



Published in final edited form as:

J Infect Dis. 2010 June 15; 201(12): 1839–1848. doi:10.1086/652781.

The role of CD4⁺FOXP3⁺ regulatory T cells in suppression of host responses to *Haemophilus ducreyi* during experimental infection of human volunteers

Wei Li^{1,*}, Klara Tenner-Racz^{5,*}, Paul Racz⁵, Diane M. Janowicz¹, Kate R. Fortney¹, Barry P. Katz¹, and Stanley M. Spinola^{1,2,3,4}

¹Department of Medicine, Indiana University, School of Medicine, Indianapolis, IN 46202

²Department of Microbiology and Immunology, Indiana University, School of Medicine, Indianapolis, IN 46202

³Department of Pathology and Laboratory Medicine, Indiana University, School of Medicine, Indianapolis, IN 46202

⁴Center for Immunobiology, Indiana University, School of Medicine, Indianapolis, IN 46202

⁵Department of Pathology, Bernhard-Nocht-Institut für Tropenmedizin, Hamburg, Germany

Abstract

Haemophilus ducreyi causes chancroid, a genital ulcer disease. In human volunteers, the majority of experimentally infected individuals fail to clear the bacteria and form pustules. Here, we investigated the role of CD4⁺FOXP3⁺ regulatory T (Treg) cells in pustule formation. In pustules, there was a significant enrichment of CD4⁺FOXP3⁺ T cells compared to peripheral blood. The majority of the lesional FOXP3⁺ T cells were CD4⁺, CD25⁺, CD127^{lo/-} and CTLA-4⁺. FOXP3⁺ T cells were found throughout pustules but were most abundant at the base of the pustules. Significantly less lesional CD4⁺FOXP3⁺ T cells expressed IFN- γ than lesional CD4⁺FOXP3⁻ effector T cells. Depletion of CD25⁺ CD4⁺ T cells from the peripheral blood of infected and uninfected volunteers significantly enhanced proliferation of *H. ducreyi*-reactive CD4⁺ T cells. Our results indicate that CD4⁺CD25⁺CD127^{lo/-}FOXP3⁺ Treg cells are expanded at *H. ducreyi*-infected sites and may play a role in suppressing the host immune response to the bacterium.

Haemophilus ducreyi is the causative agent of the sexually transmitted disease chancroid, a genital ulcer disease that facilitates the acquisition and transmission of HIV-1 [1]. In order to understand the immunopathogenesis of *H. ducreyi* infection, we developed a human challenge model, in which the skin of the upper arm of healthy adult volunteers is inoculated with the *H. ducreyi* strain 35000HP (HP, human passaged) or its derivatives [2]. After inoculation, papules form within 24 hours and either spontaneously resolve or evolve into pustules within 2 to 5 days.

Experimental pustules and natural ulcers are identical histologically and signify immunological failure. Both innate and adaptive immune cells including neutrophils, macrophages, myeloid

Reprints or correspondence: Wei Li, Ph: 317-278-4691, FAX: 317-274-1008, wl1@iupui.edu.

*Equal contribution

Potential conflicts of interest: no conflicts.

Presented in part:

The 10th International Symposium on *Haemophilus ducreyi* Pathogenesis and Chancroid, London, UK, June 28, 2009.

dendritic cells (DC), NK cells and memory/effector T cells are recruited to experimental pustules and natural ulcers [3–5]. In pustules, neutrophils coalesce to form an epidermal abscess, and macrophages form a collar at the base of the abscess. Below the collar, there is a dermal infiltrate of T cells, NK cells and macrophages. Despite this response, *H. ducreyi* replicates and persists at infected sites. In pustules and natural ulcers, the bacterium colocalizes with neutrophils and macrophages and remains extracellular [6,7]. Thus, evasion of phagocytosis is a major mechanism of bacterial survival in experimental and natural infection.

Regulatory T (Treg) cells actively suppress the function of the adaptive and innate immune systems [8]. Treg cells are essential for maintaining self-tolerance and immune homeostasis. Treg cells use cell-contact inhibition or soluble factors to control collateral tissue damage mediated by immune responses. However, they also prevent sterilizing antimicrobial immunity and promote pathogen persistence during infection.

Two major types of Treg cells have been described based on their origin of generation [9]. Naturally occurring Treg (nTreg) cells are generated in the thymus and express CD25⁺ and the transcription factor forkhead box P3 (FOXP3), which is critical for their development and function. Treg cells can also be converted from mature CD4⁺CD25⁻ T cells in peripheral tissues under immunosuppressive conditions, such as exposure to IL-10, transforming growth factor- β (TGF- β) or indoleamine 2,3-dioxygenase (IDO) made by APC [10–14]. These inducible Treg (iTreg) cells are classified as IL-10-producing Tr1 cells, TGF- β -producing Th3 cells and inducible FOXP3⁺ Treg cells. After infection with pathogens, Treg cells accumulate at infected sites through recruitment, retention, proliferation and/or conversion [15].

Clinical and laboratory data suggest that Treg cells may be involved in the formation of *H. ducreyi*-induced pustules. Approximately 16% of volunteers develop hypertrophic scars at *H. ducreyi*-infected sites that are biopsied [2]. Hypertrophic scar formation is associated with production of TGF- β , which promotes the development of Treg cells. Upon exposure to *H. ducreyi*, monocyte-derived DC from pustule formers upregulate transcripts of markers that foster Treg development [16]. Indeed, some *H. ducreyi*-specific T cell lines and clones isolated from pustules have characteristics of Treg cells in that they produce IL-10 and IFN- γ [17].

Here, we tested the hypothesis that Treg cells accumulate in *H. ducreyi*-infected pustules. We found that CD4⁺FOXP3⁺ T cells were enriched in experimental pustules and were mainly located at the base of pustules. Most of the CD4⁺FOXP3⁺ T cells expressed phenotypic markers characteristic of circulating CD4⁺FOXP3⁺ Treg cells. We also showed that the CD4⁺FOXP3⁺ T cells were unable to produce effector cytokines and that depletion of CD4⁺CD25⁺ T cells from PBMCs of persons who formed pustules and uninfected donors increased proliferative response of CD4 T cells to *H. ducreyi*.

MATERIALS AND METHODS

Human inoculation experiments

H. ducreyi strain 35000HP and its isogenic mutants were grown on chocolate agar plates and GC medium broth as described [2,18]. Preparation of *H. ducreyi*, inoculation procedures, and enrollment and exclusion criteria for the human challenge experiments were reported previously [18]. Informed consent was obtained from the volunteers in compliance with the human experimental guidelines of the US Department of Health and Human Services and the Institutional Review Board of Indiana University-Purdue University at Indianapolis.

Flow cytometry—Twenty-six healthy adult volunteers, who were infected with *H. ducreyi* for a mean \pm SD of 7.2 ± 1.6 days, contributed biopsies of pustules for flow cytometry. Biopsies were obtained from 9 subjects who were infected with only 35000HP and from 17 volunteers

who participated in 5 mutant-parent comparison trials (Table 1). Since the immunopathology caused by mutant strains that form pustules is indistinguishable from that caused by 35000HP [3], we analyzed pustules from both parent- and mutant-infected sites.

Paired samples of tissue and peripheral blood were obtained on the day of biopsy from each subject. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS gradient centrifugation. Single cell suspensions were obtained from the biopsies as described [4].

Cells were stained with the following antibodies obtained from BD Biosciences, unless indicated otherwise: allophycocyanin (APC) conjugated antibodies to CD25 (clone M-A251), IFN- γ (clone 4S.B3; eBioscience, CA) and FOXP3 (clone 236A/E7; eBioscience); isothiocyanate (FITC) conjugated antibodies to CD25 (clone M-A251) and CD4 (clone RPA-T4); phycoerythrin (PE) conjugated antibodies to CD127 (clone HIL-7R-M21), CTLA-4 (clone BNI3) and FOXP3 (clone 259D/C7), peridinin chlorophyll protein (PerCP) conjugated antibodies to CD4 (clone SK3) and CD8 (SK1); Alex-647-conjugated antibody to FOXP3 (clone 259D/C7). Cells stained with antibodies to surface markers were washed, fixed, permeabilized, and stained with anti-FOXP3, anti-CTLA-4, and anti-IFN- γ antibodies using the anti-human FOXP3 Fix/Perm Buffer Set according to the manufacturer's recommendations (BioLegend, CA). To measure intracellular production of the cytokine IFN- γ , biopsy cells and PBMCs were stimulated with 1 μ g/ml ionomycin and 2 ng/ml phorbol 12-myristate 13-acetate (PMA) for 6 hours in the presence of 3 μ M monensin prior to antibody staining.

Cells were analyzed using a FACSCalibur flow cytometer and the BD CellQuest Pro version 4.0.1 software (BD Biosciences). To calculate the percentages of CD4⁺FOXP3⁺ T cells, the lymphocyte gate was set based on forward and side scatter characteristics of PBMCs, followed by gating on CD4⁺ cells. CD4⁺FOXP3⁺ T cells were then analyzed for the expression of the surface markers CD25 and CD127 and intracellular CTLA-4 and IFN- γ .

Proliferation assays—Total and CD25-depleted (CD25⁻) CD4 T cells were isolated from PBMCs obtained from infected volunteers at the time of biopsy or from leukopacks derived from anonymous uninfected donors (the Central Indians Regional Blood Center) by using the CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec). Generation of monocyte-derived DC has been described previously [4]. Purified T cells were labeled with 5 μ M carboxyfluorescein diacetate succinimidyle ester (CFSE) (Invitrogen, CA) in PBS for 10 minutes at room temperature. Approximately 1–2 \times 10⁵ total or CD25⁻ CD4 T cells were stimulated with 1–2 \times 10⁴ DC cells pulsed with heat-killed *H. ducreyi* (MOI=20:1) and 5 μ g/ml phytohemagglutinin (PHA). After 5 days of incubation, the cells were harvested and T cell proliferation was measured by CFSE dilution.

Immunohistochemical analysis—Archived pustules were obtained from seven asymptomatic HIV-seropositive volunteers whose CD4 T cell counts were >350 cells/ μ L and 14-seronegative volunteers (Table 2). The biopsies were fixed in 4% paraformaldehyde (pH 7) and embedded in paraffin. Dewaxing, antigen retrieval, and staining of tissue sections were described previously [19]. Briefly, treated sections were incubated with antibody to CD4 (clone: NCL-CD4-IF6, Novocastra, Newcastle upon Tyne, UK), CD25 (clone NCL-CD25-305, Novocastra), or CD8 (clone: CB/144B, DakoCytomation, Denmark) overnight. Binding of antibody was detected with the StreptABCComplex/HRP (Code K0391, DakoCytomation, Denmark) using 3-amino-9-ethylcarbazole (Sigma-Aldrich, MO) as the substrate. The sections were then heat treated with sodium citrate solution and incubated with anti-FOXP3 antibody (clone 236a/E7, Cambridge, UK) overnight at 4°C. This second antibody was visualized with the alkaline phosphatase anti-alkaline phosphatase (APAAP) method using Fast Blue salt.

Semiquantitative analysis of FOXP3⁺ cells in tissue sections—Analysis of tissue sections was done with a Zeiss AxioImager M1 microscope equipped with an AxioCam MRc5 digital camera (Carl Zeiss, Germany). To estimate the numbers of FOXP3⁺ cells, a unit area of 160 × 215 μm was set using a 40× objective. In areas with inflammatory infiltrates, 10 non-overlapping areas of the dermis were selected and photomicrographs were taken. Positive cells within these unit areas were determined by using the AxioVision Rel 4.5 software (Carl Zeiss). The values were then pooled and the mean number of FOXP3⁺ cells per unit area per specimen was calculated.

Statistical analysis—Paired t-tests were used for within subject comparisons and Student's t-tests were used to analyze data from independent groups of subjects. We report the nominal P value for each comparison. A P-value that is equal to or smaller than 0.05 was considered statistically significant.

RESULTS

Enrichment of CD4⁺FOXP3⁺ T cells in *H. ducreyi*-infected skin

To examine whether Treg cells were recruited to experimental pustules, we used the expression of FOXP3 as a marker for Treg cells. Fifteen paired samples of infected sites and peripheral blood were analyzed by flow cytometry. Almost all the FOXP3⁺ cells in both peripheral blood and lesions were CD4 T cells (Figure 1A). Few FOXP3⁺ CD8 T cells were detected in either PBMCs or the biopsies (Figure 1A). As shown in Figure 1B, there was a significant enhancement in the percentage of CD4⁺FOXP3⁺ T cells recruited to lesions compared to peripheral blood.

Phenotypic characterization of CD4⁺FOXP3⁺ T cells in pustules—Expression of FOXP3 alone may not be sufficient to confer suppressive function to human T cells. Therefore, we examined the expression of phenotypic and suppressive markers associated with Treg function on CD4⁺FOXP3⁺ T cells in *H. ducreyi*-infected tissues and peripheral blood. Since Treg cells constitutively express the high affinity IL-2 receptor α chain CD25, CD4⁺FOXP3⁺ T cells from 15 pairs of PBMCs and pustule samples were analyzed for the expression of CD25. A representative result is shown in Figure 2A. The majority of CD4⁺FOXP3⁺ T cells in both PBMCs and tissue expressed CD25 (Figure 2A). In contrast, the majority of CD4⁺FOXP3⁻ effector T cells did not express CD25 (Figure 2A), and the differences in CD25 expression between CD4⁺FOXP3⁺ and CD4⁺FOXP3⁻ T cells were significant (Figure 2A).

Human Treg cells can generally be distinguished from effector T cells on the basis of low surface expression of CD127 (IL-7R α) [20,21]. CD4⁺FOXP3⁺ T cells from 4 paired samples of PBMCs and pustules were assessed for CD127 expression. A representative result is shown in Figure 2B. The majority of peripheral blood and lesional CD4⁺FOXP3⁺ T cells were CD127^{lo/-} (Figure 2B). The percentages of the peripheral blood and tissue CD127^{lo/-} CD4⁺FOXP3⁺ T cells were significantly higher than those of the CD4⁺FOXP3⁻ cells (Figure 2B). The percentage of CD127^{lo/-} CD4⁺FOXP3⁻ T cells was significantly higher in pustules than that in PBMCs (Figure 2B, right panel), consistent with our previous studies showing that CD4 memory/effector cells are enriched in pustules [22].

CTLA-4 is a marker associated with and required for regulatory T cell function [23]; ectopic expression of CTLA-4 is sufficient to confer regulatory function to CD4⁺CD25⁻ effector human T cells [24]. CD4⁺FOXP3⁺ T cells from five paired samples of PBMCs and pustules were assessed for CTLA-4 expression. A representative result is shown in Figure 2C. CD4⁺FOXP3⁺ T cells from both peripheral blood and biopsies expressed CTLA-4 at significantly higher percentages than their CD4⁺FOXP3⁻ T cell counterparts (Figure 2C).

CTLA-4 expression was significantly higher in the CD4⁺FOXP3⁺ T cells from pustules than peripheral blood (Figure 2C).

FOXP3⁺ cells accumulate at the base of pustules—To visualize the location of FOXP3⁺ cells in pustules, tissue sections were stained with an anti-FOXP3 antibody. Consistent with results from FACS analysis, the vast majority of the FOXP3⁺ cells co-stained with anti-CD4 and anti-CD25 (Figure 3A and 3B). There were no detectable CD8 and FOXP3 double positive cells (Figure 3C). FOXP3⁺ cells were present in the dermal infiltrates and the epidermis but were mostly found near the bottom of and in the wall of pustules and were rare in the upper region of the abscess (Figure 3D). Thus, a CD4⁺ T cell population carrying typical Treg markers is enriched at the base of *H. ducreyi*-induced pustules.

CD4⁺FOXP3⁺ T cells in pustules have limited capacity to make IFN- γ —An intrinsic characteristic of Treg cells is their relative inability to express effector cytokines, such as IFN- γ . Therefore, we assayed the CD4⁺FOXP3⁺ T cells for their ability to produce IFN- γ . PBMCs and lesional cells were stimulated with PMA and ionomycin in the presence of monensin. In contrast to the CD4⁺FOXP3⁻ effector T cells, which produced IFN- γ , a significantly lower percentage of CD4⁺FOXP3⁺ T cells made IFN- γ (Figure 4). Thus, similar to the circulating CD4⁺FOXP3⁺ Treg cells, most CD4⁺FOXP3⁺ T cells at *H. ducreyi*-infected sites were functionally anergic and likely represented Treg cells, not activated effector T cells.

Effects of depletion of CD4⁺CD25⁺ Treg cells on proliferative response to *H. ducreyi*—Due to the limited number of recoverable CD4 T cells from pustules, it was not possible to measure whether lesional Treg cells could down modulate T cell responses to *H. ducreyi*. Therefore, we used PBMCs, obtained at the time of biopsy from 8 subjects who formed pustules, as surrogates for lesional T cells. Treg suppressive activity was measured by comparing the proliferative responses to *H. ducreyi* antigens of total CD4 T cells and CD4 T cells depleted of CD25⁺ cells. As shown in Figure 5, the depletion of CD25⁺ cells did not significantly enhance CD4 T cell proliferation in response to PHA ($P = 0.69$). However, depletion of CD25⁺ cells led to a trend of enhanced autoreactivity in the media controls ($P = 0.052$) and significantly higher proliferation of CD4 T cells in response to heat-killed *H. ducreyi* ($P = 0.02$), suggesting that the CD4⁺CD25⁺ population contained cells that inhibit proliferation to autoantigens and *H. ducreyi*. The depletion of CD25⁺ cells also significantly enhanced proliferation of CD4 T cells from uninfected donors to *H. ducreyi* ($P = 0.03$) (Figure 5C). Taken together, these data suggest that Treg cells may inhibit CD4 responses to specific as well as cross-reactive epitopes of *H. ducreyi*.

Hypertrophic scar formation is not associated with accumulation of FOXP3⁺ cells in lesions—Hypertrophic scar formation is associated with TGF- β production. Since TGF- β can induce the generation of FOXP3⁺ iTreg cells, we investigated whether there were more FOXP3⁺ Treg cells in pustules obtained from 7 volunteers who formed hypertrophic scars compared to 7 volunteers who did not. Tissue sections of pustules were stained with anti-FOXP3 antibody and scored for the distribution of FOXP3⁺ cells. There was no significant difference in the mean number (\pm SD) of FOXP3⁺ cells between subjects who formed hypertrophic scars and those who did not (8.2 ± 4.5 and 7.8 ± 3.3 ; $P = 0.86$).

Effect of HIV infection on accumulation of FOXP3⁺ cells at *H. ducreyi*-infected skin—Cells expressing the Treg markers FOXP3, CD25 and CTLA-4 are enriched in lymphoid and mucosal tissues in HIV infected patients [25–27]. We investigated whether Treg cell accumulation in *H. ducreyi* infected skin was affected by HIV infection. Tissue sections of pustules from 7 HIV-seropositive volunteers, one of whom formed a hypertrophic scar, were stained with anti-FOXP3. Since hypertrophic scar formation did not have an effect on

accumulation of FOXP3⁺ cells, the number of FOXP3⁺ cells in the specimens obtained from the 7 HIV-seropositive volunteers was compared to that obtained from all 14 HIV-seronegative volunteers. There was no significant difference in the mean number (\pm SD) of FOXP3⁺ cells between the biopsy samples from the HIV-seropositive and HIV-seronegative subjects (10.5 ± 2.2 and 7.9 ± 3.6 ; $P = 0.099$).

DISCUSSION

We found that CD4⁺FOXP3⁺ T cells expressing typical markers of Treg cells were enriched in *H. ducreyi*-induced pustules compared to peripheral blood. Compared to lesional FOXP3⁻ effector CD4 T cells, lesional FOXP3⁺ CD4 T cells had a reduced capacity to produce the effector cytokine IFN- γ , which is a characteristic of Treg cells. Although FOXP3 is a hallmark for murine Treg cells, its role in distinguishing human Treg cells from in vitro-activated effector T cells is controversial. Recent reports on accumulation of CD4⁺FOXP3⁺ T cells in human tissues from tumors and autoimmune diseases indicate that FOXP3 expression serves as an accurate indicator of functional Treg cells [28,29].

The gold standard to confirm the identity of Treg cells is to test the suppressive function of the CD4⁺FOXP3⁺ T cells in lesions. Due to limited cell recovery of CD4⁺CD25⁺ T cells from biopsies, we used PMBC from infected and uninfected volunteers and showed that depletion of CD4⁺CD25⁺ T cells significantly enhanced *H. ducreyi*-stimulated proliferation of CD4 T cells. However, the degree of enhancement of proliferation was modest. Stimulation of human CD4⁺CD25⁻ T cells also induces FOXP3⁺ Treg cell development [30], and induction of Treg cells might have minimized the apparent enhancement of T cell proliferation to *H. ducreyi*. Previously, we were not able to detect *H. ducreyi*-specific proliferative responses in PBMCs obtained from volunteers who were infected twice [16,31]. We speculate that the lack of proliferative responses may have been due to the suppressive activity of circulating Treg cells. Taken together, our data suggest that CD4⁺FOXP3⁺ T cells in *H. ducreyi*-infected skin likely represent bona fide suppressive Treg cells and may contribute to the persistence of the bacterial infection by dampening anti-*H. ducreyi* immunity, either to specific or cross-reactive epitopes.

Due to the lack of definitive biomarkers that can distinguish between nTreg and iTreg cells, the origin of the CD4⁺FOXP3⁺ T cells in pustules is unknown. However, multiple mechanisms may account for the enrichment of the CD4⁺FOXP3⁺ T cells in *H. ducreyi*-infected skin. CD4⁺FOXP3⁺ nTreg are recruited from blood through the chemokine receptors CCR4, CCR7, CCR8, CXCR4 and CXCR5, and skin homing receptor CLA [32–34]. The CCR7 ligand, CCL19, is upregulated in *H. ducreyi* lesions and most skin-infiltrating lesional lymphocytes express both CCR4 and CLA [16,35]. Bacterial antigens, cytokines and other factors at infected sites could convert CD4⁺CD25⁻ CD4 T cells to iTreg cells. DC derived from pustule formers and infected with *H. ducreyi* express high levels of IDO transcripts [16]. IDO-expressing human DC induce the conversion of CD4⁺CD25⁻ T cells to CTLA-4- and FOXP3-expressing CD4⁺CD25⁺ Treg cells [10–13]. Therefore, the lesional CD4⁺FOXP3⁺ T cells could be composed of both nTreg and iTreg cells.

The CD4⁺FOXP3⁺ Treg cells were found mainly at the base of pustules. The position of the FOXP3⁺ cells at the base of the pustule suggests that they are likely located within a macrophage collar demonstrated by confocal microscopy [6]. In experimental lesions, the majority of effector/memory T cells lie below the macrophage collar in dermis [6]. A similar spatial arrangement of neutrophils, macrophages and lymphocytes is observed in suppurative granulomas induced by the intracellular bacterium *Listeria monocytogenes* [36]. In those granulomas, IDO-expressing DC and macrophages constitute a major part of the ring-wall structure that surrounds the neutrophils. IDO inhibits T cell activation/proliferation and promotes T cell death through tryptophan depletion and the production of pro-apoptotic

metabolites from the catabolism of tryptophan, preventing T cells from gaining access to the abscess. As stated above, IDO also promotes Treg cell expansion and iTreg development. Thus, IDO expression by the macrophage collar might prevent the effector/memory T cells from migrating into pustules, but allow Treg cells to accumulate at the wall of pustules. Unfortunately, efforts to confirm the colocalization of IDO-expressing macrophages with CD4⁺FOXP3⁺ Treg cells were technically unsuccessful due to high background staining in the tissue.

There were no significant differences in the abundance of FOXP3⁺ cells between the biopsy samples from the HIV-seropositive and HIV-seronegative subjects. However, we previously reported the recruitment of CD4 T cells to *H. ducreyi*-infected skin of HIV-seropositive subjects is significantly lower than that of HIV-seronegative volunteers [19]. Thus, the proportion of FOXP3⁺ cells in the CD4 population in pustules of HIV-seropositive volunteers is likely higher than that of HIV-seronegative volunteers. This presumed enrichment might be the result of the survival and/or proliferative advantages of Treg cells over effector T cells during HIV infection [26,37,38]

In the human challenge model, some subjects infected with *H. ducreyi* repeatedly clear the organism, while others repeatedly form pustules [39]. Although it is not known how infection is resolved, it is likely the bacteria are ingested and killed by phagocytes. In pustule formers, *H. ducreyi* evades phagocytosis and persists extracellularly. Differences in Treg activity may influence phagocytosis and confer differential host susceptibility to *H. ducreyi* infection. We previously reported that DC from resolvers and pustule formers have distinct transcriptional profiles in response to *H. ducreyi* infection. DC from resolvers have a transcript response that should promote Th1 and Th17 differentiation of CD4 T cells, while DC from pustule formers upregulate transcripts of markers that should foster Th1 and Treg responses. In pustule formers, immune suppression mediated by enhanced Treg activity might decrease the production of the T cell and NK cell effector cytokine IFN- γ and dampen the phagocytosis and bactericidal activity of neutrophils and macrophages. Similarly, in human experimental *Plasmodium falciparum* infection, an early burst of TGF- β production by monocytes correlates with the upregulation of Treg cells and differential host susceptibility to malaria [40]. We are currently determining whether DC and macrophages from resolvers and pustule formers respond differently to *H. ducreyi* infection in their production of TGF- β and other Treg-promoting factors and whether there is a preferential accumulation of Treg cells at sites infected with *H. ducreyi* in pustule formers as compared to the resolvers.

Acknowledgments

We thank Petra Meyer and Gudrun Großschupff for their excellent immunohistochemical assistance, Dr. Byron E. Batteiger for his critical review of the manuscript, Sheila Ellinger for preparation of regulatory documents, and the volunteers for their participation in these studies.

Financial Support: This work was supported by grants AI27863, AI31494, and AI059384 from the National Institute of Allergy and Infectious diseases, a grant from German Ministry of Education and Research, and FKZ 01KI0501 from Competence Network for HIV/AIDS. The human challenge trials were supported by grant UL RR025761 to the Indiana CTSI and the Indiana Clinical Research Center.

REFERENCES

1. Spinola, SM. Chancroid and *Haemophilus ducreyi*. In: Holmes, KK.; Sparling, PF.; Stamm, WE., et al., editors. Sexually Transmitted Diseases. 4th ed.. New York: McGraw-Hill; 2008. p. 689-699.
2. Janowicz DM, Ofner S, Katz BP, Spinola SM. Experimental infection of human volunteers with *Haemophilus ducreyi*: 15 years of clinical data and experience. *J Infect Dis* 2009;199:1671–1679. [PubMed: 19432549]

3. Spinola SM, Bauer ME, Munson RS Jr. Immunopathogenesis of *Haemophilus ducreyi* infection (chancroid). *Infect. Immun* 2002;70:1667–1676. [PubMed: 11895928]
4. Banks KE, Humphreys T, Li W, Katz BP, Wilkes DS, Spinola SM. *Haemophilus ducreyi* partially activates human myeloid dendritic cells. *Infect. Immun* 2007;75:5678–5685. [PubMed: 17923525]
5. Li W, Janowicz DM, Fortney KR, Katz BP, Spinola SM. Mechanism of human natural killer cell activation by *Haemophilus ducreyi*. *J Infect Dis* 2009;200:590–598. [PubMed: 19572804]
6. Bauer ME, Goheen MP, Townsend CA, Spinola SM. *Haemophilus ducreyi* associates with phagocytes, collagen, and fibrin and remains extracellular throughout infection of human volunteers. *Infect. Immun* 2001;69:2549–2557. [PubMed: 11254619]
7. Bauer ME, Townsend CA, Ronald AR, Spinola SM. Localization of *Haemophilus ducreyi* in naturally acquired chancroidal ulcers. *Microbe. Infect* 2006;8:2465–2468.
8. Shevach EM. Mechanisms of foxp3⁺ T regulatory cell-mediated suppression. *Immunity* 2009;30:636–645. [PubMed: 19464986]
9. Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3⁺ regulatory T cells: more of the same or a division of labor? *Immunity* 2009;30:626–635. [PubMed: 19464985]
10. Hill M, Tanguy-Royer S, Royer P, et al. IDO expands human CD4⁺CD25^{high} regulatory T cells by promoting maturation of LPS-treated dendritic cells. *Eur J Immunol* 2007;37:3054–3062. [PubMed: 17948274]
11. Chung DJ, Rossi M, Romano E, et al. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. *Blood* 2009;114:555–563. [PubMed: 19465693]
12. Manches O, Munn D, Fallahi A, et al. HIV-activated human plasmacytoid DCs induce Tregs through an indoleamine 2,3-dioxygenase-dependent mechanism. *J Clin Invest* 2008;118:3431–3439. [PubMed: 18776940]
13. Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. *J Immunol* 2008;181:5396–5404. [PubMed: 18832696]
14. Belkaid Y, Oldenhove G. Tuning microenvironments: induction of regulatory T cells by dendritic cells. *Immunity* 2008;29:362–371. [PubMed: 18799144]
15. Belkaid Y, Tarbell K. Regulatory T cells in the control of host-microorganism interactions (*). *Annu Rev Immunol* 2009;27:551–589. [PubMed: 19302048]
16. Humphreys T, Li L, Li X, et al. Dysregulated immune profiles for skin and dendritic cells are associated with increased host susceptibility to *Haemophilus ducreyi* infection in human volunteers. *Infect. Immun* 2007;75:5686–5697. [PubMed: 17893130]
17. Gelfanova V, Humphreys TL, Spinola SM. Characterization of *Haemophilus ducreyi*-specific T cell lines from lesions of experimentally infected human subjects. *Infect. Immun* 2001;69:4224–4231. [PubMed: 11401958]
18. Banks KE, Fortney KR, Baker B, et al. The enterobacterial common antigen-like gene cluster of *Haemophilus ducreyi* contributes to virulence in humans. *J. Infect. Dis* 2008;197:1531–1536. [PubMed: 18422457]
19. Janowicz DM, Tenner-Racz K, Racz P, et al. Experimental Infection with *Haemophilus ducreyi* in Persons Who Are Infected with HIV Does Not Cause Local or Augment Systemic Viral Replication. *J Infect Dis* 2007;195:1443–1451. [PubMed: 17436224]
20. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med* 2006;203:1701–1711. [PubMed: 16818678]
21. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006;203:1693–1700. [PubMed: 16818676]
22. Humphreys TL, Baldrige LA, Billings SD, Campbell JJ, Spinola SM. Trafficking pathways and characterization of CD4 and CD8 cells recruited to the skin of humans experimentally infected with *Haemophilus ducreyi*. *Infect. Immun* 2005;73:3896–3902. [PubMed: 15972475]
23. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3⁺ regulatory T cell function. *Science* 2008;322:271–275. [PubMed: 18845758]

24. Zheng Y, Manzotti CN, Burke F, et al. Acquisition of suppressive function by activated human CD4⁺CD25⁻ T cells is associated with the expression of CTLA-4 not FoxP3¹. *J Immunol* 2008;181:1683–1691. [PubMed: 18641304]
25. Andersson J, Boasso A, Nilsson J, et al. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *Journal of Immunology* 2005;174:3143–3147.
26. Ji J, Cloyd MW. HIV-1 binding to CD4 on CD4⁺CD25⁺ regulatory T cells enhances their suppressive function and induces them to home to, and accumulate in, peripheral and mucosal lymphoid tissues: an additional mechanism of immunosuppression. *Int Immunol* 2009;21:283–294. [PubMed: 19208751]
27. Epple H-J, Loddenkemper C, Kunkel D, et al. Mucosal but not peripheral FOXP3⁺ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 2006;108:3072–3078. [PubMed: 16728694]
28. Ahmadzadeh M, Felipe-Silva A, Heemskerk B, et al. FOXP3 expression accurately defines the population of intratumoral regulatory T cells that selectively accumulate in metastatic melanoma lesions. *Blood* 2008;112:4953–4960. [PubMed: 18820132]
29. Kryczek I, Liu R, Wang G, et al. FOXP3 defines regulatory T cells in human tumor and autoimmune disease. *Cancer Res* 2009;69:3995–4000. [PubMed: 19383912]
30. Walker MR, Kasprovicz DJ, Gersuk VH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁻ T cells. *J. Clin. Invest* 2003;112:1437–1443. [PubMed: 14597769]
31. Al-Tawfiq JA, Palmer KL, Chen C-Y, et al. Experimental infection of human volunteers with *Haemophilus ducreyi* does not confer protection against subsequent challenge. *J. Infect. Dis* 1999;179:1283–1287. [PubMed: 10191238]
32. Zou L, Barnett B, Safah H, et al. Bone marrow is a reservoir for CD4⁺CD25⁺ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res* 2004;64:8451–8455. [PubMed: 15548717]
33. Iellem A, Mariani M, Lang R, et al. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4⁽⁺⁾CD25⁽⁺⁾ regulatory T cells. *J Exp Med* 2001;194:847–853. [PubMed: 11560999]
34. Hirahara K, Liu L, Clark RA, Yamanaka K, Fuhlbrigge RC, Kupper TS. The majority of human peripheral blood CD4⁺CD25^{high}Foxp3⁺ regulatory T cells bear functional skin-homing receptors. *J Immunol* 2006;177:4488–4494. [PubMed: 16982885]
35. Soler D, Humphreys TL, Spinola SM, Campbell JJ. CCR4 versus CCR10 in human cutaneous Th lymphocyte trafficking. *Blood* 2003;101:1677–1683. [PubMed: 12406880]
36. Popov A, Abdullah Z, Wickenhauser C, et al. Indoleamine 2,3-dioxygenase-expressing dendritic cells form suppurative granulomas following *Listeria monocytogenes* infection. *J Clin Invest* 2006;116:3160–3170. [PubMed: 17111046]
37. Nilsson J, Boasso A, Velilla PA, et al. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* 2006;108:3808–3817. [PubMed: 16902147]
38. Ahmadzadeh M, Johnson LA, Heemskerk B, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood*. 2009
39. Spinola SM, Bong CTH, Faber AL, et al. Differences in host susceptibility to disease progression in the human challenge model of *Haemophilus ducreyi* infection. *Infect. Immun* 2003;71:6658–6663. [PubMed: 14573692]
40. Walther M, Tongren JE, Andrews L, et al. Upregulation of TGF- β , *FOXP3*, and CD4⁺CD25⁺ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 2005;23:287–296. [PubMed: 16169501]
41. Bauer ME, Townsend CA, Doster RS, et al. A fibrinogen-binding lipoprotein contributes to virulence of *Haemophilus ducreyi* in humans. *J Infect Dis* 2009;199:684–692. [PubMed: 19199547]
42. Fortney KR, Young RS, Bauer ME, et al. Expression of peptidoglycan-associated lipoprotein is required for virulence in the human model of *Haemophilus ducreyi* infection. *Infect. Immun* 2000;68:6441–6448. [PubMed: 11035757]

43. Bong CTH, Throm RE, Fortney KR, et al. A DsrA-deficient mutant of *Haemophilus ducreyi* is impaired in its ability to infect human volunteers. *Infect. Immun* 2001;69:1488–1491. [PubMed: 11179317]
44. Young RS, Filiatrault MJ, Fortney KR, et al. *Haemophilus ducreyi* lipooligosaccharide mutant defective in expression of B1,4-glucosyltransferase is virulent in humans. *Infect. Immun* 2001;69:4810–4814.
45. Bong CTH, Fortney KR, Katz BP, et al. A superoxide dismutase C mutant of *Haemophilus ducreyi* is virulent in human volunteers. *Infect. Immun* 2002;70:1367–1371. [PubMed: 11854222]
46. Janowicz D, Luke NR, Fortney KR, Katz BP, Campagnari AA, Spinola SM. Expression of OmpP2A and OmpP2B is not required for pustule formation by *Haemophilus ducreyi* in human volunteers. *Microb. Path* 2006;40:110–115.
47. Janowicz D, Leduc I, Fortney KR, Katz BP, Elkins C, Spinola SM. A DltA mutant of *Haemophilus ducreyi* is partially attenuated in its ability to cause pustules in human volunteers. *Infect. Immun* 2006;74:1394–1397. [PubMed: 16428791]
48. Young RS, Fortney K, Haley JC, et al. Expression of sialylated or paragloboside-like lipooligosaccharides are not required for pustule formation by *Haemophilus ducreyi* in human volunteers. *Infect. Immun* 1999;67:6335–6340. [PubMed: 10569746]
49. Al-Tawfiq JA, Bauer ME, Fortney KR, et al. A pilus-deficient mutant of *Haemophilus ducreyi* is virulent in the human model of experimental infection. *J. Infect. Dis* 2000;181:1176–1179. [PubMed: 10720550]
50. Young RS, Fortney KR, Gelfanova V, et al. Expression of cytolethal distending toxin and hemolysin are not required for pustule formation by *Haemophilus ducreyi* in human volunteers. *Infect. Immun* 2001;69:1938–1942. [PubMed: 11179379]

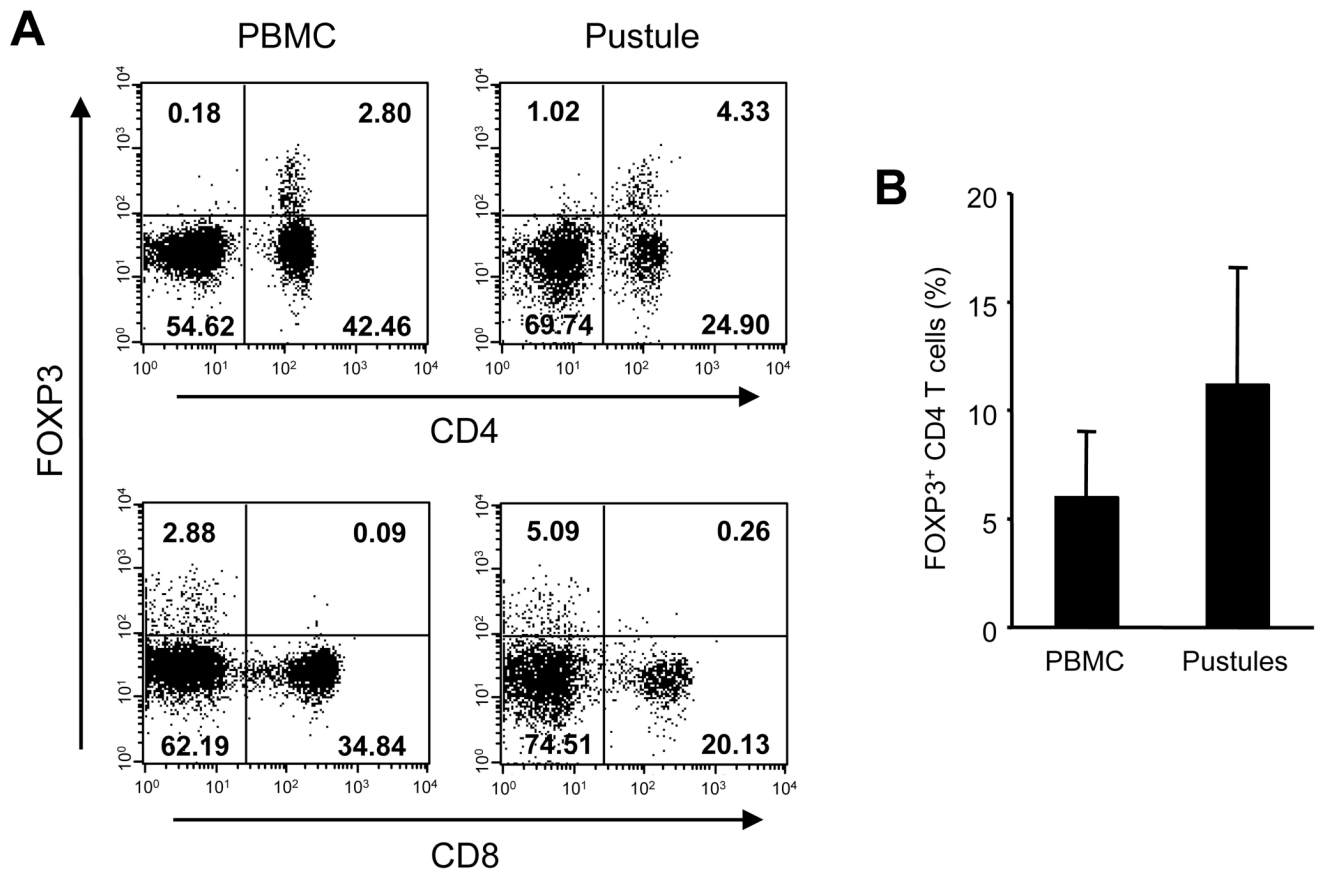


Figure 1. Characterization of CD4⁺FOXP3⁺ T cells at *H. ducreyi*-infected sites. **A**, FOXP3 versus CD4 (top row) and CD8 (bottom row) profiles of PBMCs and a pustule obtained simultaneously from 1 subject experimentally infected with *H. ducreyi*. The FACS dot plots were gated on lymphocytes and the numbers in plots represent the percentage of cells in each quadrant. **B**, Percentages of FOXP3⁺ CD4 T cells in 15 paired samples of PBMCs and pustules. The values represent the mean \pm SD. $P = 0.001$ for comparison of pustule cells with PBMCs.

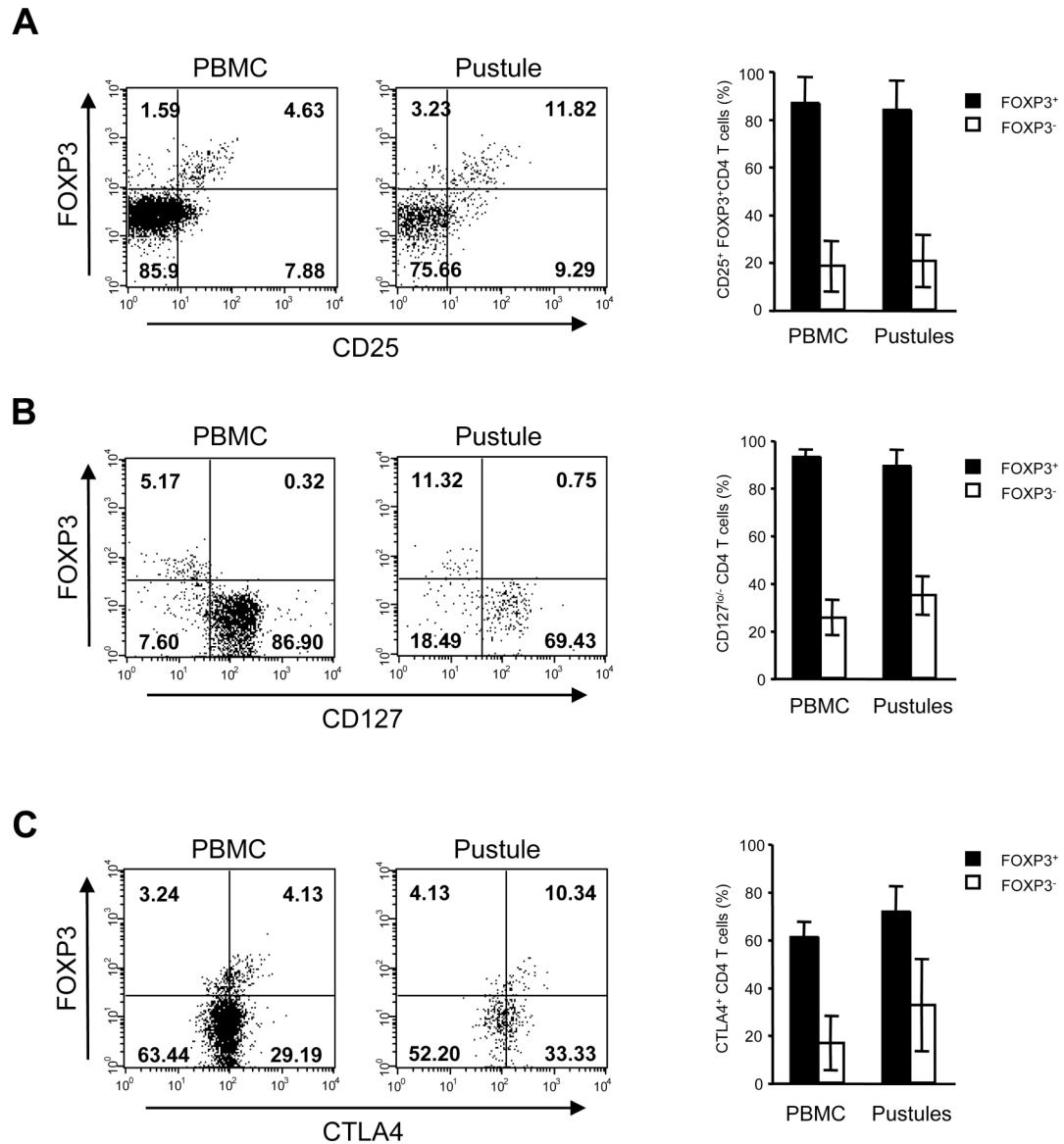


Figure 2.

Phenotypic characterization of CD4⁺FOXP3⁺ T cells. Gated CD4 T cells from PBMCs and pustules were analyzed for the expression of (A) CD25, (B) CD127 and (C) CTLA-4 on the FOXP3⁺ and FOXP3⁻ populations. The numbers in the FACS plots represent the percentage of cells in each quadrant. The values in the right panels represent the mean \pm SD of assays done on 15 (A), 4 (B), and 5 (C) pairs of samples, respectively. $P < 0.05$ for comparison of the percentage of CD25⁺, CD127^{lo/-}, or CTLA-4⁺ cells in CD4⁺FOXP3⁺ cells with that in CD4⁺FOXP3⁻ cells in both PBMCs and pustules; $P = 0.017$ for comparison of the percentage of CD127^{lo/-} CD4⁺FOXP3⁻ T cells in pustules with PBMCs; $P = 0.022$ for comparison of the percentage of CTLA-4⁺ CD4⁺FOXP3⁺ T cells in pustules with PBMCs.

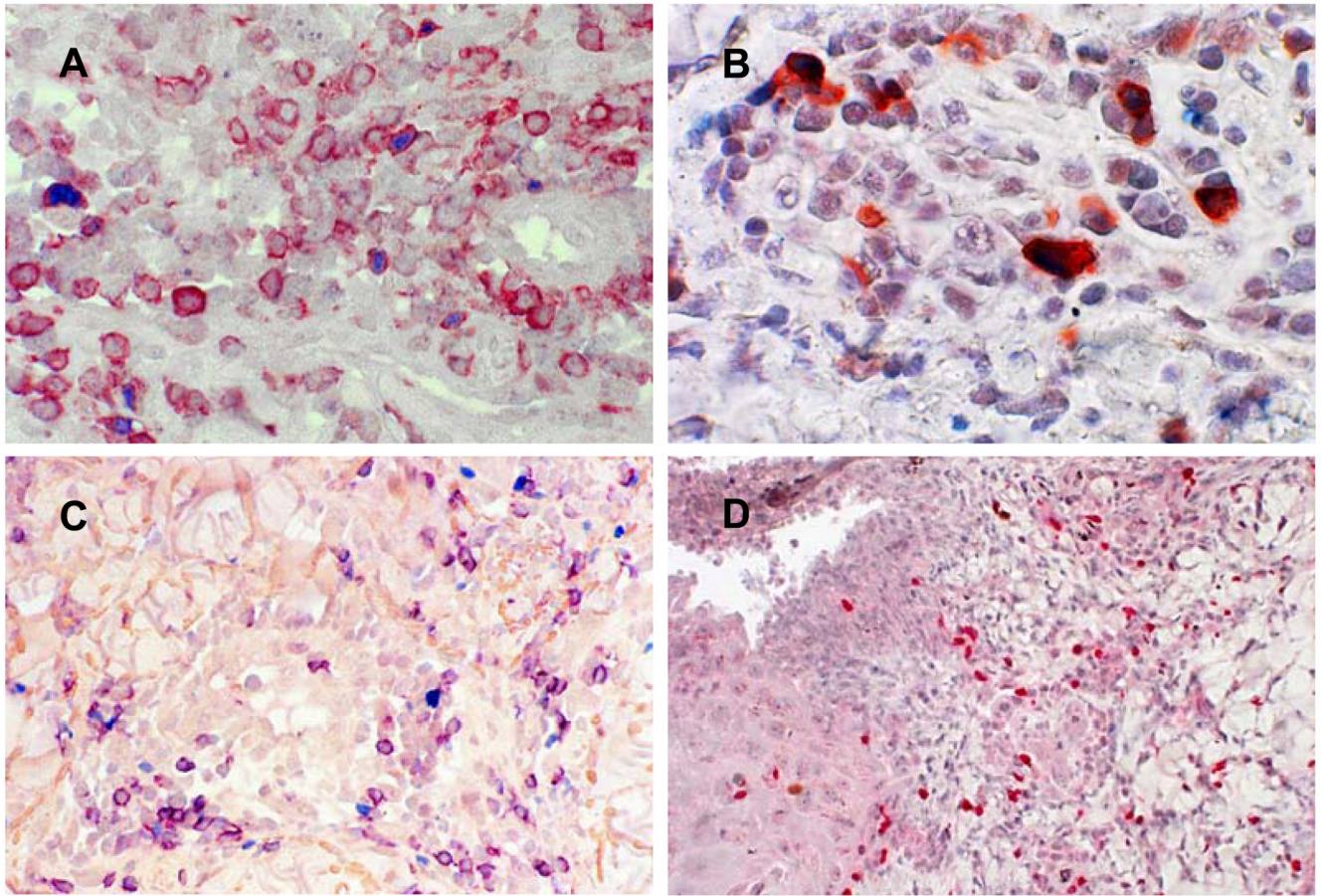


Figure 3.

Immunohistochemical analysis of FOXP3⁺ cells in pustules. Tissue sections were double stained with anti-FOXP3 and (A) anti-CD4, (B) anti-CD25, or (C) anti-CD8 antibodies. FOXP3 was stained in blue and CD4, CD25, or CD8 cells in red. (D) FOXP3⁺ cells (red) in the wall and the base of a pustule. Original magnification is 160× for A and B, 100× for C, and 50× for D.

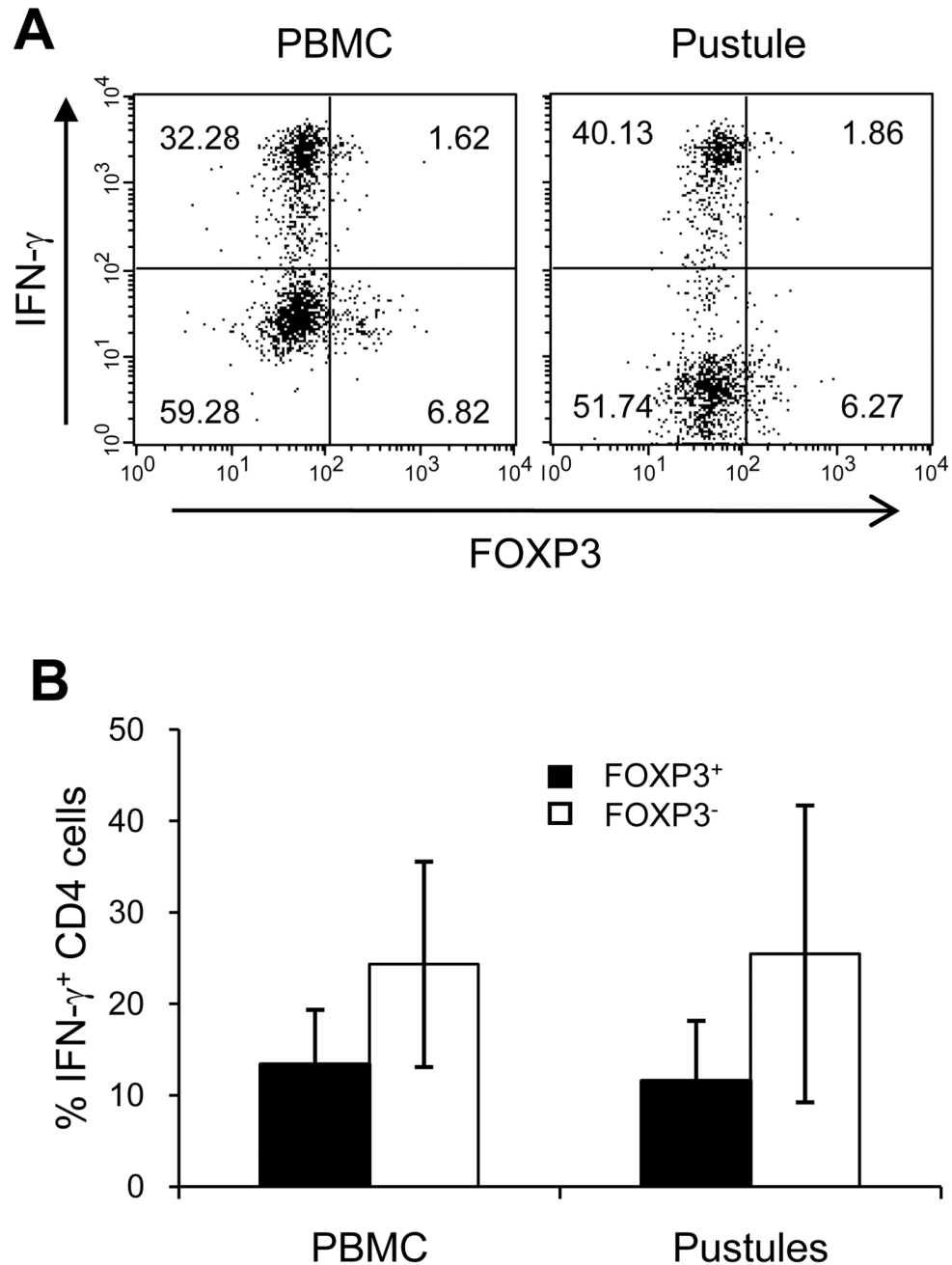


Figure 4.

Expression of IFN- γ by CD4⁺FOXP3⁺ T cells and by CD4⁺FOXP3⁻ T cells. PBMCs and pustule cells were stimulated with ionomycin and PMA in the presence of monensin. Cells were subsequently stained with anti-CD4, anti-FOXP3 and anti-IFN- γ . Gated CD4 T cells were analyzed for the expression of intracellular IFN- γ production by the FOXP3⁺ and FOXP3⁻ subsets. The numbers in the FACS plots obtained from 1 subject represent the percentage of cells in each quadrant (A). The values represent the mean \pm SD of assays done on cells from 4 and 7 volunteers for PBMCs and pustule samples, respectively (B). $P = 0.0071$ and $P = 0.016$ for comparison of the FOXP3⁺ with the FOXP3⁻ subset in PBMCs and pustules, respectively;

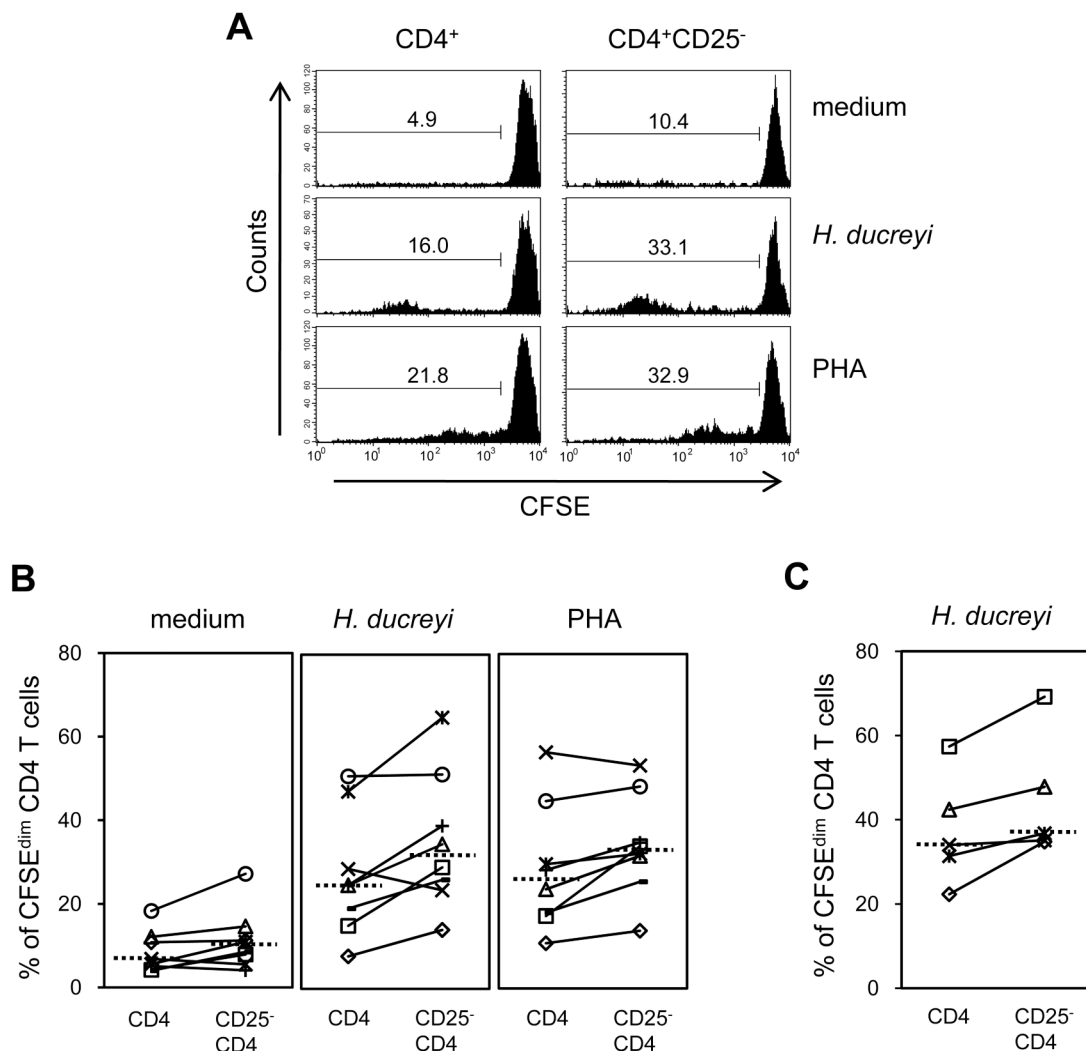


Figure 5. *H. ducreyi*-specific T cell proliferation. CFSE-labeled CD4 and CD25-depleted CD4 (CD4⁺CD25⁻) T cells were stimulated with DC pulsed with medium, heat-killed *H. ducreyi*, or PHA. Gated CD4 T cells were analyzed for proliferation by CFSE dilution. **A**, FACS histograms represent results obtained from 1 infected volunteer. The number in the histograms presents the percentage of proliferating CFSE^{dim} CD4 T cells. **B**, The percentages of CFSE^{dim} cells among the total and CD25-depleted CD4 T cells from eight *H. ducreyi*-infected subjects. Each symbol represents the response of 1 individual, and the median values are indicated by dotted lines. $P = 0.052$, 0.02 , and 0.69 for comparison of CD4 with CD4⁺CD25⁻ T cells treated with medium, *H. ducreyi* and PHA, respectively. **C**, The percentages of CFSE^{dim} cells among the total and CD25-depleted CD4 T cells from five uninfected donors. $P = 0.03$ for comparison of CD4 with CD4⁺CD25⁻ T cells treated with *H. ducreyi*.

Table 1

Sources of Tissue samples for Flow Cytometry

Subject	Sex	Inoculation strain	Infection duration, days	Data source ^a
291	M	p	7	[18]
295	F	P	8	[18]
300	M	P	8	[41]
301	M	P	7	[41]
302	M	M	7	[41]
306	F	P	6	[5]
308	F	P	7	[5]
316	F	P	7	unpublished
317	F	P	8	[5]
318	F	M	7	[5]
333	F	P	14	unpublished
335	M	P	7	unpublished
338	F	P	8	unpublished
341	M	M	8	unpublished
342	F	P	8	unpublished
344	M	P	7	unpublished
346	M	M	6	unpublished
347	M	M	6	unpublished
349	F	M/P	7	unpublished
350	M	P	6	unpublished
351	F	P	7	unpublished
353	M	P	6	This study
356	M	P	6	This study
358	M	P	6	This study
359	M	P	7	This study
360	M	P	6	This study

^aSubject 316 was infected with 35000HP and an *ompP4* mutant; subjects 333, 335, 338, 341, 342 and 344 were infected with 35000HP and a *Flp-1*, -2, -3 deletion mutant; subjects 346, 347, 349, 350 and 351 were infected with 35000HP and a *SapA* mutant.

Table 2

Sources of Tissue Samples for Immunohistochemical Analysis

Subject	Sex	Inoculated strain	Infection duration, days	HIV +/-	Hypertrophic scars	Data source
139P	F	P	9	-	-	[42]
158P	M	P	14	-	-	[43]
177P	M	P	7	-	-	[44]
185M	F	M	14	-	-	[44]
190M	F	M	10	-	-	[45]
267P	M	P	6	-	-	[46]
277M	F	M	8	-	-	[47]
91M	M	M	6	-	+	[48]
93P	F	P	10	-	+	[49]
98M	F	M	8	-	+	[49]
140P	F	P	12	-	+	[42]
148M	F	M	8	-	+	[50]
184M	M	M	13	-	+	[44]
192M	M	M	7	-	+	[45]
235	F	P	6	+	-	[19]
258	F	P	8	+	-	[19]
259	M	P	6	+	-	[19]
265	M	P	7	+	-	[19]
269	F	P	5	+	-	[19]
270	M	P	5	+	-	[19]
263	F	P	6	+	+	[19]