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Induction of a Regulatory Phenotype in Human CD4$^+$ T Cells by Streptococcal M Protein[1]

Jeffrey D. Price,*† Jessica Schaumburg,‡ Charlotta Sandin,‡ John P. Atkinson,† Gunnar Lindahl,[2]‡ and Claudia Kemper[2]†

Regulatory T cells (Tregs) participate in the control of the immune response. In the human system, an IL-10-secreting, T regulatory type 1 cell (Tr1)-like subset of Tregs can be induced by concurrent cross-linking of the TCR and CD46 on naive CD4$^+$ T cells. Because many viral and bacterial pathogens, including the major human pathogen *Streptococcus pyogenes,* bind to CD46, we asked whether this bacterium can directly induce Tr1-like cells through the streptococcal ligand for CD46, the M protein. The M5 and M22 proteins were found to induce T cells to develop into the IL-10-producing Tr1-like phenotype. Moreover, whole M5-expressing bacteria, but not isogenic M-negative bacteria, led to proliferation and IL-10 secretion by T cells. The interaction between the M5 protein and T cells was dependent on CD46 and the conserved C repeat region of M5. Supernatants derived from T cells stimulated with M proteins or M protein-expressing bacteria suppressed bystander T cell proliferation through IL-10 secretion. In addition, activation of CD46 through streptococcal M protein induced the expression of granzyme B, providing a second means for these cells to regulate an immune response. These findings suggest that binding to CD46 and exploiting its signaling pathway may represent a strategy employed by a number of important human pathogens to induce directly an immunosuppressive/regulatory phenotype in T cells. ***The Journal of Immunology,*** **2005, 175: 677–684.**

The mammalian immune system protects against microbial infections, a function fulfilled through its ability to recognize foreign Ags. Reactivity to self-Ags is prevented through central or peripheral tolerance. Although several mechanisms contribute to tolerance and immunological unresponsiveness, much interest has recently focused on regulatory T cells (Tregs).[3] These cells can be divided into two major groups: thymic (or natural) Tregs and adaptive, peripherally arising, T regulatory type 1 cells (Tr1) and Th3 cells (1–3). Natural Tregs act in a contact-dependent fashion and probably represent a distinct lineage of cells that express CD25 and the transcription factor Foxp3 (1, 4), whereas Tr1 and Th3 cells are believed to mostly function in a contact-independent manner, and may (1) or may not (5) express Foxp3, and show variable expression of CD25 (2). The adaptive Tr1 and Th3 cells preferentially synthesize the immunosuppressive cytokines IL-10 and TGF-β, respectively, and seem to be more important in the homeostasis of responses to foreign Ags (2, 3, 6). In this study, we describe a mechanism by which Tr1 cells may be directly induced by a microorganism interacting with CD46 (membrane cofactor protein) on the surface of naive human CD4$^+$ T cells.

CD46 is a complement regulatory protein expressed by most human cells. It binds C3b and C4b such that the plasma protease factor I can inactivate these fragments by limited proteolysis (7). Cross-linking of CD46 on human CD4$^+$ T cells is accompanied by a number of downstream signaling events, including phosphorylation of its cytoplasmic domain by Lck (8), the activation of Vav and Rac (9), and phosphorylation of the MAPK Erk and the adapter proteins p120CBL and Lat (9, 10). Simultaneous cross-linking of CD46 and CD3 induces Tregs with a highly proliferative, IL-10-producing and granzyme B-expressing phenotype (11–13). These cells can suppress bystander T cells through IL-10 secretion and a granzyme B/perforin-dependent mechanism (11–13).

In addition to its function(s) in the human immune system, CD46 serves as a cellular receptor for multiple viral and bacterial human pathogens (14). One of these CD46-binding microorganisms is the Gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus), a human pathogen causing multiple diseases, including pharyngitis, skin infections, the streptococcal toxic shock syndrome, and postinfectious rheumatic fever (15). The major virulence factor of *S. pyogenes* is the M protein, an antiphagocytic surface molecule (16) that also binds human CD46 (17, 18), a property that may promote bacterial adhesion. This observation led us to analyze whether M proteins may induce a Tr1 phenotype in human CD4$^+$ T cells. Although several pathogenic microorganisms, including *Bordetella pertussis* (19) and *Vibrio cholerae* (20), have recently been shown to induce Tregs by interfering with dendritic cell maturation (19–21), our data indicate that *S. pyogenes* interact with CD46 on human CD4$^+$ T cells to generate directly cells with a Tr1-like regulatory phenotype.

Materials and Methods

Media, Abs and soluble CD46

Cells were maintained in RPMI 1640 medium with 15% FCS and 200 mM L-glutamine. Abs used were described in the study by Kemper et al. (11), except the primary conjugated anti-granzyme B mAb (GB12) which was

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[3] Abbreviations used in this paper: Treg, regulatory T cell; Tr1 cells, T regulatory type 1 cell.

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purchased from Caltag Laboratories and the function-neutralizing mAb to CD18 from eBioscience). Soluble CD46 was a gift from G Yeh of Millennium Pharmaceuticals (Cambridge, MA).

Bacteria

The *S. pyogenes* M5 strain and its M-negative isogenic derivative ΔM5 have been described previously (22). Heat-inactivated bacteria were prepared from overnight cultures and grown in Todd-Hewitt broth without shaking in 5% CO_2 at 37°C. The bacteria were harvested, washed, and resuspended in PBS containing 10 μg/ml protease inhibitor E64 to 10^{10} bacteria/ml (23). The bacterial suspension was incubated at 90°C for 14 min, washed, and resuspended in PBS supplemented with E64. Heat-killed bacteria were not affected in their ability to bind radiolabeled fibrinogen, a ligand for the M5 protein, and could be kept for >9 mo at 4°C without loss of surface M5 protein.

Expression and purification of streptococcal M proteins

The M5 and M22 (Sir22) proteins were expressed in *Escherichia coli* (22, 24) and purified from whole cell extracts as described for the M22 protein (24). The mutant derivatives M5ΔB and M5ΔC were similarly purified from *E. coli* strains carrying plasmids encoding the corresponding proteins (Ref. 25 and our unpublished data). The group B streptococcal Rib protein was purified from strain BM110 as described (26).

Coating of latex beads with Ab to CD46 or with M proteins

mAbs and M proteins were coated onto latex beads as described previously (27).

Purification and sorting of CD4$^+$ lymphocytes

CD4$^+$ lymphocytes were purified from whole blood and subpopulations sorted at the Siteman Cancer Center High Speed Sorter Facility at Washington University using a MoFlo high-performance cytometer (DakoCytomation) as described elsewhere (11). The analyzed T cell populations were APC-free as shown by FACS staining for CD4.

CD4$^+$ T cell stimulation

Stimulation with plate-bound Abs and M proteins. Stimulation was performed in microtiter plate wells coated with equimolar amounts of mAbs to CD3, CD28, and/or CD46 or with a matched IgG1 isotype control (all mAbs at 10 μg/ml PBS) at 4°C overnight. Wells were similarly coated with solutions (5–10 μg/ml) of M proteins, M protein mutants, or the streptococcal control protein (Rib). Purified sorted CD4$^+$ lymphocytes (1.5–2.0 × 10^5 cells/well) were added to washed wells in 100 μl of culture medium containing 30–50 U/ml recombinant human IL-2. The plates were centrifuged at 100 × *g* for 1 min and incubated at 37°C in 5% CO_2.

Stimulation with plate-bound anti-CD3 and latex bead-bound anti-CD46 or M proteins. Purified sorted CD4$^+$ T cells (1.5 × 10^5) were incubated under slow motion (100 rpm on an orbital shaker) with latex beads coated with either anti-CD46 or the M proteins/mutants at a ratio of 15 beads:1 T cell in 50 μl of medium for 15 min at 37°C. Mixtures were centrifuged for 3 min at 100 × *g* and incubated for an additional 15 min at 37°C, with no shaking. Fresh medium (50 μl) was added, the cell:bead mixtures resuspended, and transferred (at 100 μl/well) into 96-well plates that had been coated with anti-CD3 mAbs.

Stimulation with M protein-expressing streptococci. Purified sorted T cells (1.5 × 10^5) were incubated with either the M5-expressing *S. pyogenes* or the isogenic M-negative control strain at a ratio of 20 bacteria:1 T cell in 50 μl of medium for 15 min at 37°C under slow rotation. The cell:bacteria mixtures were centrifuged for 3 min at 200 × *g* and incubated for an additional 15 min at 37°C, with no movement. Fresh medium (50 μl) was added, and the cell:bacteria mixtures were gently resuspended and transferred (at 100 μl/well) into 96-well plates coated with mAbs to CD3.

Cytokine and granzyme B analyses

CD4$^+$ cells (1.5–2.0 × 10^5 cells/well) were incubated for up to 3 days in 96-well plates coated with the mAbs. The secretion of IL-2, IL-4, IFN-γ, IL-10, and IL-12 was assessed in the supernatants by using the ProteoPlex 16-Well Human Cytokine Array (Novagen). In addition, IL-2 and IL-10 production was determined by the appropriate EASIA kits from BioSource International. To measure granzyme B expression, purified CD4$^+$ T cells (1.5 –2.0 × 10^5 cells/well) were stimulated with immobilized mAbs for 24 h. To block cytokine export/secretion, monensin (BD Biosciences) was added for the last 8–12 h of culture. After appropriate surface marker staining, cells were permeabilized, fixed, and stained for granzyme B expression.

Flow-based killing assay

The cytotoxic potential of CD3/CD46 or CD3/M5 protein-activated CD4$^+$ T cells against target cells was measured using the previously described flow-based killing assay (12). Briefly, effector cells were generated by activating purified human CD4$^+$ T cells for 48–72 h with immobilized mAbs to CD3 and CD46 or with immobilized mAbs to CD3 and purified M5 protein. The human monocytic cell line U937 and autologous CD3/CD28-activated CD4$^+$ T cells were used as target cells. Target cells were washed with PBS, resuspended at 1 × 10^6 cells/well, and labeled at 37°C for 15 min with 125 nM final concentration of CSFE (Molecular Probes). Labeled target cells were plated into 96-well V-bottom cell culture plates at 1 × 10^5 cells/well (Corning) and effector cells were added at a ratio of 20 effector cells:1 target cell in RPMI 1640 medium containing 50 U recombinant human IL-2/ml. Cell mixtures were incubated for 6–8 h. Immediately before analysis, 1 μg/ml 7-aminoactinomycin D (Calbiochem) was added to each sample. 7-aminoactinomycin D incorporation by CSFE-labeled cells was determined by FACS analysis and used as a surrogate marker for late cell death/apoptosis. EGTA or function-neutralizing mAbs to CD18 were used to demonstrate that the observed target cell killing was perforin/granzyme dependent.

Proliferation assay

Cell proliferation rates were measured using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay from Promega.

Donor selection and statistical analysis

Blood from seven healthy donors was collected and used in this study according to the Washington University Medical Center Human Studies Committee guidelines. Each experiment was performed at least three times using a different donor each time and all activation conditions were performed in triplicate. Interdonor variability for cytokine secretion and granzyme B expression ranged from 5 to 30% and the results shown are the mean of three donors from independent experiments. Statistical significance was determined using the paired Student's *t* test.

Results

Experimental system

The general design of the experiments was to incubate human CD4$^+$ T cells with streptococcal M proteins and then measure T cell proliferation, cytokine production, and suppressive properties. We have previously shown that both naive CD4$^+$ T cells (CD45RA$^+$/RO$^-$) and T cells with a CD45RA$^+$/RO$^+$ phenotype respond to CD46 activation with IL-10 production. The latter subpopulation shows an earlier and stronger response (referred to as "high responders") (11). For the experiments reported here, sorted naive and high responder T cells were combined. The structure of CD46 and the M proteins is shown in Fig. 1, *A* and *B*. The binding sites for C3b and C4b are located in the repeating modules of CD46, and the cytoplasmic tails contain motifs that mediate signaling events in several human cell types (7). The M protein has a hypervariable N-terminal region that allows classification of *S. pyogenes* isolates into ~120 M types (28). The M5 and M22 proteins used here represent two major groups of M protein, characterized by their ability to bind to human fibrinogen or the complement regulator C4b-binding protein, respectively (29–31). The ligand-binding properties of the M5 and M22 proteins are indicated in Fig. 1. Of note, M5 and M22 have been reported to be among the most common serotypes among clinical *S. pyogenes* isolates (32). The sites of interaction between M protein and CD46 have been mapped to the C repeat region in the M6 protein (17) and to the third and fourth repeat of CD46 (18). Recombinant forms of M5, M22, M5 with its B (M5ΔB) or C (M5ΔC) repeat region deleted and an unrelated group B streptococcal control protein, the surface protein Rib, were used in this study (Fig. 1*C*).

Streptococcal M proteins induce a Tr1-like cytokine pattern in CD4$^+$ T cells

Human peripheral blood CD4$^+$ T cells were activated with plate-bound anti-CD3 mAbs and M5 protein and the cytokine profile

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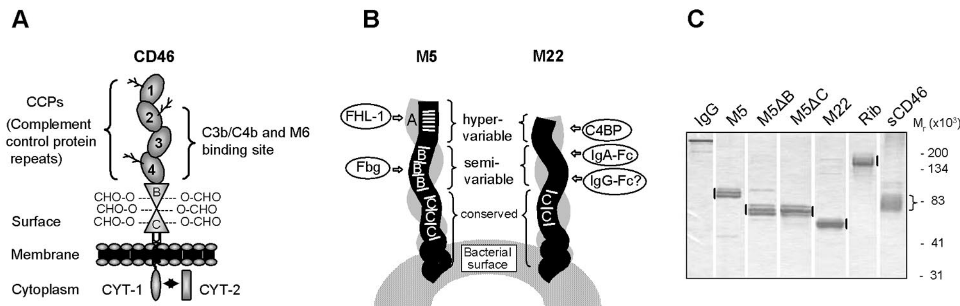

FIGURE 1. Human CD46 and streptococcal M proteins. *A,* CD46 contains four CCP domains, the alternatively spliced *O*-glycosylated region designated B and C, and a region of unknown function (U) that is followed by a transmembrane domain, cytoplasmic anchor, and one of two possible cytoplasmic tails. *B,* The M5 and M22 proteins are dimeric coiled-coils. The binding sites for human plasma proteins and of the B and C repeat regions are indicated. FHL-1, factor H-related protein 1; Fbg, fibrinogen; C4BP, C4b-binding protein. *C,* SDS-PAGE analysis (nonreducing) of proteins (2 μg/lane) used in this study. The microheterogeneity of the M proteins is characteristic of this family of proteins when produced by *E. coli* and may represent variability at the COOH terminus (16). See text for description of these proteins. A mouse mAb IgG was included as a control.

examined after 72 h (Fig. 2*A*). CD3/M5-stimulated cells produced little IL-2, intermediate amounts of IFN-γ, and large amounts of IL-10. Moreover, CD3/CD46 and CD3/M5-activated cells produce no intracellular IL-2 as determined by FACS staining (data not shown). In contrast, CD3/CD28-stimulated cells produce large amounts of IL-2, intermediate amounts of IFN-γ, and limited amounts of IL-10. No release of IL-4 or IL-12 was observed. IL-10 secretion peaked at 48 or 72 h poststimulation and then declined to baseline by day 5 or 6 (data not shown). Neutralization of IL-2 abrogated IL-10 secretion induced by the M5 protein, demonstrating that Tr1-like cells produced by interaction with the microbial ligand are IL-2 dependent (Fig. 2*B*). The M22 protein also induced IL-10 secretion by T cells, whereas only background levels of IL-10 were observed with the streptococcal control protein Rib (Fig. 3*A*). Deletion of the C repeat region of M5 (M5ΔC) decreased IL-10 in the culture supernatants, whereas removal of the B repeat region (M5ΔB) had little effect (Fig. 3*A*). This result is in agreement with previous findings for the M6 protein (17), indicating that the C repeat region of M5 is critical for binding to CD46. Because the C repeat region is present in all M proteins, this interaction may be crucial for the pathogenicity of *S. pyogenes*.

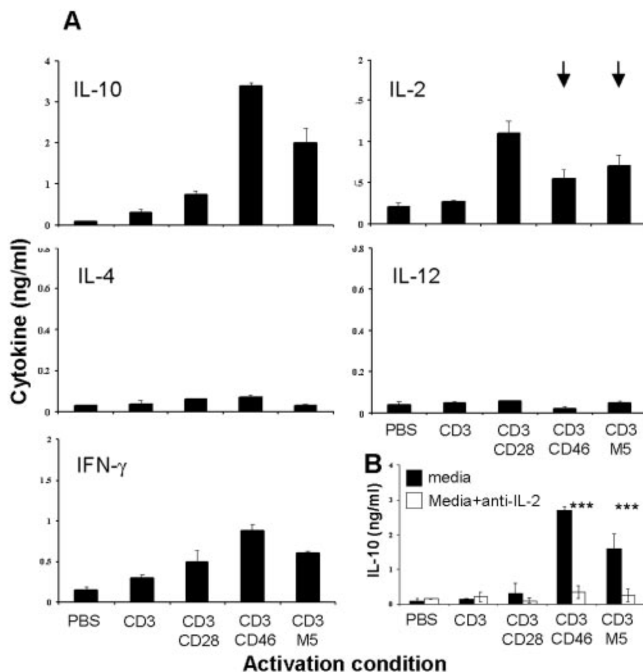

FIGURE 2. Streptococcal M proteins induce a Tr1-like cytokine secretion profile in human peripheral blood $CD4^+$ T lymphocytes. *A,* Streptococcal M5 protein induces a similar cytokine profile as mAbs to CD46. Microtiter wells were coated with mAbs to CD molecules and with pure M5 protein, as indicated, and purified T cells were seeded. The cell supernatants were analyzed at 72 h for secreted cytokines. Arrows indicate the detection of added recombinant human IL-2. *B,* M5-induced IL-10 production is IL-2 dependent. T cells were incubated under the indicated activation conditions for 72 h, with or without a neutralizing mAb to IL-2. Results are the mean ± SD of three independent experiments. **, $p < 0.001$.

Streptococcal proteins were also attached to latex beads and then incubated with $CD4^+$ T cells in the setting of plate-bound anti-CD3 (Fig. 3*B*). This led to a similar degree of IL-10 secretion as that of the plate-bound proteins, with a strong IL-10 response to M5 and M22 and a weak response to Rib and M5ΔC. M proteins introduced into the cultures in the fluid phase induced no IL-10 response (data not shown), suggesting that its arrangement on a surface is important for the M protein to induce Tr1-like cells.

Next, heat-inactivated streptococci expressing M5 or an isogenic strain lacking M5 were incubated with $CD4^+$ T cells. If stimulated simultaneously through CD3, the M5-expressing bacteria induced IL-10 production at levels comparable to those of Abs to CD46, while the M5-deleted strain induced little IL-10 (Fig. 3*C*). If T cells were simultaneously stimulated with plate-bound mAbs to CD3 and CD46 and with heat-inactivated bacteria, IL-10 secretion exceeded that induced by the plate-bound Abs. This occurred independently of bacterial M5 expression (Fig. 3*C*). This suggests that the initial M protein-CD46 interaction is crucial for IL-10 production, but that additional components on the bacterial surface can enhance the CD46-dependent development of the Tr1-like phenotype.

CD46 is essential for the initiation of a Tr1-like phenotype by streptococcal M5 protein

Addition of soluble CD46 to the culture medium during T cell activation decreased IL-10 secretion by T cells stimulated with anti-CD46 mAbs or with M proteins, whether these proteins were immobilized in microtiter plates (Fig. 3*D*) or bound to latex beads (Fig. 3*E*). Bacteria-induced secretion of IL-10 was also reduced by addition of soluble CD46 (Fig. 3*F*).

Morphological changes induced in Tr1-like cells

Cells stimulated with mAbs to CD3 and CD46 exhibit a greater and prolonged proliferation and characteristic morphological changes as compared with cells stimulated through CD3 and CD28 (9–11). To analyze whether M proteins induce such changes, we

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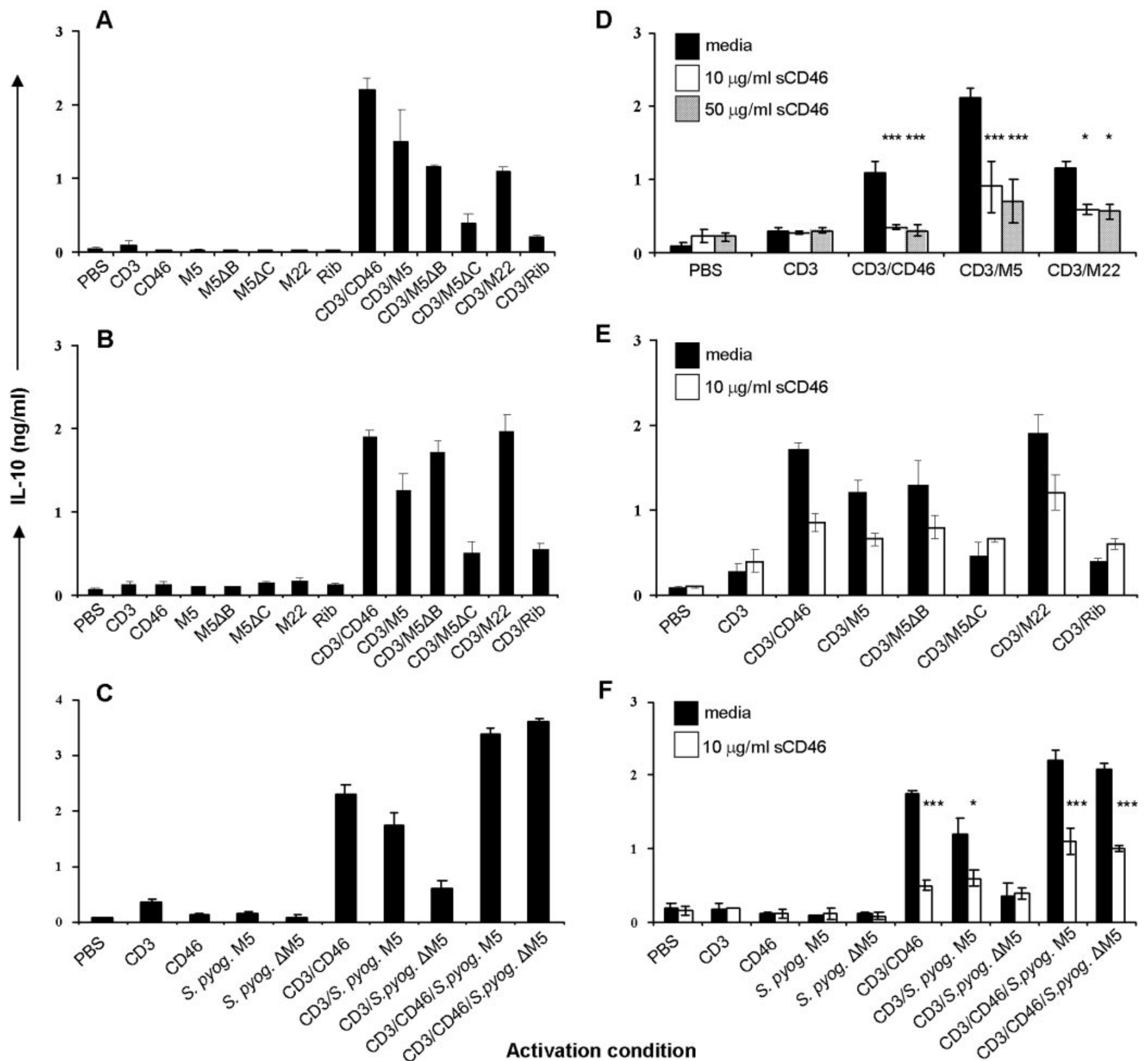

FIGURE 3. M protein-induced IL-10 production by CD4$^+$ T cells is dependent on the C repeat region of M5 and CD46. *A,* Analysis of plate-immobilized M proteins and mutant proteins for ability to induce IL-10 production. *B,* Latex bead-bound M proteins induce IL-10 production. Latex beads coated with anti-CD46 mAbs and/or streptococcal proteins were added to T cells (15 beads:1 T cell) in wells coated with or without anti-CD3 as indicated. *C,* IL-10 production induced by whole streptococci. T cells were seeded into microtiter wells coated with anti-CD3 and/or anti-CD46 and heat-inactivated bacteria were added as indicated (20 bacteria:1 T cell). *S. pyog*. M5, bacteria expressing M5; *S. pyog*. ΔM5, isogenic M5-negative mutant. *D—F,* Protocols as indicated in *A*, *B*, and *C*, respectively, except for the addition of the indicated amounts of soluble CD46 (sCD46) to the medium at time 0. All supernatants were analyzed at 72 h. Results are the mean ± SD of three independent experiments. ***, $p < 0.001$; *, $p < 0.05$.

activated CD4$^+$ T cells with immobilized anti-CD3 and M5. T cells increased in size due to anti-CD3 stimulation but, in addition, formed homotypic clusters when simultaneously stimulated through CD46 (Fig. 4). Activation with either the M5 or M22 protein plus anti-CD3 induced similar morphological changes. The clusters decreased in number and size when soluble CD46 was added to the culture.

Supernatants from M protein-induced Tr1-like cells suppress bystander T cell activation

We next asked whether supernatants from cultures of CD4$^+$ T cells stimulated through CD3 and M5 protein or with M5-expressing bacteria suppress bystander T cell activation. Transfer of such supernatants to freshly purified, activated CD4$^+$ T cells reduced proliferation of these cells in contrast to cultures incubated with the supernatant from CD3/CD28-stimulated T cells (Fig. 5, *upper panels*). Suppression was reversed by anti-IL-10 mAbs, demonstrating that the suppressive property of the supernatants is mediated by IL-10 (Fig. 5, *lower panels*).

M5 protein-induced Tr1-like cells express granzyme B and can kill target cells

In addition to driving cytokine production, activation via CD46 has recently been shown to enhance the cytotoxic potential of CD4$^+$ T

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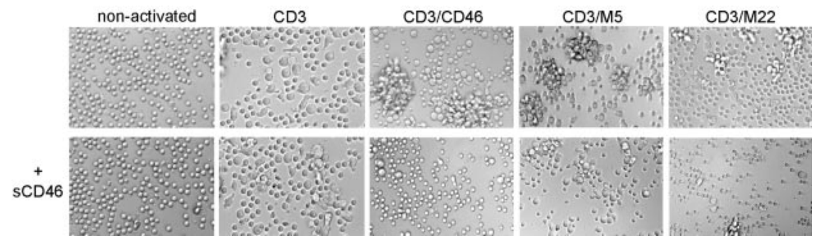

FIGURE 4. M protein induces a similar morphological phenotype in $CD4^+$ T cells as mAbs to CD46. The T cells were stimulated for 3 days with or without the addition of 10 μg/ml soluble CD46. Original magnification, ×200. Results shown are representative of three independent experiments.

lymphocytes against several human cell lines and autologous activated T cells through the expression of granzyme B (12, 13). To analyze whether the activation of CD46 through streptococcal M protein induces similar properties, we measured the expression of granzyme B in CD3/CD46 and CD3/M5 protein-activated T cells as well as their potential to kill target cells (Fig. 6). M5-activated T cells show a granzyme B expression profile comparable to that of CD3/CD46-activated T cells (Fig. 6*A*). In addition, these cells were capable of killing target cells from the human monocytic cell line U937 (Fig. 6*B*) as well as autologous CD3/CD46-activated $CD4^+$ T cells (data not shown). As previously described for CD46-induced Tr1 cells (12, 13), the M5 protein-induced killing is CD18 dependent (Fig. 6*B*) and is inhibited by the addition of EGTA (data not shown).

Discussion

Several pathogenic microorganisms have recently been shown to induce Tregs (21, 33, 34). The mechanism reported for *B. pertussis* (19) and *V. cholerae* (20) centers on the inhibition of dendritic cell maturation, which indirectly causes the induction of Tregs. A similar mechanism might operate for the other pathogens that cause immunosuppression by inducing IL-10 (33–37). Our data identify a distinct process by which a pathogen may induce human Tregs. In this report, we show that *S. pyogenes* M protein, a surface-localized virulence factor, interacts with CD46 on peripheral human $CD4^+$ T cells to induce directly Tr1-like regulatory cells. These cells suppress the proliferation of bystander T cells through IL-10. It is likely that the cross-linking of CD46 on human T cells by M proteins triggers intracellular signals that promote Tr1-like cell development.

Our studies grew out of a previous finding that cross-linking of CD46 with mAbs or C3b dimers on $CD4^+$ T cells promotes the development of adaptive Tregs with a phenotype related to that of Tr1 cells (11). Immobilized M protein had properties similar to anti-CD46 Abs and C3b dimers in its ability to induce cells with a Tr1-like phenotype. Importantly, the induction was observed not

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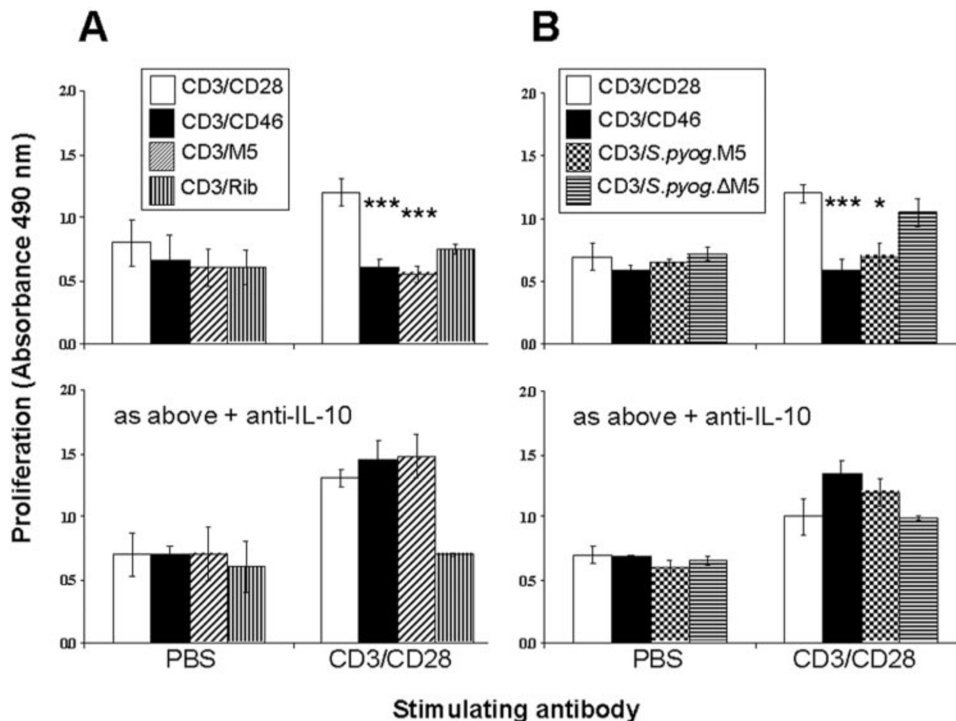

FIGURE 5. Activation of CD46 through purified M5 or M5-expressing bacteria induce functional Tr1-like cells. *A,* M protein-activated T cells inhibit the proliferation of bystander T cells through IL-10. *Upper panel,* T cells were activated for 3 days with the indicated immobilized mAbs or streptococcal proteins. Supernatants were transferred to freshly purified $CD4^+$ T cells, the mixture was incubated with stimulating Abs or PBS, and proliferation was measured at day 7. *Lower panel,* As above but with the addition of 10 μg/ml of a neutralizing mAb to IL-10. *B,* Bacteria-activated T cells inhibit the activation of bystander T cells. *Upper panel,* T cells were plated onto microtiter plate wells coated with the indicated mAbs and bacteria were added (20 bacteria:1 T cell). At day 3, supernatants were transferred to freshly purified T cells and the mixtures were incubated under the indicated stimulation conditions. Proliferation was measured at day 7. *Lower panel,* As above but with the addition of a neutralizing anti-IL-10 mAb. Results are the mean ± SD of three independent experiments. Note that, in contrast to CD3/CD28- and CD3/CD46-activated T cells, CD3/Rib-activated T cells do not produce growth factors; thus, their supernatants do not add to the proliferation of freshly purified CD3/CD28-activated T cells. This is also observed for supernatants derived from cells activated with mAbs to CD3 alone (data not shown). ***, $p < 0.001$; *, $p < 0.05$.

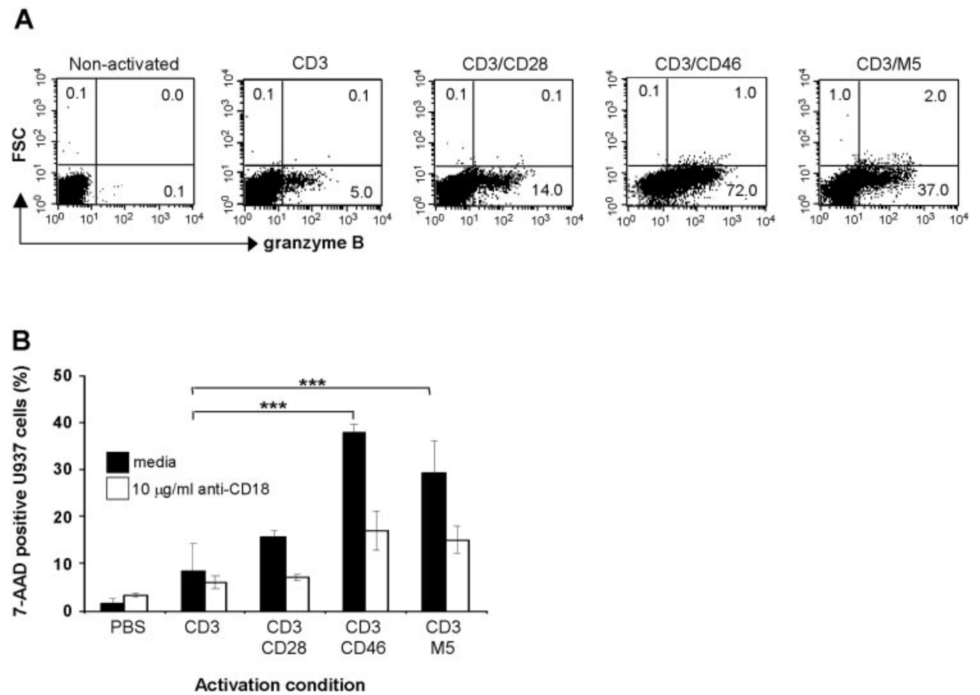

FIGURE 6. T cells activated with M protein exhibit perforin/granzyme B-dependent cytotoxicity. *A,* Activation of CD46 through M5 protein induces granzyme B expression. Purified human T cells were incubated with the indicated immobilized mAbs and/or M5 protein for 36 h and granzyme B expression was determined by FACS. Results shown are representative of three independent experiments. *B,* M protein-activated T cells kill human monocytic U937 cells. Purified T cells were activated for 60 h under the indicated conditions and used as effector cells in a flow-based killing assay against target U937 cells with or without the addition of a function-neutralizing mAb to CD18. Data are representative of three independent experiments showing similar results. ***, $p < 0.001$.

only with immobilized purified M protein but also with M protein present on the bacterial surface. It required the presence of the C repeat region which binds CD46. Because the induction was observed with immobilized but not soluble M protein, multiple-point attachment seems to be necessary. The conclusion that anti-CD46 mAbs and M protein induce Tregs by a similar mechanism is further supported by the similar morphological changes and proliferation rates.

Interestingly, CD46-induced Tr1-like cells also express granzyme B/perforin and have cytotoxic potential toward $CD4^+$ and $CD8^+$ T cells, monocytes, and both mature and immature dendritic cells (12, 13). As reported here, the Tr1-like cells induced by M protein similarly express granzyme B and can kill autologous activated $CD4^+$ T cells and monocytic U937 cells. Although the role of such cytotoxicity during an infection is not yet known, one can envisage that it might be beneficial for invading pathogens to induce a cytotoxic response that targets APCs to prevent or delay the adaptive response.

A number of microbes bind human CD46 (14). Our results suggest that this characteristic not only promotes adhesion to CD46-expressing cells, but also suppresses the human immune response by promoting the formation of Tregs. Pathogens that bind to CD46 include Gram-positive and Gram-negative bacteria and DNA and RNA viruses. The ability to trigger induction of Tr1-like cells may be a virulence mechanism of general significance. Of note, CD46 is not expressed by somatic cells in the mouse (7), a finding consistent with the notion that many immunological mechanisms exhibit important differences between the human and murine systems (38) and underlining the importance of analyzing human pathogens in the homologous system. Along this line, the measles virus (MV) has also taken advantage of the immunomodulatory properties of CD46: cross-linking of CD46 on macrophages by MV down-regulates IL-12, a putative mechanism for virus-induced immunosuppression (39).

Adaptive IL-10-secreting Tregs can be generated in vitro under a number of conditions. The original human (and murine) Tr1 cells were generated and defined by Groux et al. (6) through repeated CD3/CD28 stimulation of purified $CD4^+$ T cells in the presence of IL-10. Such cells are nonproliferative, secrete high amounts of IL-10 but no IL-4, and suppress the activation of bystander T cells through IL-10. In contrast, treatment of human and mouse $CD4^+$ T cells with a combination of the immunosuppressive drugs vitamin D_3 and dexamethasone induces a homogenous population of IL-10-secreting Tr1 cells with strong proliferative capacities (40). In addition, IL-10-secreting $CD4^+$ Tregs can be generated through an incubation with IL-10 and IFN-α (41) and through interactions with immature or tolerogenic dendritic cells (42) or natural $CD4^+CD25^+$ Tregs (43). The interrelationship of these adaptive Treg subpopulations is currently unclear, and it remains to be analyzed whether these cells represent uniform lineages at different developmental stages or whether they are unique populations with distinct origins and characteristics.

In a recent study, Viera et al. (5) reported that adaptive IL-10-secreting Tregs induced with vitamin D_3 and dexamethasone lack Foxp3 expression and IL-2 production. Moreover, these Tregs suppress T cells in both an IL-10 and cell contact-dependent manner (5). CD46-activated human $CD4^+$ T cells also lack IL-2 expression (11), do not require constitutive basal Foxp3 expression (13), and can suppress the activation/expansion of bystander T cells through either IL-10 secretion (11) or an additional cell/cell contact-dependent mechanism involving granzyme B expression and the perforin pathway (12, 13). Overall, these characteristics of CD46-activated T cells resemble most closely those of Tr1 cells; thus, we elected to label them Tr1-like cells.

The role of Tregs in infections varies among types of infection (34, 44). However, several lines of evidence indicate that Tregs contribute to a balanced immune response, particularly at mucosal surfaces (2, 21, 33, 34, 44–48). In this scenario, a lack of Tregs leads to an uncontrolled immune response that contributes to pathogen elimination but might also cause undesirable immunopathology in host tissue. In addition, rapid pathogen clearance might hamper the generation of a memory T/B cell pool to the pathogen. In contrast, an excessive suppressive response by Tregs would allow for uncontrolled growth of the pathogen. This result

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is not evolutionarily favorable for either pathogen or host. However, a balanced response may facilitate pathogen spread and persistence while simultaneously allowing for the development of a proper adaptive response, so called concomitant immunity, that protects against reinfection (21, 34, 46, 48).

The classic disease caused by *S. pyogenes* is “tonsillitis.” The presence of viable S. *pyogenes* and other bacterial species in tonsils, lymph nodes, and other lymphoid tissues has been observed (49). This implies that, in an in vivo setting, whole M protein-expressing bacteria or bacterial cell wall fragments might directly contact T cells during Ag presentation in lymphoid tissues and induce a Treg phenotype locally. Persistence of *S. pyogenes* in the human throat favors an Ab response and is required for the development of the postinfectious syndrome of rheumatic fever (50). Thus, one can envisage the paradoxical situation that the M protein of *S. pyogenes* suppresses the local immune response by inducing Tr1-like cells, thereby favoring bacterial persistence, but also favoring a subsequent immune response that may result in rheumatic fever. Consistent with this idea, the immune response to M protein is delayed, as compared with other (extracellular) *S. pyogenes* Ags (50, 51), possibly reflecting the ability of this protein to suppress the local immune response. Because *S. pyogenes* is specific for humans, in vivo studies are limited, but Chen et al. (52) reported that cell wall components of *S. pyogenes* induce the secretion of IL-10 and TNF-α by human PBMCs.

We show here that activation of human $CD4^+$ T cells with mAbs to CD3- and CD46-binding streptococcal M protein induces human Tr1-like cells in vitro. To use a more relevant model, we are in the process of establishing a human streptococcal Ag-specific APC/T cell system. Thus, to mimic Tr1 cell induction in vivo, we are replacing the anti-CD3 stimulation with autologous streptococcus-pulsed APCs. In an initial set of experiments, we could generate streptococcal Ag-specific T cells that produce IL-10 and display suppressive properties (C. Kemper, J. D. Price, D. F. Hoft, and J. P. Atkinson, unpublished data). This type of experimental system has been developed for bacillus Calmette-Guérin (53) and vaccinia. We will address questions regarding Ag specificity of Tr1-like cells, potential generation of a Tr1-like cell memory pool, and interactions of Tr1-like cells with other immunocompetent cell populations.

In summary, we present evidence that *S. pyogenes* M protein, a major bacterial virulence factor, interacts with CD46 on human $CD4^+$ T cells to induce directly IL-10-secreting/granzyme B-expressing T cells with a Tr1-like phenotype. Thus, in addition to its well-known ability to evade innate immunity by blocking phagocytosis (16, 25, 31), M protein may exploit the immunomodulatory properties of CD46 to create a local environment that delays the adaptive immune response and is permissive to the establishment of an infection. These findings focus interest on interactions between CD46 and human pathogens and suggest that further analysis of this interplay may provide novel information about multiple immunological phenomena.

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Disclosures

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References

1. Fehérvari, Z., and S. Sakaguchi, 2004. Development of $CD25^+CD4^+$ regulatory T cells. *Curr. Opin. Immunol.* 16: 203–208.
2. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253–257.
3. Jonuleit, H., and E. Schmitt. 2003. The regulatory T cell family: distinct subsets and their interrelations. *J. Immunol.* 171: 6323–6327.
4. Ramsdell, F. 2003. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19: 165–168.
5. Vieira, P. L., J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthlott, B. Stockinger, D. C. Wraith, and A. O'Garra. 2004. IL-10-secreting regulatory cells do not express Foxp3 but have comparable regulatory function to naturally occurring $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 172: 5986–5993.
6. Groux, H., A. O'Garra, M. Bigler, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
7. Riley-Vargas, R. C., D. B. Gill, C. Kemper, M. K. Liszewski, and J. P. Atkinson. 2004. CD46: expanding beyond complement regulation. *Trends Immunol.* 25: 496–503.
8. Wang, G., M. K. Liszewski, A. C. Chan, and J. P. Atkinson. 2000. Membrane cofactor protein (MCP; CD46): isoform-specific tyrosine phosphorylation. *J. Immunol.* 164: 1839–1846.
9. Zaffran, Y., O. Destaing, A. Roux, S. Ory, T. Nheu, P. Jurdic, C. Rabourdin-Combe, and A. L. Astier. 2001. CD46/CD3 costimulation induces morphological changes of human T cells and activation of Vav, Rac, and extracellular signal-regulated kinase mitogen-activated protein kinase. *J. Immunol.* 167: 6780–6785.
10. Astier, A. L., M.-C. Trescol-Biemont, O. Azocar, B. Lamouille, and C. Rabourdin-Combe. 2000. Cutting edge: CD46, a new costimulatory molecule for T cells, that induces $p120^{CBL}$ and LAT phosphorylation. *J. Immunol.* 164: 6091–6095.
11. Kemper, C., A. C. Chan, J. M. Green, K. A. Brett, K. M. Murphy, and J. P. Atkinson. 2003. Activation of human $CD4^+$ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 421: 388–392.
12. Grossman, W. L., J. W. Verbsky, B. L. Tollefsen, C. Kemper, J. P. Atkinson, and T. J. Ley. 2004. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* 104: 2840–2848.
13. Grossman, W. J., J. W. Verbsky, W. Barchet, M. Colonna, J. P. Atkinson and T. J. Ley. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21: 589–601.
14. Cattaneo, R. 2004. Four viruses, two bacteria, and one receptor: membrane cofactor protein (CD46) as pathogens' magnet. *J. Virol.* 78: 4385–4388.
15. Mitchell, T. J. 2003. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat. Rev. Microbiol.* 1: 219–230.
16. Fischetti, V. A. 1989. Streptococcal M protein: molecular design and biological behavior. *Clin. Microbiol. Rev.* 2: 285–314.
17. Okada, N., M. K. Liszewski, J. P. Atkinson, and M. Caparon. 1995. Membrane cofactor protein (MCP; CD46) is a keratinocyte receptor for the M protein of group A streptococcus. *Proc. Natl. Acad. Sci. USA* 92: 2489–2493.
18. Giannakis, E., T. S. Jokiranta, R. J. Ormsby, T. G. Duthy, D. A. Male, D. Christiansen, V. A. Fischetti, C. Bagley, B. E. Loveland, and D. L. Gordon. 2002. Identification of the streptococcal M protein binding site on membrane cofactor protein (CD46). *J. Immunol.* 168: 4585–4592.
19. McGuirk, P., C. McCann, and K. H. G. Mills. 2002. Pathogen-specific T-regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis. J. Exp. Med.* 195: 221–231.
20. Lavelle, E. C., E. McNeela, M. E. Armstrong, O. Leavy, S. C. Higgins, and K. H. G. Mills. 2003. Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. *J. Immunol.* 171: 2384–2392.
21. Mittrücker, H.-W., and S. H. E. Kaufmann. 2004. Regulatory T cells and infection: suppression revisited. *Eur. J. Immunol.* 34: 306–312.
22. Johnsson, E., K. Berggård, H. Kotarsky, J. Hellwage, P. Zipfel, U. Sjöbring, and G. Lindahl,. 1998. Role of the hypervariable region in streptococcal M proteins: binding of a human complement inhibitor. *J. Immunol.* 161: 4894–4901.
23. Pandiripally, V., E. Gregory, and D. Cue. 2002. Acquisition of regulators of complement activation by *Streptococcus pyogenes* serotype M1. *Infect. Immun.* 70: 6206–6214.
24. Stenberg, L., P. W. O'Toole, J. Mestecky, and G. Lindahl. 1994. Characterization of protein Sir, a streptococcal cell surface protein that binds both immunoglobulin A and immunoglobulin G. *J. Biol. Chem.* 269: 13458–13464.
25. Carlsson, F., C. Sandin, and G. Lindahl. 2005. Human fibrinogen bound to *Streptococcus pyogenes* M protein inhibits complement deposition via the classical pathway. *Mol. Microbiol.* 56: 28–39.
26. Stålhammar-Carlemalm, M., L. Stenberg, and G. Lindahl. 1993. Protein Rib: a novel group B streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections. *J. Exp. Med.* 177: 1593–1603.
27. Dombek, P. E., D. Cue, J. Sedgewick, H. Lam, S. Ruschkowski, B. B. Finlay, and P. P. Cleary. 1999. High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements. *Mol. Microbiol.* 31: 859–870.
28. Facklam, R. F., D. R. Martin, M. Lovgren, D. R. Johnson, A. Efstratiou, T. A. Thompson, S. Gowan, P. Kriz, G. J. Tyrrell, E. Kaplan, and B. Beall. 2002. Extension of the Lancefield classification for group A streptococci by addition of 22 new M protein gene sequence types from clinical isolates: emm103 to emm124. *Clin. Infect. Dis.* 34: 28–38.

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29. Kehoe, M. A. 1994. Cell-wall-associated proteins in Gram-positive bacteria. *New Comp. Biochem.* 27: 217–261.
30. Thern, A., L. Stenberg, B. Dahlbäck, and G. Lindahl. 1995. Ig-binding surface proteins of *Streptococcus pyogenes* also bind human C4b-binding protein (C4BP), a regulatory component of complement system. *J. Immunol.* 154: 375–386.
31. Carlsson, F., K. Berggard, M. Stålhammer-Carlemalm, and G. Lindahl. 2003. Evasion of phagocytosis through cooperation between two ligand-binding regions in *Streptococcus pyogenes* M protein. *J. Exp. Med.* 198: 1057–1068.
32. Johnson, D. R., D. L. Stevens, and E. L. Kaplan. 1992. Epidemiologic analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis. *J. Infect. Dis.* 166: 374–382.
33. McGuirk, P., and K. H. Mills. 2002. Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. *Trends Immunol.* 23: 450–455.
34. Mills, K. H. G. 2004. Regulatory T cells: friend or foe in immunity to infection? *Nat. Rev. Immunol.* 4: 841–855.
35. Redpath, S., P. Ghazal, and N. R. J. Gascoigne. 2001. Hijacking and exploitation of IL-10 by intracellular pathogens. *Trends Microbiol.* 9: 86–92.
36. Sing, A., D. Rost, N. Tvardovskaia, A. Roggenkamp, A. Wiedemann, C. J. Kirschning, M. Aepfelbacher, and J. Heeseman. 2002. Yersinia V-antigen exploits Toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J. Exp. Med.* 196: 1017–1024.
37. Diterich, I., C. Rauter, C. J. Kirschning, and T. Hartung. 2003. *Borrelia burgdorferi*-induced tolerance as a model of persistence via immunosuppression. *Infect. Immun.* 71: 3979–3987.
38. Mestas, J., and C. C. W. Hughes. 2004. Of mice and not men: differences between mouse and human immunology. *J. Immunol.* 172: 2731–2738.
39. Karp, C. L., M. Wysocka, L. M. Wahl, J. M. Ahearn, P. J. Cuomo, B. Sherry, G. Trinchieri, and D. Griffin. 1996. Mechanism of suppression of cell-mediated immunity by measles virus.[Published erratum appears in *Science 1997 275: 1053*.] *Science* 273: 228–231.
40. Barrat, F. J., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory $CD4^+$ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 195: 603–616.
41. Levings, M. K., R. Sangregorio, F. Galbiati, S. Squadrone, R. de Waal Malefyt, and M. G. Roncarolo. 2001. IFN-α and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* 166: 5530–5539.
42. Wakkach, A., N. Fournier, V. Brun, J. P. Breittmayer, F. Cottrez, and H. Groux. 2003. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18: 605–617.
43. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier and D. A. Horwitz. 2004. Natural and induced $CD4^+CD25^+$ cells educate $CD4^+CD25^-$cells to develop suppressive activity: the role of IL-2, TGF-β, and IL-10. *J. Immunol.* 172: 5213–5221.
44. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. $CD4^+CD25^+$ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 420: 502–507.
45. Weiner, H. L. 2001. The mucosal milieu creates tolerogenic dendritic cells and T_R1 and T_H3 regulatory cells. *Nat. Immunol.* 2: 671–672.
46. Rouse, B. T., and S. Suvas. 2004. Regulatory cells and infectious agents: detentes cordiale and contraire. *J. Immunol.* 173: 2211–2215.
47. Raghavan, S., M. Fredriksson, A.-M. Svennerholm, J. Holmgren, and E. Suri-Payer. 2003. Absence of $CD4^+CD25^+$ regulatory T cells is associated with a loss of regulation leading to increased pathology in *Helicobacter pylori*-infected mice. *Clin. Exp. Immunol.* 132: 393–400.
48. Sakaguchi, S. 2003. Regulatory T cells: mediating compromises between host and parasite. *Nat. Immunol.* 4: 10–11.
49. Park, H. S., K. P. Francis, J. Yu, and P. P. Cleary. 2003. Membranous cells in nasal-associated lymphoid tissue: a portal of entry for the respiratory mucosal pathogen group A streptococcus. *J. Immunol.* 171: 2532–2537.
50. Ashbaugh, C. D., T. J. Moser, M. H. Shearer, G. L. White, R. C. Kennedy, and M. R. Wessels. 2000. Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. *Cell. Microbiol.* 2: 283–292.
51. Lancefield, R. C. 1959. Persistence of type-specific antibodies in man following infection with group A streptococci. *J. Exp. Med.* 110: 271–292.
52. Chen, T., P. Isomaki, M. Rimpilainen, and P. Toivanen. 1999. Human cytokine responses induced by Gram-positive cell walls of normal intestinal microbiota. *Clin. Exp. Immunol.* 118: 261–267.
53. Worku, S., and D. F. Hoft. 2003. Differential effects of control and antigen-specific T cells on intracellular mycobacterial growth. *Infect. Immun.* 71: 1763–1773.

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