for 30 min with anti-IL-10, anti–TGF-β, anti–CD152 (CTLA-4), anti–B7-1 (PDL-1, CD274), anti–B7-DC (PDL-2, CD273), anti–PD1 (CD279), A2A antagonist, IL-2, or the different intracellular pathway inhibitors described above.

**Flow cytometric analysis of lymphocytes**

Cells were washed in PBS, fixed in human FOXP3 buffer A, and frozen at −80°C, according to the manufacturer’s manual. The samples were later thawed and stained with FOXP3, according to the manufacturer’s manual, and Abs to cell surface markers. The samples were next subjected to analysis by flow cytometry (FACSCalibur or FACScan; BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Results**

**Heterogeneity in nTreg activation requirements in healthy blood donors**

Individual variation among healthy blood donors in the stimulation requirements for CD4+CD25+CD127+ Tregs to acquire immunosuppressive capacity was examined (Figs. 1, 2). Tregs were tested for their suppressive capacity in add-back experiments by assessment of effector functions in T cells in Treg-depleted PBMC as responder cells. Cells were stimulated with anti-CD3/CD2/CD28 beads, and T cell proliferation and cytokine production were measured by CFSE proliferation and intracellular cytokine assays, respectively. The suppressive activity was assessed by direct add-back of untreated Tregs or paraformaldehyde-fixed Tregs with or without prestimulation. When prestimulated, Tregs were routinely stimulated with anti-CD3/CD2/CD28 beads for 6 h.
prior to fixation in paraformaldehyde and add-back. Effector T cells treated in the same manner with paraformaldehyde fixation with or without prestimulation were used as controls to exclude the possibility that preactivation and fixation could elicit activation-induced cell death or stimulate nonstimulated responding T cells on their own (Fig. 1A). As shown in Fig. 1A (donors 1–3), the suppressive activity of unfixed Tregs varied widely among the donors. Furthermore, when unfixed Tregs were activated prior to add-back, little or no changes in suppressive activity were observed (data not shown), and when the suppressive activity of unstimulated, fixed Tregs (no stimulation prior to fixation = 0 h) was compared with that of unfixed Tregs from the same donor, levels of suppression before and after fixation did not correlate (Fig. 1A). However, when Tregs were stimulated with anti-CD3/CD2/CD28 beads for 6 h and fixed prior to add-back, Tregs from all the donors were fully suppressive with near-complete suppression of cell proliferation (Fig. 1A). Similar results were obtained assessing intracellular IFN-γ and TNF-α expression (Fig. 2). Interestingly, differences between donors in activation requirements for Tregs to acquire suppressive activity were observed (Figs. 1, 2A). Fifty-four percent ($n = 50$) had fully suppressive Tregs without the need for ex vivo stimulation prior to fixation and add-back (0 h) (hereafter called spontaneously active Tregs), whereas the others (46%) required ex vivo stimulation for 6 h (hereafter called inactive Tregs) (Fig. 1B). After 6 h of prestimulation prior to fixation, Tregs from all the donors had acquired full suppressive activity. Together, our observations suggest that healthy blood donors can be stratified into groups, as follows: those with spontaneously active Tregs and those with

**FIGURE 1.** Variable suppressive activity of human Tregs. (A) Ability of Tregs to suppress anti-CD3/CD2/CD28–induced proliferation (CFSE assay) of CD3+ T cells in PBMC depleted of CD4+CD25+CD127+ Tregs. Suppressive activity of unfixed CD4+CD25+CD127+ Tregs compared with that of Tregs fixed in paraformaldehyde prior to add-back, and Tregs stimulated with anti-CD3/CD2/CD28 for 6 h prior to fixation and add-back. Individual data from three blood donors are shown. In donor 1, right three panels, PBMC depleted of CD4+CD25+CD127+ Tregs (Teff cells) were fixed in paraformaldehyde directly or stimulated with anti-CD3/CD2/CD28 beads for 6 h prior to fixation and add-back to either stimulated or unstimulated autologous PBMC depleted of CD4+CD25+CD127+ Tregs. (B) Amalgamated data ($n = 18$) from experiments, as in (A), in which suppressive activity of unstimulated (0-h) Tregs and Tregs stimulated with anti-CD3/CD2/CD28 for 6 h prior to fixation and add-back was examined. Right panel, Compounded data on FOXP3 expression for unstimulated Tregs from 9 donors with active Tregs and 19 donors with inactive Tregs are shown.

**FIGURE 2.** Variable suppressive activity of human Tregs on cytokine production. (A) Treg-mediated suppression of IFN-γ and TNF-α production in anti-CD3/CD2/CD28–activated CD3+ T cells assessed by intracellular cytokine flow cytometry. Individual data from two donors are shown. (B) Amalgamated data in which Treg-mediated suppression of IFN-γ production was analyzed separately in CD4 and CD8 T cell subsets.
inactive Tregs that acquire immunosuppressive activity after stimulation. Surprisingly, this appeared to be a binary phenomenon, as no donors with populations of partially active or mixed active/inactive Tregs were observed (Fig. 1B). The difference in suppressive activity in spontaneously active and inactive Tregs was not reflected in the expression of FOXP3 (Fig. 1B, right panel).

Kinetic properties of nTreg and effector T cell interaction define a window of suppression

To further investigate the activation requirements, isolated Tregs were stimulated with anti-CD3/CD2/CD28 beads for different time periods prior to fixation and assessment of their suppressive effect on effector T cells (Fig. 3A). Cells from blood donors with inactive Tregs were used in these experiments. As shown in Fig. 3A, Tregs from different donors acquired suppressive activity between 1 h (donor 1) and 5 h (donor 2) of activation. All donors examined acquired full suppressive activity after 6 h of stimulation, which was chosen as the routine stimulation time in subsequent experiments. This procedure of activation and fixation allows an isolated investigation of the contact-dependent suppressive activity of Tregs, which excludes dynamic changes in cell surface receptor repertoire and cytokine production from the Tregs, and also enabled us to examine for how long time after activation effector T cells were sensitive to suppression. Our results demonstrate that effector T cells were sensitive to inhibition by activated, fixed Tregs from the time of activation and up to 24 h postactivation (Fig. 3B).

It has been suggested that Tregs suppress effector T cells through interaction with APCs, for instance through a CTLA-4–dependent mechanism (17). Therefore, we investigated whether activated, fixed Tregs could suppress effector T cells also in isolated T cell cultures. Sorted CD4+ T cells were used as effector T cells, and, as seen in Fig. 3C, the Treg-mediated T cell suppression did not depend on the presence of APCs. We also tested whether the suppressive effect of activated, fixed Tregs is exclusive to autologous effector cells or acts across donors in an allogenic experiment. As seen in Fig. 3D, activated and fixed Tregs from donor A effectively suppressed effector T cells from both donors A and B.

FIGURE 3. Activation requirements of human Tregs define a window of suppression. Proliferation (CFSE assay) of anti-CD3/CD2/CD28–stimulated CD3+ T cells in PBMC depleted of CD4+CD25+CD127– Tregs or purified CD4+ T cell cultures. CD3+ T cell proliferation was assessed in the absence or presence of CD4+CD25+CD127– Tregs from autologous (A-C) or allogeneic (D) donors in a 3:1 ratio. (A) Tregs were fixed in paraformaldehyde directly after isolation (0 h) or stimulated for indicated time periods prior to fixation and add-back. Data from two individual donors are shown. (B) Tregs stimulated with anti-CD3/CD2/CD28 for 6 h and then fixed were added back into the PBMC culture at indicated times postactivation of the effector cells. (C) Suppression of CD3+ T cell proliferation in the presence or absence of anti-CD3/CD2/CD28–activated (6 h), fixed Tregs in purified CD4+ T cell cultures. (D) The inhibitory activity of Tregs stimulated with anti-CD3/CD2/CD28 for 6 h and then fixed prior to add-back was examined in an allo-experimental setup in which Tregs from donor A were tested for suppressive function on CD3+ T cells stimulated with anti-CD3/CD2/CD28 from donor B.
Phenotypic characterization of ex vivo stimulated nTregs

We next characterized Tregs from donors with spontaneously active and inactive Tregs with respect to their expression of FOXP3 and CD45RA. The analysis was performed according to the previously defined Treg subsets that identify resting Tregs (FOXP3⁺, CD45RA⁻), activated Tregs (FOXP3⁺⁺, CD45RA⁻), and cytokine-producing effector T cells (FOXP3⁺, CD45RA⁻) (Fig. 4A, left panel) (19). Spontaneously suppressive and inactive Tregs did not significantly differ in phenotype prior to stimulation. Furthermore, the activation and fixation protocol did not lead to any significant changes in Treg subsets, as defined by CD45RA and FOXP3 status neither when applied on spontaneously active nor inactive Tregs (Fig. 4A, right panel). In conclusion, the resting and active Treg subsets useful to determine Treg activity in disease did not correlate with the heterogeneity in suppressive activity observed in this study. Neither did this subset analysis serve to identify the activating events induced by stimulation of inactive Tregs, although that could be due to a time lag in the shift from expression of CD45RA to expression of CD45RO. Furthermore, when the expression of CTLA-4 and Helios was analyzed within each of the defined Treg subsets, the frequencies of CTLA-4⁺ and Helios⁺ cells were similar in spontaneously active and inactive Tregs (Fig. 4B). The frequency of CTLA-4⁺ cells increased within the resting Treg and cytokine-producing effector T cell subsets upon stimulation in both the spontaneously active and inactive Tregs, whereas no changes were observed analyzing Helios expression.

nTreg and effector T cell activation depend on different signal pathways

The procedure of stimulation and subsequent paraformaldehyde fixation of Tregs prior to add-back allowed us separately perturb signal events in Tregs and effector T cells without affecting the other subset. In a series of experiments, we treated effector T cells with a range of inhibitors of intracellular pathways important for T cell functional responses. Inhibitors of Src (SRC-1, PP2), PI3K (PI-103), MEK (MEK-1), mTOR (rapamycin), and calcineurin (cyclosporine) all inhibited effector T cell activation assessed as CFSE proliferation (Fig. 5A, 5D). We next examined the effect of the same set of inhibitors on spontaneously active Tregs and found that none of the inhibitors were able to reverse the suppressive activity (Fig. 5B). More surprisingly, however, was the observation that none of the inhibitors were able to prevent activation of inactive Tregs and their acquisition of suppressive activity (Fig. 5C, 5E). These results indicate that the pathway(s) involved in activation of Tregs differs from those of conventional T cell activation, or that pathway redundancy exists in Tregs. As an aside, we also tested signaling inhibitors that did not interfere with activation of conventional T cells (Akt, BCR-Abl, Jak3, protein kinase A, glycogen synthase kinase inhibitors), but none of these consistently affected Treg activation (data not shown).

Ex vivo stimulated nTregs suppress effector T cells by a contact-dependent mechanism that does not require de novo protein synthesis

The procedure of separate stimulation of Tregs prior to fixation and add-back effectively excludes the production and secretion of cytokines and freezes the Tregs as mediators of contact-dependent immune suppression. As expected, Abs to IL-10 or TGF-β did not reverse the suppressive activity of the active Tregs (Fig. 6A, left panel). Furthermore, consistent with the finding that activated, fixed Tregs were suppressive in isolated T cell cultures (Fig. 3C), CTLA-4 blocking Abs did not interfere with Treg immunosuppressive activity (Fig. 6A). Similar results were obtained using blocking Abs to FasL and PD-1 in addition to an adenosine A2A receptor antagonist (Fig. 6A). Induction of cell death of the responding T cells in the presence of active Tregs was excluded by propidium iodide staining, and corresponding isotype controls were included for all the mAbs (data not shown).

Next, cells from donors with inactive Tregs were activated and fixed, and the nature of the contact-dependent suppression was...
examined. As seen in Fig. 6B, incubation of activated Tregs with trypsin after fixation completely abolished the suppressive activity, indicating that the contact-dependent Treg function requires a membrane-bound protein factor. To determine whether stimulation of inactive Tregs to induce suppressive activity depends on de novo protein synthesis and transport by an exocytotic pathway, cells were treated with brefeldin A, which disrupts transport through the Golgi apparatus and leads to intracellular accumulation of proteins targeted to the cell membrane and exocytosis. Treatment with brefeldin A during the 6 h of stimulation prior to fixation and add-back did not reverse the immunosuppressive activity of the Tregs (Fig. 6B), which indicates that the protein(s) directly involved in the immunosuppressive mechanism may reside in post-Golgi vesicles transported to the cell membrane when Tregs receive the proper stimulus or may already be situated at the cell membrane and subject to conformational changes upon activation.

To assess whether the combination of anti-CD3 and the coactivators anti-CD28 and anti-CD2 was required for stimulation, inactive Tregs were also separately activated by anti-CD3 alone, which was sufficient to induce suppressive activity (Fig. 6C). In contrast, anti-CD28 alone did not induce active Tregs.

**Discussion**

nTregs represent a T cell subset absolutely critical for maintaining peripheral immune tolerance. Tregs suppress CD4+ and CD8+ T cell immune responses such as proliferation, cytokine production, and CD8+ T cell cytotoxicity in a dominant manner, and also regulate other immune cells, including B cells and NK cells. Our knowledge of Treg physiology and modes of action has increased rapidly after identification of this T cell subset in mice in 1995 (2). However, several key questions remain to be answered. The molecular mechanism of the contact-dependent immune suppression is still not revealed, although several lines of evidence indicate that well-known cell surface proteins such as CTLA-4 may be involved under certain circumstances. The level of involvement of cytokines in the immunoregulatory action of Tregs is also a subject of discussion, and special attention has been paid to IL-10, TGF-β, and IL-35 (20–23). Functional studies of Tregs represent many challenges relating to experimental conditions and cell
preparation, and it appears that nTregs operate mainly through a contact-dependent immunosuppressive mechanism, whereas inducible Tregs use both contact-dependent and contact-independent mechanisms of immune suppression (8). It is also apparent that there are important differences between species, so that results obtained from studies of Tregs from rodents may not be applicable to human physiology and vice versa (24). In this study, we have explored the temporal regulation of human nTreg function in the interaction with effector T cells in coculture experiments with PBMC or in sorted T cell cultures. In most experiments, Tregs were fixed with paraformaldehyde prior to add-back, which allowed separate manipulation of the Tregs without affecting the effector T cells and vice versa.

It has previously been reported that Tregs from both mice and humans need to be activated through the TCR to attain inhibitory function. Activation of Tregs is Ag specific, but once activated, the Tregs can suppress other immune cells in an Ag-nonspecific manner (15). However, Tregs from mice and humans have some clear differences. Immune cells isolated from humans have an Ag exposition history, whereas Tregs from laboratory animals with limited exposure to foreign pathogens may not have been previously activated. Indeed, our findings in this study are consistent with the hypothesis that Tregs from humans demonstrate a greater functional and phenotypic variability reflecting previous Ag exposure. Indeed, our findings in this study are consistent with the hypothesis that Tregs from humans demonstrate a greater functional and phenotypic variability reflecting previous Ag exposure. Interestingly, this may manifest itself in functional assays as a split in the population of healthy blood donors with respect to the ex vivo immunosuppressive activity of the Tregs. The blood donors included in this study were recruited according to standard health requirements, and samples were consecutively included in the study. For that reason, we could not obtain data indicating whether the observed differences in immunosuppressive activity are permanent and a consequence of the long-term Ag exposition history of the individuals included in the study, or whether they are transient and a result of a recent or acute Ag challenge such as a recently experienced intercurrent infection or allergy. Addressing this question would require a future longitudinal study with a specifically recruited blood donor cohort. Experimentally, however, the phenomenon of varying suppressive activity may be important to take into account when studying human Tregs. Furthermore, the variability in Treg-suppressive function observed may reflect normal physiological variance in humans or could be associated with the propensity to immune-related disease such as autoimmunity, risk of infectious diseases, or cancer. Furthermore, we were surprised to find that the procedure of fixating the Tregs prior to add-back to the T cell cultures revealed a split in the immunosuppressive capacity rather than a continuum in inhibitory activity, as observed using nonfixed Tregs in such assays. This could be due to the fixation protocol excluding the contribution of Treg-derived cytokines or that dynamic changes in Treg surface protein exposure are avoided. Both these effects may contribute to the observation that the Tregs from some donors are fully active or completely inactive, and therefore, require stimulation to become immunosuppressive.

A particular emphasis was given in this study to the conversion of inactive Tregs to actively suppressing Tregs. To identify important intracellular pathways downstream of the TCR that regulate the activation of Tregs, we performed a screen of known inhibitors of T cell activation. Surprisingly, none of the inhibitors that were able to suppress proliferation of effector T cells were able to prevent activation of Tregs and the acquisition of regulatory function in a consistent manner. Protein kinase B/Akt has previously been shown to enhance Treg-suppressive function in protein kinase B

![FIGURE 6. Characterization of the fixation-resistant Treg-suppressive activity.](http://www.jimmunol.org/)

![Image](http://www.jimmunol.org/)
transgenic mice (25), whereas activation of the Akt-mTOR pathway by the receptor for sphingosine 1-phosphate has been shown to impede the development and suppressive function of Tregs (26, 27). However, in the current study, protein kinase B/Akt inhibitors were added during the 6-h stimulation period required for activating the Tregs. By isolating this process, none of the inhibitors of conventional T cells impaired the activation of the Tregs. Taken together, this may suggest that multiple, redundant pathways orchestrate the Treg activation process, or the involvement of other unique pathways yet to be identified. However, treatment of active, paraformaldehyde-fixed Tregs with trypsin completely abrogated the immunosuppressive activity. The finding that treatment with brefeldin A that blocks the Golgi apparatus did not reverse the activation of Tregs indicates that the cell surface protein(s) involved in the inhibitory mechanism is (are) stored in secretory vesicles and redistribute(s) to the cell surface upon the proper activation signal.

In conclusion, our findings point to heterogeneity with respect to the activation status of nTregs in humans. When a contact-dependent mechanism of immune regulation was isolated by fixation of Tregs by paraformaldehyde, it appeared that the Tregs from individual healthy blood donors were either fully active or nearly completely inactive. However, the latter acquired full immunosuppressive capacity within 6 h of stimulation. Furthermore, it appeared that by isolating the Treg activation process, inhibitors of conventional T cells were not sufficient to block the activation of Tregs and thereby prevent the acquisition of suppressive function. The mechanism of immune suppression of Tregs appeared to be dependent on a cell surface protein either unveiled upon activation or stored intracellularly and translocated to the cell membrane. Our study reveals key aspects of Treg activation requirements, and the heterogeneity revealed in human Treg endogenous activity may have important implications for the propensity and risk for autoimmunity and other immune-related diseases.

Disclosures
The authors have no financial conflicts of interest.

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