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CD55 Costimulation Induces Differentiation of a Discrete T Regulatory Type 1 Cell Population with a Stable Phenotype

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Unlike other helper T cells, the costimulatory ligands responsible for T regulatory type 1 (Tr1) cell differentiation remain undefined. Understanding the molecular interactions driving peripheral Tr1 differentiation is important because Tr1s potently regulate immune responses by IL-10 production. In this study, we show that costimulation of human naive CD4+ cells through CD97/CD55 interaction drives Tr1 activation, expansion, and function. T cell activation and expansion was equipotent with CD55 or CD28 costimulation; however, CD55 costimulation resulted in two IL-10–secreting populations. Most IL-10 was secreted by the minor Tr1 population (IL-10highIFN-γlow IL-4<5% cells) that expresses Tr1 markers CD49b, LAG-3, and CD226. This Tr1 phenotype was not restimulated by CD28. However, on CD55 restimulation, Tr1s proliferated and maintained their differentiated IL-10high phenotype. The Tr1s significantly suppressed effector T cell function in an IL-10–dependent manner. The remaining (>95%) cells adopted a Th1-like IFN-γ phenotype. However, in contrast to CD28-derived Th1s, CD55-derived Th1s demonstrated increased plasticity with the ability to coexpress IL-10 when restimulated through CD55 or CD28. These data identify CD55 as a novel costimulator of human Tr1s and support a role for alternative costimulatory pathways in determining the fate of the growing number of T helper populations. This study demonstrates that CD55 acts as a potent costimulator and activator of human naive CD4+ cells, resulting in the differentiation of a discrete Tr1 population that inhibits T cell function in an IL-10–dependent manner and maintains the Tr1 phenotype upon restimulation. The Journal of Immunology, 2013, 191: 5895–5903.

T regulatory type 1 (Tr1) cells play a major role in maintenance of immune homeostasis and tolerance by limiting activation of effector cells to prevent autoimmune reactions. They are peripherally induced regulatory T cells (Tregs), which mediate suppression mainly by IL-10 production (1, 2). They are elevated in cancer, with evidence of tumor Ag–specific Tr1s (3), and in autoimmune disease where the presence of Tr1s correlates with a favorable outcome (4–6).

Similar to other CD4+ phenotypes, they lack a unique surface marker and have been characterized by their cytokine profile (high IL-10, TGF-β, and IL-5; low IFN-γ and IL-2; and no IL-4) (1, 7), although Gagliani et al. (8) recently demonstrated coexpression of CD49b, LAG-3, and CD226 by both murine and human Tr1 cells. Their induction in murine and human systems is broadly based on the use of immature APCs (9), the presence of immune inhibitors, including high-dose IL-10 (10, 11), IL-10 with IFN-α (11), vitamin D3 plus dexamethasone (12), and rapamycin (13, 14), through tolerogenic dendritic cells (DCs) (15) and DC-10 (16), or under the influence of IL-27 (17, 18) and IL-6 (19). Furthermore, in the human system, costimulation through CD2 (20) or CD46 (21, 22) results in the induction of a Tr1 phenotype from CD4+ cells. However, characterization of human Tr1s has thus far been limited by the inability to expand them in vitro. Although these studies imply a role for alternative costimulatory receptors in induction of IL-10 from CD4+ cells, none has addressed the role of costimulatory molecules in Tr1 differentiation.

Costimulation is an absolute requirement for T cell differentiation (23). This is classically achieved with CD28 engagement by CD80/86 on DCs (24), which results in activation, differentiation, and licensing of a T helper effector phenotype (23, 25), with the phenotype being heavily influenced by a third signal, the cytokine milieu (26, 27). Although CD28 is a potent inducer of CD4+ cell differentiation, costimulation through other surface receptors (25, 28), including 4-1BB, ICAM-1, CD2, CD44, CD9, and CD5, has been shown to act as alternative pathways for Th1, Th2, and Th17 differentiation (25, 28, 29).

CD55 was characterized as a complement regulatory protein (CRP) (30). Subsequently, CD97 was identified as a cellular ligand for CD55 (31). We have previously shown that interaction of CD55 with CD97 is independent of its role in complement regulation (32). Engagement of CD55 on CD4+ cells by CD97 results in activation, proliferation, and IL-10 production by these cells (33). Prior to this, another CRP, CD46, was shown to induce IL-10 production by CD4+ cells (21) following engagement of complement (22). Unlike CD46, CD55-induced IL-10 production was CD97-dependent (34).

In this study, we demonstrate that CD55 costimulation of naive human CD4+ cells results in their activation, induction of IL-2, and proliferation, comparable with CD28 costimulation. However, the phenotypes of the cells differentiated under the two costimuli differ. CD55 induces a discrete IL-10 single-positive Tr1 pop-

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I.S. and L.G.D. designed the research; I.S., R.V.S., R.G.B., and J.M.R. performed experiments; and R.V.S., I.S., A.M.J., L.G.D., R.G.B., and J.M.R. designed experiments, analyzed results, and wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CRP, complement regulatory protein; DC, dendritic cell; iTreg, inducible regulatory T cell; Tr1, T regulatory type 1; Treg, regulatory T cell.

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ulation, which is superimposable on Tr1 cells as defined by other groups, and a Th1 population, whereas CD28 only induces Th1s.

On subsequent stimulation, only CD55 is able to restimulate Tr1s. Although CD28 has no effect on Tr1s, it restimates CD55-induced Th1s and promotes IL-10 coexpression in these cells (IL-10+/IFN-γ double-positive).

Materials and Methods

Reagents

Lymphocyte separation medium and FBS were purchased from PAA Laboratories. All other reagents were purchased from Sigma-Aldrich. Anti-CD3 mAb (clone OKT3; American Type Culture Collection) and anti-CD55 mAb (clone 791T/36; in-house Ab) were purified in-house. EMR2-Fc and CD97-Fc fusion proteins were purified as previously described (33). Fluorophore-conjugated Abs were CD4-PE-Cy7 (RPA-T4; BD Biosciences), CD45RA-FITC (HI100; eBioscience), CD45RO-PE (UCHL1; BioLegend), CCR7-FITC (3D12; eBioscience), CD62L-PE (DREG-56; eBioscience), CD25-PE-Cy5 (BC96; BioLegend), CD69-FITC (FN50; AbD Serotec), Foxp3-Alexa Fluor 488 (259D; BioLegend), CD49b-FITC (AK7; Bio-Legend), LAG-3-FITC (17B4; AdipoGen), and CD226-allophycocyanin (11A8; Bio.Legend).

Isolation of naive CD4+ cells

PBMCs were purified from fresh blood of healthy donors by density gradient centrifugation and following the recommended protocol. All blood donors provided written informed consent in accordance with the Declaration of Helsinki and the rules of the Nottingham Regional Ethics Committee, which approved this study. Naive CD4+ cells were isolated from PBMCs by negative selection for CD45RA expression using the naive CD4+ T cell isolation kit II (Miltenyi Biotec). Purity of naive CD4+ cells was routinely assessed by flow cytometry.

In vitro culture of naive CD4+ cells

Cells were cultured in flat-bottom 96-well (Costar 3361) or 48-well (Costar 3548) tissue culture plates in T cell medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% 2 mM L-glutamine, 2% HEPES, 1% sodium pyruvate, and 1% nonessential amino acids).
acids) with IL-2 (50 U/ml) at 37°C and 5% CO₂. Cells were stimulated with plate-bound anti-CD3 mAb (1 µg/ml) plus plate-bound costimulatory Ab (5 µg/ml): anti-CD55 mAb, anti-CD28 mAb (clone CD28.2; BD Biosciences), isotype control IgG1 (AbD Serotec), isotype control IgG2b (BD Biosciences), or fusion proteins (5 µg/ml) EMR2-Fc or CD97-Fc, as indicated. Images of cells were captured using the ×20 objective lens of a Nikon Eclipse TS100 microscope (Nikon Instruments Europe) with a Nikon Digital Sight DS-Fi1 camera. Images were cropped to size using Adobe Photoshop CS5 software.

**T cell proliferation assay**

Cells (1 × 10⁵/well) were cultured in 96-well plates for 5 d, unless indicated otherwise. Cells were pulsed with 0.5 µCi [³H]thymidine (Amersham Biosciences) for the final 15 h, harvested on a 96-well plate harvester (Filtermate 196; Packard/PerkinElmer), and the incorporated radioactivity was measured as described in the proliferation protocol (Miltenyi Biotec). Purity of isolated IL-10⁺ and IL-10⁻ populations was assessed by flow cytometry.

**Cytokine assays (ELISA and Bio-Plex)**

Culture supernatants were harvested on day 5 unless indicated otherwise and assessed for the presence of IL-10, IFN-γ, IL-4, and IL-2 using the respective ELISA (R&D Systems) or Bio-Plex (Bio-Rad) kits, following the recommended protocols.

**Cell suppression assay**

Naive CD4⁺ cells (5 × 10⁵ cells/well in a 48-well plate) were differentiated with plate-bound anti-CD3 and anti-CD55 mAbs, and culture supernatant was harvested on day 5 (Tr1 supernatant), following secondary stimulation, or day 17 (Tr1E supernatant), following Tr1 cell enrichment. Subsequently, naive CD4⁺ cells (1 × 10⁵ cells/well in a 96-well plate) were stimulated with anti-CD3 and anti-CD28 mAbs in the presence or absence of Tr1 supernatant, with or without neutralizing anti–IL-10 Ab (BD Biosciences) for 72 h. Cultures were pulsed with [³H]thymidine, and incorporated radioactivity was measured as described in the proliferation assay protocol.

**IL-10 capture**

Naive CD4⁺ cells were stimulated with plate-bound anti-CD3 and anti-CD55/CD28 mAbs, for 72 h, as indicated. Cells (5 × 10⁵ per condition) were assessed for IL-10 production, by flow cytometry, using an IL-10 enrichment and detection kit (PE) and following the recommended protocol (Miltenyi Biotec). Matched cell samples without the IL-10 capture Ab were used as controls.

**Sorting of IL-10⁺ cells**

A minimum of 1 × 10⁵ cells were stained for IL-10 as described above and IL-10 PE cells were isolated using anti-PE microbeads and following the recommended protocol (Miltenyi Biotec). Purity of isolated IL-10⁺ and IL-10⁻ populations was assessed by flow cytometry.

**Tr1 enrichment**

Naive CD4⁺ cells were differentiated with anti-CD3/anti-CD55 for 5 d, rested for 5 d, restimulated with anti-CD3/anti-CD55 for 3 d, and rested overnight. Tr1 cells were enriched either on primary stimulation (day 3) or secondary stimulation (day 14) following reactivation with anti-CD3/anti-CD55 for 6 h. The enriched Tr1 cells were rested overnight and restimulated with anti-CD3/anti-CD55 for 48 h.

**IL-10 and IFN-γ cocapture**

Naive CD4⁺ cells were stimulated as described in the IL-10 capture assay. Cells were simultaneously assessed for IL-10, as above, and IFN-γ using an IFN-γ detection kit (FITC) and following the recommended cocapture protocol (Miltenyi Biotec). Controls were as described above.

**FIGURE 2.** CD55 costimulation induces a Tr1 phenotype that mediates bystander cell suppression in an IL-10-dependent manner. (A) CD55 costimulation induced a characteristic Tr1 cytokine profile (IL-10[^a] IFN-γ[^b] IL-4[^c]) whereas CD28 costimulation induced a Th1 cytokine profile (IL-10[^a] IFN-γ[^b] IL-4[^c]). Statistical significance was determined by a two-tailed, unpaired Student t test (n = 6). (B) IL-10 production was induced specifically in response to CD55 costimulation. Statistical significance was determined by a two-tailed, unpaired Student t test (n = 6). (C) Presence of IL-10 was assessed in the culture supernatants of primary CD55 costimulation or CD28 costimulation. Statistical significance was determined by a Wilcoxon matched-pairs signed rank test in both panels (n = 13 and 7, respectively). (D and E) Presence of IL-10 and IFN-γ was assessed in the culture supernatants of primary CD55 costimulation or CD28 costimulation. Statistical significance was determined by a Wilcoxon matched-pairs signed rank test in both panels (n = 13 and 7, respectively). (F and G) Supernatant (Tr1 sup) was harvested from cells following a secondary stimulation with CD55 (day 13). Tr1 supernatant mediated bystander cell suppression in an IL-10-dependent manner, as assessed on day 3 of the suppression assay. Statistical significance was determined by a two-tailed, unpaired Student t test in (F) and by a two-tailed, paired Student t test in (G) (n = 8). Data shown [means ± SD of triplicates in (A), (B), and (F)] are either representative of or summarized from n independent experiments. Assays were performed on day 5 of primary stimulation unless otherwise stated.

*p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.
CFSE assay
Naive CD4+ cells (1 × 10^6) were resuspended in 1 ml AIM V media at 37˚C (Invitrogen), and 1 ml 1 μM CFSE/AIM V was added dropwise under gentle mixing, continued for 5 min. Labeling was quenched with 1 ml cold FBS, and cells were washed twice with 10 volumes of T cell medium. CFSE-labeled cells were cultured, as indicated, and cell proliferation, along with CD25 expression or IL-10 capture, was assessed by flow cytometry.

Flow cytometry
For staining other than by cytokine capture or CFSE, 1 × 10^5 cells/sample were stained with the relevant Ab combinations, following recommended protocols. Matched cell samples were used for fluorescence minus one controls. Data were acquired on an FC500 (Beckman Coulter) or MACSQuant (Miltenyi Biotec).

Data analysis
Cell proliferation, suppression assay, ELISA, and Bio-Plex cytokine assay data were analyzed with GraphPad Prism 5 software. Flow cytometry data were analyzed with FlowJo software (Tree Star) or MACSQuantify software (Miltenyi Biotec) and gates were set on fluorescence minus one controls.

Statistical analysis
GraphPad Prism 5 software was used for all statistical analyses. Two-tailed, unpaired Student t tests were used for analyzing data from a single donor. Two-tailed, paired Student t tests were used for analyzing data from multiple donors with normal distribution. Wilcoxon matched-pairs signed rank tests were used for analyzing data from multiple donors with skewed distribution. One-way ANOVA was used for comparing more than two groups. A p value ≤0.05 was considered statistically significant.

Results
CD55 costimulates peripheral blood naive CD4+ cells
Characterization of the Tr1 phenotype has historically been hampered by the lack of appropriate means to expand this population. We have previously reported that CD55 costimulation of CD4+ cells stimulated a population of cells with a pronounced capacity to secrete IL-10 (33), when compared with costimulation through CD28. In the present study we tested the hypothesis that costimulation of naive CD4+ cells through CD55 results in differentiation of Tr1 cells.

Using highly pure (>99% CD45RA⁺CD45RO⁻) naive CD4+ cells (Fig. 1A) the influence of CD55- or CD28-mediated costimulation on their activation and proliferation was compared. Their naive status was confirmed by CD62L⁺/CCR7⁺ expression and their inactive state by a CD69⁻/CD25⁻ phenotype (Supplemental Fig. 1). Costimulation through CD55 or CD28 elicited similar levels of blast formation (Fig. 1B), with a corresponding increase in expression of activation markers CD25 and CD69 (Supplemental Fig. 2), and proliferation of the cells (Fig. 1C). CD55 costimulation consistently resulted in a similar proliferative response to that induced by CD28 costimulation, typically driving cells through four cell divisions (Fig. 1D–G). Substitution of anti-CD55 mAb with CD97-Fc, the natural cellular ligand for CD55, also resulted in a significant increase in proliferation (Fig. 1C), indicating a physiological relevance for CD55 costimulation and making CD55 as potent a costimulator of naive CD4+ cells as CD28.

Induction of IL-2 is necessary for successful differentiation of naive CD4+ cells. Cells were stimulated in the absence of exogenous IL-2, and induction of IL-2 was monitored. As was observed with CD28, both CD55 mAb and CD97-Fc fusion protein stimulated IL-2 production from naive CD4+ cells (Fig. 1H). The response to CD55 costimulation using anti-CD55 and CD97-Fc, even in the absence of exogeneous IL-2, was highly reproducible. Naive CD4+ cells from all donors proliferated upregulated activation markers and produced IL-2. Although donor variation was observed, activation of naive CD4+ T cells induced by CD55 costimulation was comparable to that induced by CD28 costimulation.
CD55 costimulation induces Tr1s

CD55 costimulation of naive CD4+ cells induced an IL-10highIL-4low cytokine profile, characteristic of Tr1s (2) (Fig. 2A, 2B). This was unlike CD28 costimulation that results in induction of a Th1 phenotype (IFN-γhighIL-10lowIL-4+). The Tr1 cytokine profile was consistently observed with anti-CD55 mAb and CD97-Fc ligand–mediated costimulation, with a very high level of IL-10 being secreted (Fig. 2B, 2D). This was independent of exogenous IL-2, and even in its absence a high level of IL-10 was detected (Fig. 2C). Comparison of the cytokine profiles induced by CD28 and CD55 costimulation revealed that CD28 induced a high level of IFN-γ whereas CD55 only stimulated IL-10 production (Fig. 2C–E), supporting our hypothesis of Tr1 induction.

Tr1s are known to mediate suppression via IL-10 (1). A Tr1 phenotype was confirmed by determining the ability of CD55-derived Tr1 supernatant to suppress proliferation of CD28-costimulated cells in suppression assays. Tr1 supernatant inhibited proliferation of CD28-costimulated naive CD4+ cells, in reproducible experiments (Fig. 2F, 2G). This inhibition was dependent on IL-10, as neutralization of this cytokine with Abs restored proliferation to normal levels.

Tr1s are a discrete population of cells

Treg populations invariably appear as small populations, making up 1–5% of T cells (35). Examination of CD55 cultures by IL-10 ELISPOT revealed that the large amount of IL-10 in the culture supernatant was secreted by a small percentage (0.4–3%) of cells (Supplemental Fig. 3). To identify the Tr1 population, cells were assessed for IL-10 production during the course of primary CD55 costimulation using an IL-10 capture assay. PMA/ionomycin stimulation of naive CD4+ cells was used as a control. PMA/ionomycin stimulation failed to induce IL-10 from naive CD4+ cells, confirming their uncommitted, naive phenotype. However, on CD55 costimulation IL-10 secretion by the cells (2.86% of cells) reached a peak at day 3 of primary stimulation (Fig. 3A, 3B). Following 5 d of rest, the cells no longer secreted IL-10 until they were restimulated either nonspecifically with PMA/ionomycin or with CD55. Both stimuli resulted in IL-10 production within 24 h (Fig. 3A). Although IL-10 was produced by a small percentage of cells, the concentration of IL-10 in the supernatant was high (Fig. 3B, 3C). Although the percentage of IL-10–secreting cells varied among donors, the kinetics of IL-10 production on primary and secondary stimulation remained constant between donors (Fig. 3B). A defining characteristic of differentiated T cells is the shorter response time on restimulation. Maintenance of IL-10 production and a shorter response time on restimulation indicated differentiation into a stable Tr1 phenotype (Fig. 3A-C).

Tr1s are IL-10 single-positive cells with a stable phenotype

In the context of emerging knowledge of T cell plasticity, an interesting and important question is this: How do costimulatory molecules influence phenotypes acquired under different priming conditions? We compared classical Th1s, differentiated under CD28 costimulation, with Tr1s, differentiated under CD55 costimulation, for their response to secondary stimulation with CD55 or CD28. CD55 restimulation resulted in an increase in IL-10+ cells in CD55-primed cultures (from 4 to 11%). Although CD28 restimulation resulted in a small increase in IL-10+ cells in CD55-primed cultures, primary costimulation with CD28 did not induce IL-10+ cells (Fig. 4A). This supported earlier observations that CD55 costimulation resulted in differentiation of Tr1s whereas CD28 costimulation did not, but it also showed that Tr1s were more responsive to CD55 restimulation.

FIGURE 4. Tr1 cells are differentiated IL-10 single-positive cells with a stable phenotype. (A) Tr1 cells were induced specifically in response to primary CD55 costimulation of naive CD4+ cells, but IL-10 secretion was maintained on secondary CD55 or CD28 restimulation (day 12). Cells were assessed for IL-10 production at the indicated time points by flow cytometry and following the IL-10 capture protocol (n = 6). (B) The experiment shown (A) was repeated following an IL-10 and IFN-γ dual capture protocol. CD55-induced IL-10+ single-positive Tr1 cells were restimulated only in response to CD55, whereas CD28 restimulation only produced IL-10+/IFN-γ+ double-positive cells (n = 3). (C) IL-10+ cells induced on primary (day 3) CD55 costimulation were maintained on secondary (day 12) CD55 costimulation (n = 7). (D) and (E) The percentage of IL-10+ or IFN-γ+ single-positive cells was measured in CD55 or CD28 primary costimulated cultures, showing that CD55 costimulation gave significantly more Tr1 cells whereas Th1 cells were obtained by CD28 costimulation. Statistical significance was determined by a two-tailed, paired Student t test in both panels (n = 8). Data shown are either representative of or summarized from n independent experiments. *p ≤ 0.05.
Recent reports on T cell plasticity implied that coexpression of IL-10 and IFN-γ (IL-10+/IFN-γ- double-positive cells) indicated a plasticity of T cell subsets (36). We therefore assessed IL-10 and IFN-γ production simultaneously. In CD55-costimulated cultures, by day 3 of primary stimulation, two main populations of cells were seen, one IL-10+ and the other IFN-γ-. Both of these populations expanded on restimulation with CD55 and a double-positive population became apparent (Fig. 4A). Unlike CD55 restimulation, CD28 restimulation did not reactivate the IL-10+ single-positive cells but maintained the IFN-γ+ cells and the IL-10+/IFN-γ- double-positive cells. The percentage of double-positive cells was comparable between CD55 and CD28 restimulated cultures. These data implicate CD55 as the driving force for IL-10 production in the single-positive cells. This also accounted for the difference in the percentage of IL-10+ cells between CD55 and CD28 restimulation when assessing IL-10 alone (Fig. 4A). In comparison, CD28 costimulation had more of an influence on driving IFN-γ+ cells at each time point compared with CD55, even when restimulating the CD55-primed cells (Fig. 4B). CD55 costimulation consistently gave ~0.5–4.5% IL-10+ single-positive cells on primary stimulation, which increased upon restimulation with CD55 (Fig. 4C), whereas CD28 costimulation gave significantly more IFN-γ+ cells (Fig. 4D, 4E). Taken together, these data suggest that CD55 costimulation has a predominant influence on T1 polarization and that CD28 does not induce IFN-γ production by Tr1s.

To confirm the phenotype of the Tr1s, they were sorted on the basis of IL-10 secretion and their purity was assessed by flow cytometry (Fig. 5A, 5C). On restimulation, the IL-10-enriched population consistently produced large amounts of IL-10 but no IFN-γ (Fig. 5B, 5D). Whereas the IL-10-depleted population failed to produce IL-10 and only produced IFN-γ (Fig. 5B, 5D). Only supernatant from the IL-10-sorted cells was able to inhibit cell proliferation in an IL-10-dependent manner (Fig. 5E). CD55-induced Tr1s express the Tr1 markers CD49b, LAG-3, and CD226 (Fig. 6A). Tr1s were examined for their proliferative potential. In CFSE dye dilution assays, Tr1s showed a similar number of cell divisions as IL-10– Th1s (Fig. 6B). This was supported by the thymidine incorporation assay of purified Tr1s (Fig. 6C). Also, Tr1s maintained secretion of high levels of IL-10 during multiple rounds of stimulation (Fig. 6D). This corroborates our previous observations that Tr1s are not anergic and have a stable phenotype.

In this study, we have shown that the alternate costimulator CD55 can induce activation and IL-2 production by naive CD4+ cells. This also results in the differentiation of a small population of these cells into IL-10+ single-positive (Tr1) cells that express Tr1 markers CD49b, LAG-3, and CD226. These proliferate in culture, maintain a differentiated Tr1 phenotype, and continue to secrete IL-10. They show a preferential response to CD55 as a costimulator and are not induced by CD28, supporting the role of CD55/CD97 interaction as an alternate costimulatory pathway for human Tr1 differentiation.

**Discussion**

In this study, we identify a novel role for CD55 in costimulating naive CD4+ cells, and we show that CD55 costimulation induces a discrete Tr1 population. On CD55 costimulation, all naive CD4+ cells are activated, proliferate, secrete IL-2, and acquire differentiated phenotypes. On subsequent stimulation, the Tr1 cells...
maintain their phenotype over multiple rounds of CD55 costimulation. These data identify that CD55 is as potent a costimulatory molecule for naive CD4+ cells as CD28, 4-1BB, ICAM-1, and CD5 (25, 29, 37).

CD55 costimulation results in the differentiation of a small (1–5%) and discrete population of Tr1s (IL-10+IFN-γ+IL-4+) that express the Tr1 markers CD49b, LAG-3, and CD226 and inhibit bystander T cells in an IL-10–dependent manner. Tr1s can be restimulated with CD55 and they continue to proliferate and secrete IL-10. Interestingly, the Tr1 phenotype does not respond to CD28 restimulation, implying that there is a specific CD55–mediated costimulation requirement for the maintenance of this population. The remaining (95–99%) cells acquire a Th1 phenotype that is comparably maintained with CD55 or CD28 restimulation, but these cells can also secrete IL-10 on restimulation.

On CD55 costimulation, a large amount of IL-10 is produced along with small amounts of IL-2 and IFN-γ. The cytokine capture experiment shows that the small Tr1 population is the main source of IL-10 in the culture supernatant, whereas the Th1s are the source of the small amount of IFN-γ. Comparing IFN-γ secretion by surface capture with intracellular IFN-γ staining (data not shown) showed that the Tr1s neither produced nor secreted IFN-γ, and, more importantly, although the Th1s produced IFN-γ, its secretion was inhibited by IL-10 from the Tr1s. Depletion of the Tr1s and restimulation of the remaining Th1s result in a significant increase in IFN-γ secretion by a larger number of these cells. These data demonstrate the regulatory function of the induced Tr1s and corroborate their phenotype as Tr1.

Little is known about the role of costimulatory molecules in the differentiation, maintenance, and regulation of any Treg population. A number of studies have implicated CD28 in the control of naturally occurring Tregs (38). From a recent study it can be inferred that CD28 costimulation could indirectly regulate inducible Tregs (iTregs) by influencing TCR signaling (39). Low levels of CD28 in noninflammatory conditions allowed for iTreg induction. However, higher levels of CD28 in inflammatory conditions lowered the T cell activation threshold, promoting other effector phenotypes. In another study, Semple et al. showed that

FIGURE 6. Tr1 cells proliferate and maintain IL-10 production during multiple rounds of stimulation. (A) CD55-induced Tr1 cells express CD49b, LAG-3, and CD226 (n = 3). (B) Naive CD4+ cells were labeled with CFSE and were costimulated with CD55 for 72 h. Proliferation was assessed by CFSE dye dilution in the context of IL-10 secretion from unsorted (B i) and sorted populations (B ii) (n = 4). (C) Naive cells were differentiated with CD55 and Tr1s were sorted on day 14, following a secondary CD55 stimulation. Proliferation of Tr1E and Tr1D cells was assessed by [3H] thymidine incorporation following 48 h restimulation with CD55. Statistical significance was determined by a two-tailed, unpaired Student t test (n = 3). (D) IL-10 was assessed by ELISA during 17 d, as indicated, and following three stimulations with anti-CD55 (n = 3). Data shown are representative of n independent experiments. ***p ≤ 0.0005.
low CD28 levels resulted in the stimulation of iTregs, but at higher CD28 levels it resulted in the induction of T effector cells mediated by increased signaling via lck and recruitment to the immunological synapse (40). Because natural and induced Tregs exist alongside effector T cell populations, mechanisms for their induction and maintenance must exist. Both of the above studies show that strong CD28 signaling favors a Th effector phenotype, and they support a role for alternate costimulatory molecules in the induction and maintenance of iTregs. This suggests a mechanism for the induction of iTregs with alternatively activated DCs (15, 16). Our data show that CD55 fits the role of an alternate costimulatory molecule for the induction of Tr1s.

Two other costimulatory molecules, CD2 (20) and CD46 (21), have been shown to induce IL-10 production from CD4+ cells, but in the present study we show that CD55 induces differentiation of an IL-10 single-positive (Th1) phenotype. Also, unlike CD2 or CD46 costimulation, CD55-induced Tr1 differentiation is not dependent on exogenous IL-2. CD46 is a CRP similar to CD55, and Kemper et al. (22) showed that binding of CD46 by the active C3b component of complement induced IL-10 production. Recently, they also reported CD46-mediated regulation of IL-10 production during the Th1 lifecycle and demonstrated CD46-regulated plasticity of Th1 cells (36). This IL-10+ Th1 population is distinct from the CD55-induced Tr1 (IL-10+IFN-γ+IL-4−) population, because the Tr1s maintain a differentiated phenotype, but it is similar to the CD55-induced IL-10+IFN-γ+ population. With regard to their role as CRPs, similar to CD46, CD55 accelerates the decay of C3 and C5 convertases, preventing C3b deposition and downstream assembly of the membrane attack complex (41). However, unlike CD46, CD55 also binds to the leukocyte activation Ag CD97, its cellular ligand. Our previous study showed that engagement of CD55 by CD97, but not by complement, costimulated human CD4+ cells (33). In the present study, we show that CD97 engagement of CD55 on naive CD4+ cells drives the differentiation of a T1p population and also induces IL-10 production by Th1s. Thus, although both CD46 and CD55 regulate IL-10 production by Th1s, only CD55 can drive the induction of a stable Tr1 population.

A number of groups have reported induction of “Tr1 cells” and have focused on the role of cytokines in the induction of these cells (11, 12, 17–20, 22, 42). Similarly, other groups have identified regulatory DC phenotypes that promote Tr1 induction (15, 16, 43–45). In the absence of lineage-specific markers, it has been difficult to differentiate between true Tr1s and IL-10–producing Th1 cells (46). Recently, however, both genotype and phenotype analyses of Tr1 cells have identified CD49b, LAG-3, and CD226 expression as markers of human and mouse Tr1 cells (8). In all studies, the two common identifying features of a Tr1 phenotype are the characteristic cytokine profile, specifically IL-10high but IL-4−, and IL-10–mediated suppressive function.

The CD55-derived Tr1s have the above attributes and this corroborates their phenotype as Tr1 cells. Furthermore, we can isolate Tr1s on the basis of their phenotype and regulatory function. We show that they are a discrete, stable, differentiated population of IL-10+ single-positive cells. We also confirm previous observations of Tr1s being IFN-γ+ and Foxp3− (Supplemental Fig. 4) (47). Two studies reported Tr1s as being anergic (1, 36). In contrast to this, we show that Tr1s are highly proliferative cells and respond to restimulation. Importantly, this occurs specifically in response to CD55 costimulation.

Currently, there is debate over whether Tr1s are a differentiated population or a regulatory phenotype acquired by effector T helper subsets. A recent study by Cope et al. (36) supports the latter argument, showing that Th1s acquire a transient Th1 phenotype in certain stimulatory conditions. Furthermore, they proposed that Th1s can be “locked” in a Tr1 phenotype and this gives rise to Tr1 cells, suggesting that Tr1s are an IL-10–producing regulatory phenotype acquired by T helper cells (36). The authors also speculated that a change in the stimulatory environment of Tr1s would “unlock” their phenotype, allowing them to revert to another effector phenotype. The absence of a lineage-specific marker (2), the IL-10–producing capability of effector T cells (48), and the growing evidence that shows plasticity of cells (49–52) lend support to this argument. However, our data suggest that CD55-derived Tr1s are a stable and discrete population, differentiated from naive CD4+ cells. They are also superimposable on Tr1 cells as defined by other groups due to their CD49b, LAG-3, and CD226 expression. This study identifies that the alternate costimulatory pair, CD55/CD97, is involved in the differentiation of Tr1 cells from naive precursors.

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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1

Isolated naïve CD4+ cells expressed CCR7 and CD62L but not CD69 or CD25.

The expression of phenotypic markers on freshly isolated naïve CD4+ cells was assessed by flow cytometry. Antibody binding (black histogram) was compared to respective controls (grey histograms), (n = 5).
Figure S2. CD55 co-stimulation activated naïve CD4+ cells, comparable to CD28 co-stimulation and higher than CD3 stimulation alone.

Naïve CD4+ cells were stimulated for 24 h, as indicated. Expression of activation markers, CD25 and CD69, on freshly isolated naïve CD4+ cells and stimulated cells was assessed by flow cytometry, (n = 3).
Figure S3

![Figure S3](image)

**Figure S3. IL-10 was produced by a small number of cells.**

A 96 well Immobilin-P plate (R&D systems) was coated with anti-IL-10 capture Ab (R&D systems) and stimulatory mAbs as indicated. Naïve CD4+ cells were stimulated with mAb combinations as indicated, for 48 h. 5x10^4 stimulated cells/ well were seeded in triplicate, in the pre-coated Immobilin-P plate, keeping the stimulatory conditions constant, and cells were cultured for a further 72 h at 37 °C and 5% CO₂. The captured IL-10 was detected with biotinylated anti-IL-10 Ab. The plate was developed with streptavidin alkaline phosphatase and a chromogenic substrate. Spots were analysed and counted using an automated plate reader (C.T.L. Europe), (n = 3).
Figure S4. Tr1 cells do not express Foxp3 constitutively.

Naïve CD4+ cells were co-stimulated with CD55 for 72 h and cells were simultaneously assessed for IL-10 production, by IL-10 capture, and Foxp3 expression, by intracellular staining, by flow cytometry. After IL-10 capture, cells were fixed and permabilised using the FOXP3 Fix/Perm Buffer Set (BioLegend) and following the recommended protocol. Cells were co-stained with Foxp3 at the recommended dilution and at least $5 \times 10^4$ events were acquired, (n = 3).