Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes

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Published online: 19 February 2002, DOI: 10.1038/ni769

Infection with *Neisseria gonorrhoeae* can trigger an intense inflammatory response, yet there is little specific immune response or development of immune memory. In addition, gonorrhea typically correlates with a transient reduction in T lymphocyte counts in blood, and these populations recover when gonococcal infection is resolved. Such observations suggest that the gonococci have a suppressive effect on the host immune response. We report here that *N. gonorrhoeae* Opa proteins were able to bind CEACAM1 expressed by primary CD4+ T lymphocytes and suppress their activation and proliferation. CEACAM1 bound by gonococcal Opa_{52} associated with the tyrosine phosphatases SHP-1 and SHP-2, which implicates the receptor's ITIM (immunoreceptor tyrosine-based inhibitory motif) in this effect.

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Despite the availability of effective antibiotic therapies with which to combat infection, *Neisseria gonorrhoeae* causes ~78 million infections globally each year (see World Health Organization: *World Health Report* (1995) at http://www.who.int/whr/1995/state.html). Gonorrhea is characterized by an intense inflammatory response that leads to the liberation of large amounts of urethral or cervical pus, which consists primarily of neutrophils with extracellular- and intracellular-associated *N. gonorrhoeae*. Despite this fact, up to 15% of infected men and 80% of infected women remain asymptomatic[1]. In such situations, infection tends to be prolonged and is consistently transmissible, both vertically (to the neonates of infected mothers) and horizontally (to sexual partners). If undetected, such infections are a source of significant morbidity, including conjunctivitis in neonates, disseminated gonococcal infection, pelvic inflammatory disease and sterility through fallopian tube scarring[2].

The persistence of *N. gonorrhoeae* within the population relies on the fact that gonorrhea can be contracted repeatedly, and there is little evidence that the infection reduces an individual's susceptibility to subsequent infection[1]. This is at least partially attributable to the antigenic variation of gonococcal surface epitopes[3]; however, individuals can be reinfected by the same serotype of *N. gonorrhoeae*[4–6], which indicates that immunoevasion is not the only survival strategy used by this pathogen. Although gonococci-specific immunoglobulins (Igs) can be detected in serum and mucosal secretions, their concentration is typically low and short-lived[7,8]. In addition, the antibody response that does occur is neither protective nor higher during subsequent gonococcal infections, which suggests that immunological memory is not induced[8]. These phenomena are unlikely to result from a general inability to develop an immune response within the urogenital tract because marked vaginal and cervical antibody responses can be generated by intravaginal immunization with appropriate epitopes[9]. In addition, the antibody response to gonococcal infection of the rectum, which contains lymphoid follicles that resemble Peyer's patches, is also weak[8]. It thus appears likely that *N. gonorrhoeae* possess some mechanism by which to subvert the natural immune response. Such an immunosuppressive effect would also help to explain other clinical observations. For example, there is a transient decline in $CD4^+$ T cell counts[10] and $CD8^+$ T cell responses (R. Kaul *et al.*, unpublished data) in blood during gonococcal infection, which resolves after clearance of the bacterial infection. Whether these effects help to explain why gonococcal infection also increases an individual's susceptibility to subsequent infection by both *Chlamydia trachomatis*[11] and HIV-1[12], or why gonococci markedly increases viral shedding by HIV-1–infected individuals[12,13], is still uncertain. However, collectively, these observations are consistent with *N. gonorrhoeae* being able to directly influence the immune response. The mechanisms that determine such effects have yet to be identified.

The neisserial colony opacity-associated (Opa) proteins govern bacterial adhesion to, and uptake into, host cells[14]. A single strain of *N. gonorrhoeae* encodes up to 11 different *opa* alleles and expression of each locus is phase-variable: that is, it is turned on and off at a rate of ~1 per 10^3 cells/generation/locus. The natural ligands of most Opa variants have been well defined. Some variants, typified by the Opa_{50} variant of gonococcal strain MS11, bind to heparan sulfate proteoglycans (HSPG), including cell surface–expressed syndecan receptors, and to the extracellular matrix proteins vitronectin and fibronectin[14]. A second class of Opa variants—which includes the antigenically distinct, but functionally conserved, Opa_{52} and Opa_{57} variants of strain MS11—are specific for various members of the carcinoembryonic antigen–related cellular adhesion molecule (CEACAM, also known as CD66) receptor family. This is a highly specific, protein-protein interaction that allows individual Opa variants to

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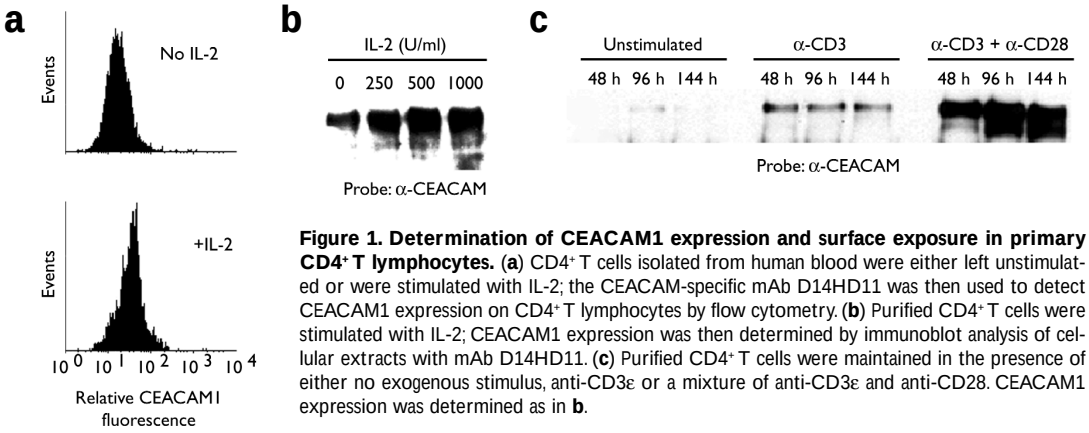

Figure 1. Determination of CEACAM1 expression and surface exposure in primary CD4⁺ T lymphocytes. (**a**) CD4⁺ T cells isolated from human blood were either left unstimulated or were stimulated with IL-2; the CEACAM-specific mAb D14HD11 was then used to detect CEACAM1 expression on CD4⁺ T lymphocytes by flow cytometry. (**b**) Purified CD4⁺ T cells were stimulated with IL-2; CEACAM1 expression was then determined by immunoblot analysis of cellular extracts with mAb D14HD11. (**c**) Purified CD4⁺ T cells were maintained in the presence of either no exogenous stimulus, anti-CD3ε or a mixture of anti-CD3ε and anti-CD28. CEACAM1 expression was determined as in **b**.

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bind various combinations of CEACAM1, CEACAM3, CEACAM5 and/or CEACAM6[14]. Although nonopaque gonococcal isolates can establish an infection after urethral challenge in human male volunteers, the bacteria recovered are predominantly Opa^{+15-17}. Attempts have been made to relate size and/or immunological reactivity to clinical symptoms associated with individual gonococcal infections[4,18], but neither of these characteristics correlate with the receptor specificity of individual Opa variants[19]. However, ~94% of a diverse set of gonococcal isolates obtained from mucosal infections bind CEACAM1[20]. Together, these studies suggest that the expression of CEACAM1-specific Opa phase variants is strongly favored *in vivo*.

CEACAM proteins are members of the Ig superfamily; individual family members are differentially expressed on various tissues *in vivo*[21]. CEACAM1 (also known as CD66a or BGP) is unique within this group because it contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain[21,22]. The ITIM is present in various coinhibitory receptors that function to antagonize kinase-dependent signaling cascades initiated by lymphocyte activation[23]. This inhibitory effect is triggered by the phosphorylation of tyrosine residues within the ITIM, which results in recruitment of the Src homology 2 (SH2) domain–containing tyrosine phosphatases SHP-1[24] and SHP-2[25] and the SH2-containing inositol phosphatase SHIP[26]. Consistent with these attributes, CEACAM1 associates with SHP-1[27] and SHP-2[28] after pervanadate treatment of cells or in the presence of a constitutively active tyrosine kinase[27,28], and CEACAM1 recruitment of these phosphatases appears to mediate the receptor's ability to arrest tumor cell growth[29,30].

CEACAM1 is the only receptor within the CEACAM family that is expressed by human lymphocytes[31]. The influence of this receptor on lymphocyte activation is, however, unclear. It has been reported that CEACAM1-specific antibodies either enhance[31,32] or reduce[33] the activation of T lymphocytes in response to T cell receptor (TCR) cross-linking *in vitro*. Consequently, we speculated that gonococcal Opa protein binding to CEACAM1 may also modulate the effector function of lymphocytes in response to activating stimuli. Such an effect could directly affect the development of specific immunity to infection *in vivo*, by either increasing or decreasing the rate and/or magnitude of the immune response. We show here that gonococci that were expressing CEACAM1-specific Opa proteins inhibited the activation and proliferation of primary human CD4⁺ T lymphocytes in response to otherwise activating stimuli. The specific contribution of Opa-CEACAM1 interactions to this effect was evident because isogenic strains of *N. gonorrhoeae* expressing adhesins that do not bind to CEACAM1 did not induce comparable inhibition. In addition, suppression of the T lymphocyte response was mimicked by CEACAM-specific antibody. This represents an example of bacterial suppression of lymphocyte activation through ligation of a coinhibitory receptor.

Results

CEACAM1 expression by primary CD4⁺ T cells

Lymphocyte expression of CEACAM1 correlates with activation state[31–33]. Consistent with this, after interleukin-2 (IL-2) treatment of primary CD4⁺ T lymphocytes, increased expression of CEACAM1 was observed both by flow cytometry (**Fig. 1a**) and immunoblot analysis (**Fig. 1b**). Receptor expression was stimulated in a dose-dependent manner by the addition of IL-2 (**Fig. 1b**). Ligation of CD3ε also induced CEACAM1 expression within 48 h, and no further increases in expression were detected after 96 or 144 h (**Fig. 1c**). Coligation of CD3ε and CD28 induced more CEACAM1 expression than was observed after ligation of CD3ε alone (**Fig. 1c**). In this case, induction of CEACAM1 expression was notable after 48 h and reached maximal and sustained amounts after 96 h (**Fig. 1c**).

Inhibition of CD69 expression

CD69 is a well characterized marker of lymphocyte activation; it is typically expressed 6–24 h after exposure to either mitogens or recall antigens[34,35]. We evaluated the effect of gonococcal infection and immunological challenge on lymphocyte expression of CD69 in

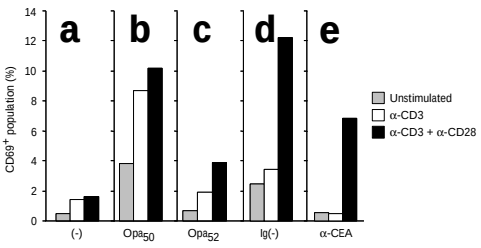

Figure 2. Determination of CD69 surface expression by primary CD4⁺ T lymphocytes. Purified CD4⁺ T cells were left unstimulated or were stimulated with anti-CD3ε or mixtures of antibodies to CD3ε and CD28 in the presence of (**a**) nothing, (**b**) Opa_{50}-expressing *N. gonorrhoeae*, (**c**) Opa_{52}-expressing *N. gonorrhoeae*, (**d**) control Ig or (**e**) CEACAM-specific Ig. The proportion of the CD4⁺ T cell population that expressed the CD69 early activation marker was determined by flow cytometry; at least 5000 cells were analyzed for each sample. Data are from one representative of at least three independent experiments. For each condition, the probability that CD69 expression in response to Opa_{52}-expressing gonococci was the same as that observed in response to Opa_{50}-expressing gonococci was $P \leq 0.015$. The probability that CEACAM-specific Ig samples were the same as control Ig samples was, in each case, $P \leq 0.009$. The exception was for the control Ig *versus* CEACAM-specific Ig samples that were stimulated with CD3 alone, which was $P = 0.04$.

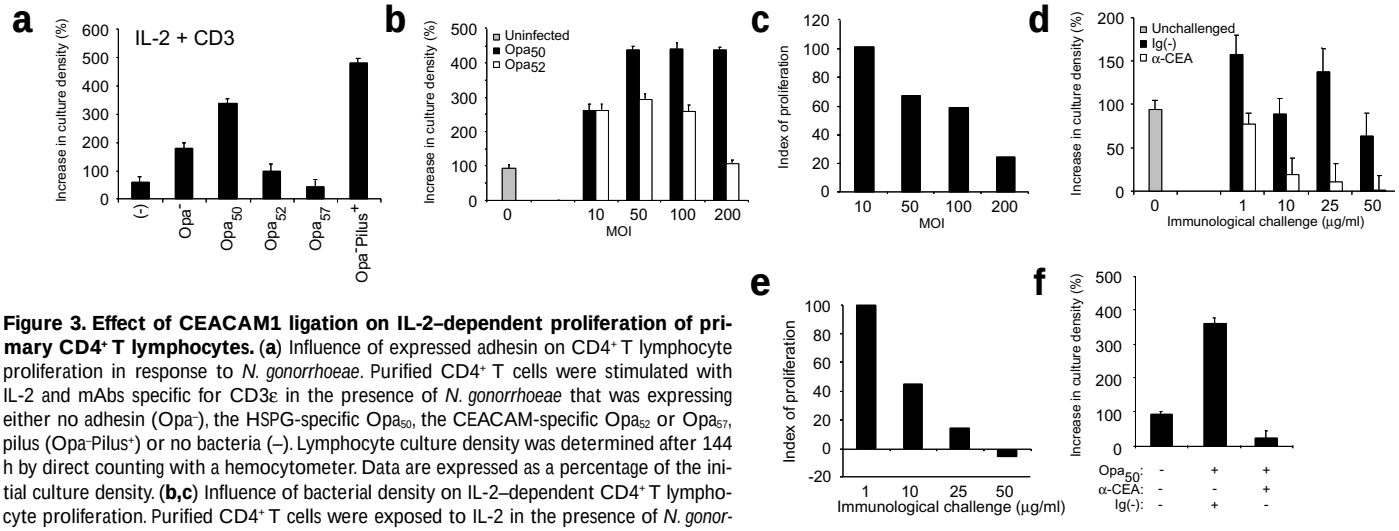

Figure 3. Effect of CEACAM1 ligation on IL-2–dependent proliferation of primary CD4⁺ T lymphocytes. (**a**) Influence of expressed adhesin on CD4⁺ T lymphocyte proliferation in response to *N. gonorrhoeae*. Purified CD4⁺ T cells were stimulated with IL-2 and mAbs specific for CD3ε in the presence of *N. gonorrhoeae* that was expressing either no adhesin (Opa⁻), the HSPG-specific Opa_{50}, the CEACAM-specific Opa_{52} or Opa_{57}, pilus (Opa⁻Pilus⁺) or no bacteria (–). Lymphocyte culture density was determined after 144 h by direct counting with a hemocytometer. Data are expressed as a percentage of the initial culture density. (**b,c**) Influence of bacterial density on IL-2–dependent CD4⁺ T lymphocyte proliferation. Purified CD4⁺ T cells were exposed to IL-2 in the presence of *N. gonorrhoeae* expressing either Opa_{50} or Opa_{52}; densities are expressed as bacteria per lymphocyte (or MOI). Lymphocyte culture densities and the index of proliferation (which indicates the relative increase in culture density in the presence of Opa_{52}-expressing gonococci as compared to the increase occurring in the presence of Opa_{50}-expressing bacteria) are shown. (**d,e**) Influence of CEACAM-specific (α-CEA) *versus* control (Ig(–)) Ig on IL-2–dependent proliferation of CD4⁺ T cells. Purified CD4⁺ T cells were exposed to IL-2 in the presence of antibody and (**d**) the culture density and (**e**) index of proliferation (which indicates the relative increase in lymphocyte culture density after exposure to anti-CEACAM *versus* control Ig) were assessed. (**f**) Influence of anti-CEACAM *versus* control Ig on lymphocyte proliferation in response to *N. gonorrhoeae* that was expressing Opa_{50}. Lymphocytes were exposed to IL-2, Opa_{50}-expressing gonococci and either anti-CEACAM or control antibodies. Proliferation was determined as in **a**. Data are mean±s.d. from at least four samples and are representative of at least two independent experiments.

response to various stimuli. In uninfected and unstimulated CD4⁺ lymphocytes, CD69 was expressed by <1% of the cell population (**Fig. 2a**). The number of CD69⁺ cells increased coincident with ligation of CD3ε and was increased further by coligation of CD3ε and CD28. However, in the absence of other stimuli, <2% of lymphocytes expressed CD69 in each of these conditions (**Fig. 2a**). Consistent with the reported influence of bacterial products, including lipopolysaccharides[35], on CD69 expression, infection with *N. gonorrhoeae* expressing the HSPG-specific Opa_{50} protein increased the proportion of CD69⁺ cells to 3.9–10.1% of the total population, depending on the method of stimulation (**Fig. 2b**). Comparable infection with an isogenic *N. gonorrhoeae* strain expressing the CEACAM-specific Opa_{52} protein resulted in a much lower stimulatory effect. The influence of Opa_{52} expression was most marked when cells were either left unstimulated or were stimulated by ligation of CD3ε alone. Under these conditions, cells infected with Opa_{52}-expressing gonococci were essentially indistinguishable from uninfected populations (compare unstimulated and anti-CD3 in **Fig. 2a–c**). After coligation of CD3ε and CD28, some lymphocyte stimulation was apparent, even in the presence of gonococci expressing Opa_{52}. However, the relative number of CD69⁺ cells was reduced by >60% in comparison to populations infected with gonococci expressing Opa_{50} (compare anti-CD3 + anti-CD28 in **Fig. 2a–c**).

To ascertain whether the difference between the gonococcal strains could result from an inhibitory effect of Opa_{52} binding to CEACAM1 on T cell activation, we tested the effect of CEACAM-specific antibodies on CD69 expression. Ligation of CEACAM1 with antibodies produced a similar result: CEACAM-specific antibodies inhibited lymphocyte activation in comparison to the control antibody (compare **Fig. 2d,e**). CEACAM-specific antibody completely abrogated any increase in CD69 expression in response to CD3ε ligation and reduced the number of CD69⁺ cells after coligation of CD3ε and CD28 by ~45% (**Fig. 2d,e**).

Inhibition of CD4⁺ T lymphocyte proliferation

Subsequent to CD69 expression, clonal proliferation of activated CD4⁺ T lymphocytes results in an increased number of effector cells capable of propagating the immune response[34]. Consequently, we investigated whether gonococcal infection also influenced CD4⁺ T lymphocyte proliferation in response to activating stimuli. Initially, lymphocytes stimulated with IL-2 and through ligation of CD3 were challenged with gonococci expressing either pilus, the HSPG-specific Opa_{50}, the CEACAM-specific Opa_{52} or Opa_{57}[14] or no adhesin. In experiments of this type, gonococci expressing the antigenically distinct, but functionally conserved, Opa_{52} or Opa_{57} protein variants inhibited lymphocyte proliferation, whereas comparable challenge with other gonococcal strains stimulated lymphocyte proliferation relative to uninfected controls (**Fig. 3a**). Having established this trend, subsequent analyses used only *N. gonorrhoeae* expressing Opa_{50} as a control for Opa_{52}-expressing gonococci. The Opa_{50}-expressing strain was selected because Opa_{50} is closely related to Opa_{52}, but binds to HSPG rather than CEACAM receptors. Yet, our microscopic analyses established that these two strains were bound and internalized by primary CD4⁺ T cells at broadly comparable amounts. [Opa_{50}: mean bacteria associated/lymphocyte, 20; mean intracellular bacteria/lymphocyte, 10 (50%). Opa_{52}: mean bacteria associated/lymphocyte, 35; mean intracellular bacteria/lymphocyte, 13 (37%).] Differences seen in the lymphocyte response were not, therefore, attributable to differences in bacterial association.

IL-2 treatment of the purified CD4⁺ T cells caused the population to double in size by 144 h (**Fig. 3b**). At a low multiplicity of infection (MOI) of 10, *N. gonorrhoeae* increased lymphocyte proliferation regardless of the Opa variant expressed. However, at an MOI of 50, infection with Opa_{52}-expressing gonococci reduced lymphocyte proliferation by 34% relative to infection with gonococci expressing Opa_{50}. At a higher MOI, a similar effect was noted: Opa_{52} reduced lymphocyte proliferation by 51% at an MOI of 100 and by 76% at an MOI of 200, essentially abrogating the stimulatory effect otherwise associated with

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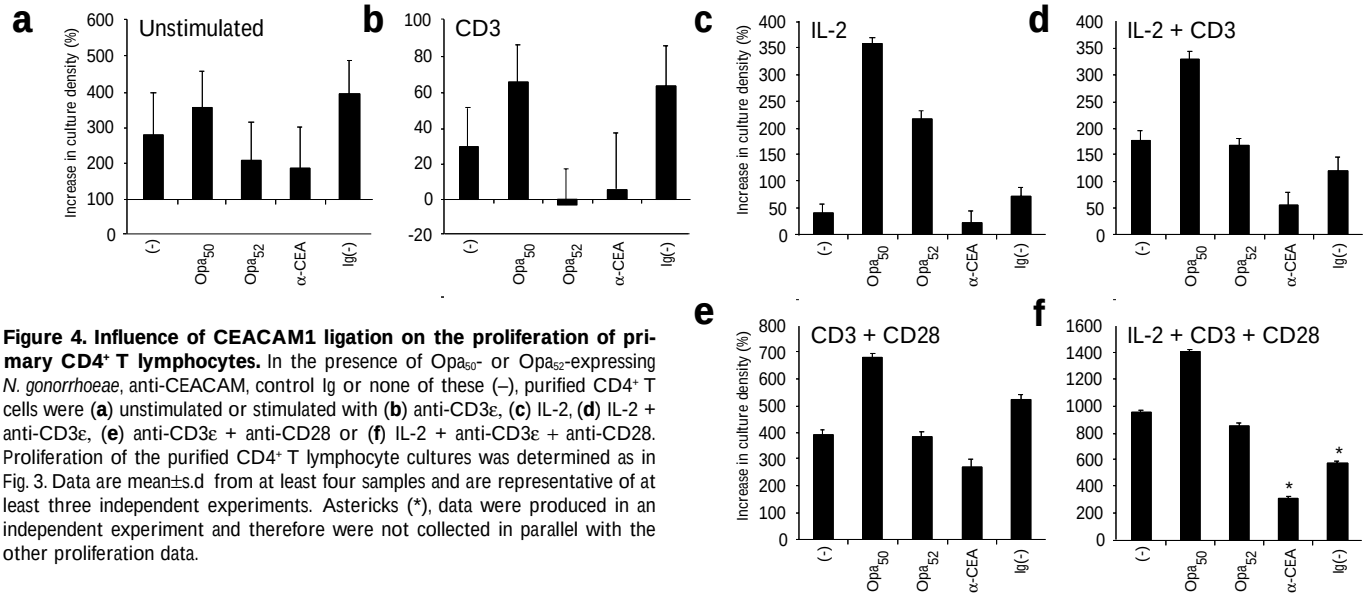

Figure 4. Influence of CEACAM1 ligation on the proliferation of primary CD4⁺ T lymphocytes. In the presence of Opa_{50}- or Opa_{52}-expressing *N. gonorrhoeae*, anti-CEACAM, control Ig or none of these (–), purified $CD4^+$ T cells were (**a**) unstimulated or stimulated with (**b**) anti-CD3ε, (**c**) IL-2, (**d**) IL-2 + anti-CD3ε, (**e**) anti-CD3ε + anti-CD28 or (**f**) IL-2 + anti-CD3ε + anti-CD28. Proliferation of the purified $CD4^+$ T lymphocyte cultures was determined as in Fig. 3. Data are mean±s.d from at least four samples and are representative of at least three independent experiments. Astericks (*), data were produced in an independent experiment and therefore were not collected in parallel with the other proliferation data.

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gonococcal infection (**Fig. 3b**). Plotting the index of proliferation (proliferation in response to Opa_{52}/proliferation in response to Opa_{50}) showed a dose-dependent inhibition of lymphocyte growth that correlated with gonococcal expression of Opa_{52} (**Fig. 3c**).

To ascertain whether CEACAM1 ligation can itself influence the proliferation of primary $CD4^+$ T lymphocytes, we used an immunological challenge with CEACAM-specific and control antibodies. Proliferation was typically lower in the absence of bacterial infection (**Fig. 3d**). However, CEACAM-specific antibody consistently reduced culture growth in comparison to treatment with control antibody. This effect was dose-dependent: the CEACAM-specific antibody reduced proliferation by 54–100%, depending upon the concentration used (**Fig. 3d**). Consistent with bacterial infections, plotting the index of proliferation in response to CEACAM-specific *versus* control antibody showed the dose-dependent nature of this inhibitory effect (**Fig. 3e**).

To confirm that CEACAM1 ligation affected the stimulatory effect of gonococci on $CD4^+$ T cells, we exposed the lymphocytes to Opa_{50}-expressing *N. gonorrhoeae* in the presence of control or CEACAM-specific antibodies. Lymphocyte exposure to Opa_{50}-expressing bacteria and control antibody stimulated proliferation by 365% relative to uninfected cells. In contrast, challenge with this strain plus CEACAM-specific antibody reduced lymphocyte proliferation by ~95% relative to that observed in the presence of control antibody (**Fig. 3f**). These results showed that the observed differences in lymphocyte proliferation in response to Opa_{50} *versus* Opa_{52} were due to the ability of Opa_{52} to ligate CEACAM1 and that this effect can overcome the stimulatory effect otherwise associated with Opa_{50}-expressing bacteria.

Effect of CEACAM1 ligation on stimulus responses

We next determined whether Opa-CEACAM1 interactions could suppress lymphocyte proliferation in response to other stimuli (**Fig. 4**). Phosphorylation of tyrosine residues within an ITIM is required for the inhibitory function of coinhibitory receptors[36]. Lymphocyte activation by the ligation of immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors (for example, the CD3ε component of the TCR) potently activates Src family tyrosine kinases that can phosphorylate the ITIM of adjacent receptors[36]. Subsequent recruitment and activation of phosphatases effectively increase the threshold of activating signals required to induce an effector response, and the relative strength of activating (ITAM) and antagonistic inhibitory (ITIM) signals determines the ultimate cellular response to stimulation. Consistent with such a model, we observed that ligation of CEACAM1 by either *N. gonorrhoeae* Opa_{52} or CEACAM-specific antibody had the most marked effect after coligation of the TCR (**Fig. 4b**). Lymphocytes were exposed to various combinations of IL-2, CD3ε-specific antibodies and CD28-specific antibodies in the presence of *N. gonorrhoeae* expressing either Opa_{50} or Opa_{52}, or to CEACAM-specific or control antibodies. In each condition, infection with gonococci expressing the HSPG-specific Opa_{50} variant increased the proliferation of T cell cultures compared to the uninfected control (**Fig. 4**). In contrast, gonococcal expression of the CEACAM-specific Opa_{52} consistently abrogated this effect, and generally limited proliferation to amounts observed in uninfected samples (**Fig. 4d–f**). In the case of stimulation by CD3ε ligation in the absence of IL-2 or anti-CD28, Opa_{52} completely inhibited growth of the lymphocyte culture (**Fig. 4b**). In each case, CEACAM-reactive or control antibodies had effects similar to those observed for the Opa_{52}- or Opa_{50}-expressing bacteria, respectively. This suggests that the suppression of T cell growth by Opa_{52}-expressing gonococci was due to the bacteria's ability to ligate CEACAM1.

Effect of CEACAM1 ligation on lymphocyte death

The lower number of activated and proliferating lymphocytes present in samples that contained either Opa_{52}-expressing gonococci or CEACAM-specific antibody could result from either an increase in lymphocyte death or a decrease in the rate of proliferation among an otherwise viable population. Therefore, we characterized and quantified the effects of gonococcal infection and immunological challenge on lymphocyte viability to determine whether ligation of CEACAM1 increased cell death. In general, different stimuli influenced lymphocyte viability even in the absence of gonococcal infection or immunological challenge (compare control Ig samples in **Fig. 5**). Specifically, costimulation, through CD3ε and CD28, marginally reduced cell viability, which was consistent with the fact that profound lymphocyte activation can induce cell death[37]. However, after 48 h no strain- or antibody-dependent differences were apparent, regardless of the

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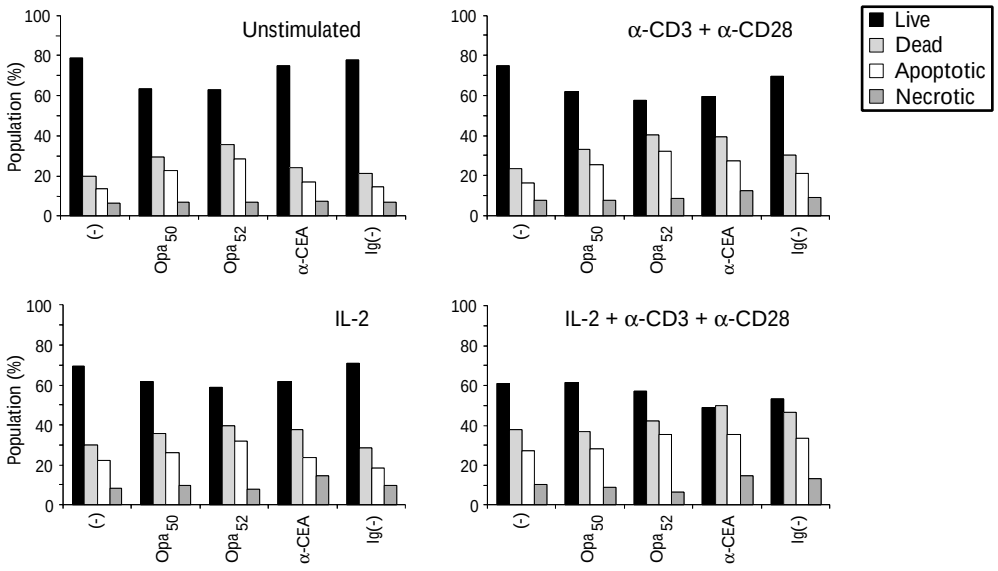

Figure 5. Characterization and quantification of cell death in primary CD4⁺ T lymphocytes. Together with Opa_{50}- or Opa_{52}-expressing *N. gonorrhoeae*, anti-CEACAM or control Ig, or none of these, purified CD4⁺ T cells were (**a**) left unstimulated or were stimulated with (**b**) anti-CD3ε + anti-CD28, (**c**) IL-2 (**d**) or IL-2 + anti-CD3ε + anti-CD28. Viability staining was done with FLUOS-conjugated annexin V and propidium iodide; live, dead, apoptotic and necrotic cellular populations were quantified by flow cytometry. Data are representative of three independent experiments.

method of stimulation used (**Fig. 5**). After 72 h, the relative proportions and patterns of cell viability were broadly consistent with the earlier timepoint, although necrosis was proportionally greater in each activation state (data not shown). Consistent with the observation made after 48 h (**Fig. 5**), ligation of CEACAM1 by either gonococcal Opa_{52} or CEACAM-specific antibody did not induce lymphocyte death relative to the appropriate controls after 72 h (data not shown).

Association of CEACAM1 with SHP-1 and SHP-2

As indicated above, reduced proliferation of CD4⁺ T lymphocytes was not coincident with reduced cell viability. Consequently, we proposed that suppression of lymphocyte activation and proliferation might result from CEACAM1 recruitment of effector molecules, which antagonize otherwise activating stimuli. Association of the tyrosine phosphatases SHP-1 and SHP-2 with ITIM-containing cellular receptors is critical to their function in down-regulating lymphocyte activation[38–40]. Therefore, we examined the association of these enzymes with CEACAM1. To recover CEACAM1 that was associated with Opa_{52} rather than total cellular CEACAM1, we developed a "bacterial precipitation". This involved the differential solubilisation of host cell, but not gonococcal, membranes, and then centrifugal recovery of intact bacteria with associated host proteins. CEACAM1 was selectively bound by Opa_{52}, with little receptor evident in the pellet containing Opa_{50}-expressing bacteria (**Fig. 6a**), which thus reflected the established receptor specificities of these Opa variants[14]. Consistent with our previous results (**Fig. 1**), treatment with increasing amounts of cross-linked anti–CD3ε IgG increased expression of CEACAM1. In this assay, induction was evident with 0.25 μg/ml of anti–CD3ε IgG, and maximal expression was induced by 0.50 μg/ml of this antibody (**Fig. 6a**). No additional increases were noted using higher concentrations of this antibody. SHP-1 and SHP-2 were recovered coincident with CEACAM1, and increasing recovery was evident in the presence of higher concentrations of anti–CD3ε IgG. SHP-1 was precipitated after TCR stimulation with 0.5 μg/ml of anti–CD3ε IgG, and increased progressively at higher concentrations of this antibody, despite no obvious increase in total CEACAM1 within the bacterial pellet (**Fig. 6b**). Coprecipitation of SHP-2 was also observed under these conditions; however, maximal recovery was achieved after TCR stimulation with 1.0 μg/ml of anti–CD3ε IgG (**Fig. 6c**).

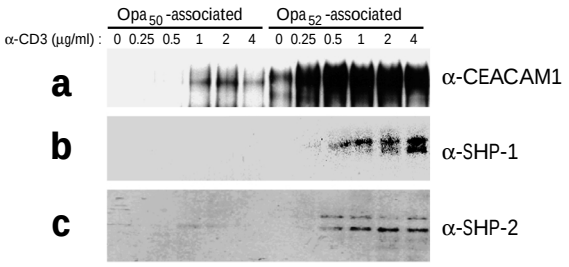

Figure 6. SHP-1 and SHP-2 tyrosine phosphatases associate with CEACAM1 that is bound by Opa_{52}-expressing *N. gonorrhoeae*. After stimulation with anti-CD3ε, purified CD4⁺ T cells were infected with *N. gonorrhoeae* that was expressing either the HSPG-specific Opa_{50} or CEACAM-specific Opa_{52}. Lymphocyte membranes were then solubilized and gonococci recovered by differential centrifugation of the lysates. Component proteins in the bacteria-containing pellets were then analyzed by immunoblot analysis to detect (**a**) CEACAM1, (**b**) SHP-1 and (**c**) SHP-2 that remained associated with each *N. gonorrhoeae* strain.

Discussion

We have established here that infection by gonococci expressing CEACAM-specific Opa proteins suppressed expression of the early activation marker CD69 and the subsequent proliferation of CD4⁺ T cells in response to various activating stimuli. Infection with isogenic strains that do not bind CEACAM instead stimulated the lymphocytes, which indicated that the expression of Opa variants that bound to CEACAM1 was required for this effect. Lymphocyte exposure to CEACAM-specific antibodies also suppressed the T cell response. This showed that CEACAM1 ligation alone is sufficient to suppress CD4⁺ T cell activation and proliferation. In addition, lymphocyte exposure to a combination of anti-CEACAM1 and *N. gonorrhoeae*, which was unable to bind CEACAM1, inhibited lymphocyte proliferation to the same extent as that observed in response to gonococci expressing the CEACAM-specific Opa_{52} (that is, in the absence of antibody). Together these findings indicate that Opa-mediated ligation of CEACAM1 is responsible for the gonococci's ability to inhibit CD4⁺ T cell activation and proliferation. These effects were not attributable to strain- or antibody-specific differences in cell viability or adhesin- or strain-specific differences in bacterial internalisation

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by the lymphocytes. This showed that the CEACAM1-dependent effects resulted from a specific arrest in cell division rather than from infection-induced cytotoxicity.

Such inhibition is consistent with the presence of an ITIM sequence within the cytoplasmic domain of CEACAM1. ITIM phosphorylation allows the recruitment of the SH2-containing phosphatases[38–40]. This results in antagonism of kinase-dependent events, which increase the intensity of the activating stimulus required to induce a lymphocyte response. We observed that CEACAM1 bound by Opa_{52}-expressing gonococci was associated with SHP-1 and SHP-2, which suggests that these tyrosine phosphatases may be involved in the Opa_{52}-dependent suppression of T cell activation and division. Consistent with this, SHP-1 and SHP-2 both contribute to the inhibition of intracellular calcium flux observed in response to ligation of chimeric receptors containing the cytoplasmic tail of CEACAM1[41].

The inhibitory effect of CEACAM1 ligation, either by CEACAM-specific antibody or gonococci expressing Opa_{52}, was consistently greater after coligation of the ITAM-containing CD3ε chain of the TCR. This was likely due to increased activity among Src family kinases, which can phosphorylate CEACAM1[42]. This effect was evident when analyzing CD69 expression and lymphocyte proliferation. When CD3ε ligation was coincident with the presence of IL-2 and/or CD28-ligating antibodies, the inhibitory effect of CEACAM1 ligation became less marked. This is consistent with the threshold activation model, as such costimulation increases the relative magnitude of activating stimulus, thereby overcoming the otherwise inhibitory signal mediated by CEACAM1. Although the suppressive effect of Opa_{52} and CEACAM-specific antibody were still evident in the presence of multiple stimuli, CEACAM1 ligation no longer abrogated activation. In this regard, it should be noted that we used high doses of IL-2 (1000 U/ml) and stimulatory antibodies (1 μg/ml each of anti-CD3ε and anti-CD28) throughout this study. Previous studies showed that the coinhibitory effect of other ITIM-containing receptors is more marked if less potent lymphocyte stimulation is used[36,43–48], and it is possible that the inhibitory effect of CEACAM1 would be even more pronounced under such conditions. It will be useful to determine the effect of Opa_{52}-expressing bacteria and CEACAM1-specific antibody on the response of $CD4^+$ T lymphocytes exposed to antigen presented in the context of major histocompatibility complex class II.

Although our results showed an inhibitory role for CEACAM1, other groups have reported the opposite effect. It has been shown that ligation of CEACAM1 enhances the proliferation and interferon-γ release by primary lymphocytes[31,32]. In contrast, we observed that the CEACAM-specific Opa_{52} protein expressed on the surface of *N. gonorrhoeae* and CEACAM-specific antibody both suppressed T cell activation and proliferation in response to IL-2, CD3ε and/or CD28 receptor–mediated stimulation. Such an inhibitory role is consistent with the ability of CEACAM1 to block the growth of transformed cells[30] and down-regulate the cytolytic function of intestinal intraepithelial lymphocytes[33]. In addition, ligation of chimeric receptors that contain the cytoplasmic domain of CEACAM1 inhibits the calcium flux that is otherwise apparent after B cell receptor ligation[41]. Such an effect has been used to help establish the inhibitory role of other ITIM-containing receptors[47,49]. In the analysis of lymphocyte function, the apparent contradictions associated with CEACAM1 are not without precedent. Depending on the conditions used, the ITIM-containing receptors CD5[46,47], CD72[45,50] and PECAM1 (also known as CD31)[36,49] have all been described as mediating both the activation and inhibition of cellular responses. Consequently, receptor density, degree of cross-linking, nature of the cross-linking ligand and/or the pre-existing state of cellular activation may all contribute to the apparent function of these coinhibitory receptors.

$CD4^+$ T lymphocytes are often overlooked as a normal and key constituent of the submucosa, yet their density is roughly equivalent to that of $CD8^+$ T lymphocytes in the endocervix[51]. $CD4^+$ T cells normally constitute ~2.5% of all cells recovered by endocervical cytobrush, with further recruitment occurring coincident with nonulcerative sexually transmitted diseases, including gonococcal infection[51]. Because gonococci are evident in the subepithelial spaces after infection[52], they come into direct contact with these cells. Such interactions would presumably allow Opa binding to CEACAM1, as ~94% of gonococcal isolates obtained from mucosal infections recognize CEACAM1[20].

The Opa-CEACAM1–induced immunosuppression we describe here may have several potential benefits to *N. gonorrhoeae*. $CD4^+$ T lymphocytes effectively control the development of a humoral response. Inhibiting the activation and proliferation of $CD4^+$ T cells should diminish available T cell help for B cell activation, thus reducing and/or delaying the development of a specific immunity. This may explain why local and systemic antibody responses to gonococcal infection are unexpectedly low and lack signs of developing immune memory[7,8]. In addition, CEACAM1 is not restricted to $CD4^+$ T cells: it is also expressed by other lymphocytes and professional phagocytes[21,31]. Whether Opa-dependent ligation of CEACAM1 also influences the activity of these cells during gonococcal infection remains to be investigated.

Although the lack of a nonprimate animal model precludes simple assessment of the impact of Opa-CEACAM1 interactions *in vivo*, the ability of an ITIM-containing receptor to suppress an immune response *in vivo* has been shown. Immune complex–induced inflammation is controlled by the relative intensity of activating and inhibitory signals that emanate from the ITAM-containing Fcγ *versus* ITIM-containing FcγRIIB receptors, respectively[53]. These findings have lead to the suggestion that targeted induction of ITIM-mediated inhibitory processes should provide a therapeutic strategy with which to impede undesirable inflammatory responses, such as those that occur during autoimmune disease[53]. With respect to *N. gonorrhoeae* binding to CEACAM1, even a short delay in the initiation of an immune response could potentially increase the likelihood that the infecting bacteria successfully colonizes the urethral or cervical mucosa and persist for an extended period of time. A longer delay may facilitate asymptomatic persistence of *N. gonorrhoeae* if the intense inflammatory response that typically characterizes gonorrhea is prevented. If Opa-dependent interactions also affect the local response to coincident infections, infection-induced immunosuppression could also help explain why gonococcal infection increases an individual's risk of acquiring other STDs, including chlamydia[11] and HIV[12]. Particularly relevant in this regard are reports that $CD4^+$ T cell counts[10] and HIV-1–specific $CD8^+$ T cell responses (R. Kaul *et al.*, unpublished data) decline during episodes of gonococcal infection in HIV-1–infected individuals, as Opa binding to CEACAM1 may influence both these parameters *in vivo*. Due to the strict host specificity of *N. gonorrhoeae* for humans, our ability to dissect the contribution of Opa-CEACAM1 interactions *in vivo* awaits the generation of transgenic mice that express human CEACAM1[54] and/or the use of recombinant strains that express Opa variants of defined receptor specificity in the human male urethral challenge model[55].

In conclusion, we have shown here the immunosuppressive effect that results from bacterial engagement of a coinhibitory receptor. The implications of this work are not, however, restricted to gonococcal disease. *Neisseria meningitidis*[56] and *Haemophilus influenzae*[57], both of which colonize the upper respiratory tract and can cause invasive disease, also express adhesins that bind CEACAM1. It thus seems likely that such an effect has contributed to the evolutionary success of each of these important human pathogens.

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Methods

Bacterial strains. Gonococcal strains N302 (Opa^-), N303 (Opa_{50}), N309 (Opa_{52}), N313 (Opa_{57}) and N496 (Opa^-Pilus^+), which constitutively express single Opa variants or pilus, were as described[58]. These *opa* genes are expressed in a derivative of strain MS11 with mutations that abolish the expression of HSPG receptor–specific Opa_{30}. The ligands recognized by these various Opa variants were as described[14]. Gonococci were grown from frozen stocks on GC agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 1% (v/v) IsoVitaleX enrichment (BBL); they were subcultured daily and a binocular microscope was used to monitor colony opacity phenotype. Opa expression and variant type were routinely confirmed by SDS-PAGE (10%) and resolved proteins were transferred onto Immobilon P membranes (Millipore, Bedford, MA) and probed with a Opa cross-reactive monoclonal antibody (mAb) 4B12/C11[59].

Purification of $CD4^+$ T cells. Lymphocytes were purified from citrated human peripheral blood with Ficoll paque (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec), according to the manufacturers' specifications. $CD4^+$ T lymphocytes were then isolated by negative selection with Cellect plus purification columns (Cedar Lane Laboratories, Hornby, Ontario), according to the manufacturers' specifications. Purified lymphocytes were routinely >85% $CD3^+CD4^+$, as determined by flow cytometry (data not shown). This indicated an enrichment of this cell type and established the efficacy of this purification system. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Burlington, Ontario) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL) at 37 °C in 5% CO_2 and humidified air.

Lymphocyte stimulation. Where appropriate, isolated lymphocytes were stimulated with recombinant human IL-2 (1000 U/ml) (Pharmingen, Mississauga, Ontario) for 48 h before infection or antibody challenge. TCR stimulation was induced by treatment with 1 μg/ml of mouse anti–human CD3ε IgG (clone UCHT1, Pharmingen); in some cases, costimulation was induced with 1 μg/ml of mouse anti–human CD28 (clone CD28.2, Pharmingen). Stimulatory antibodies were cross-linked with 3 μg/ml of sheep anti–mouse IgG $F(ab')_2$ (Sigma, Oakville, Ontario).

Flow cytometric analysis of cell surface proteins. Lymphocyte purification efficiency assessments were done by quantifying CD3 and CD4 coexpression on purified lymphocytes. These surface proteins were detected with fluorescein isothiocyanate–anti-CD4 (clone RPA-T4, Pharmingen) and allophycocyanin–anti-CD3 (clone UCHT1, Pharmingen). CD69 expression was revealed with phycoerythrin–anti-CD69 (clone FN50, Pharmingen) and CEACAM1 expression was revealed with the mAb D14HD11 (a gift of F. Grunert, University of Freiburg, Germany) followed by goat anti–mouse IgG conjugated to the fluorophore BODIPY-FL (Molecular Probes, Eugene, OR). In each case, 1×10^6–2×10^6 lymphocytes were resuspended in 50 μl of PBS that contained 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (PBS-Mg-Ca) with 1% FBS and 0.05% sodium azide. Samples were then incubated with various antibodies. A minimum of 5000 cells from each sample were then analyzed by flow cytometry using a FACSCalibur with CellQuest software (Becton Dickinson, San Diego, CA).

Lymphocyte proliferation assays. Purified $CD4^+$ T lymphocytes were either stimulated with IL-2 (as described above) or left unstimulated. Lymphocytes were then prepared at a standardized cell density of 0.25×10^6–0.5×10^6 cells/ml by direct counting with the use of a Levy double hemocytometer. In some instances, additional immunological stimulation was induced *via* ligation of CD3ε, either alone or with coligation of CD28. These treatments were carried out simultaneous to the addition of bacterial infection or CEACAM-specific antibody. Infections and immunological treatments were carried out in RPMI + 4 mM GlutaMAX (Gibco-BRL) supplemented with 5% (v/v) PBS-Mg-Ca and 1 U/ml of endonuclease (Sigma), which was added to prevent gonococcal aggregation mediated by DNA released through bacterial autolysis[60]. Multiplicity of infection (MOI) was 0–200 bacteria/cell and, in parallel experiments, lymphocytes were challenged with either CEACAM-specific antibody solution (anti-CEA, Dako Diagnostics, Mississauga, Ontario) or equal concentrations of nonreactive control antibody (Dako Diagnostics) at concentrations of 0–50 μg/ml. In some experiments, lymphocytes were challenged with bacteria (MOI=200) + antibody (50 μg/ml). Gentamycin (50 μg/ml, Bioshop, Burlington, Ontario) was added to each sample 3 h after the start of infection and/or immunological challenge; it was maintained throughout the experimental time-course to prevent gonococcal overgrowth during the extended proliferation experiments. In each case, lymphocyte density was assessed by direct counting with a hemocytometer at the start of the experiment and at various times after infection or challenge. A standardized counting pattern was used throughout these analyses, and at least 12 quadrants were counted for each sample.

Characterization and quantification of cell death. Purified $CD4^+$ T lymphocytes were stimulated and either infected or immunologically challenged as described above. Cells were then fluorescein-conjugated annexin V and propidium iodide (Boehringer Mannheim, Mannheim, Germany), according to the manufacturers specifications. Stained cells were analyzed by flow cytometry, which allowed the relative quantification of live, dead, apoptotic and necrotic populations.

Analysis of CEACAM1 expression and association with SHP-1 and SHP-2. In addition to analysis by flow cytometry (as described above), CEACAM1 expression was analyzed by SDS-PAGE (10%) and immunoblotting with the CEACAM-specific mAb D14HD11. In several experiments, gonococci that expressed either Opa_{50} or Opa_{52} were used to recover Opa-associated proteins from purified $CD4^+$ T lymphocytes stimulated with either IL-2 (for 48 h) or 0–4 μg/ml of anti–CD3ε IgG (for 3 h concurrent with infection) or, in separate experiments, with 1 μg/ml of anti–CD3ε IgG for 48, 96 or 144 h. Lymphocytes were infected at an MOI of 200. However, in these experiments, gentamycin treatment was omitted and cells were treated with cytochalasin D (1 μg/ml) for 30 min immediately before lysis in order to prevent cytoskeletal association of the receptors. Recovered cells were then lysed on ice with Tris buffer (50 mM, pH 7.4) that contained 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 100 mM $NaVO_4$, 10 mM H_2O_2, 1 mM NaF, 1 mM PMSF and 2 μg/ml each of aprotinin, leupeptin and pepstatin. After centrifugation at low speed, residual pellets—which included essentially intact *N. gonorrhoeae* (data not shown)—were analyzed by SDS-PAGE (10% or 7.5%) and then immunoblotted with either mAb D14DH11 or antiserum directed against either SHP-1 or SHP-2 (Santa Cruz Biotechnology, Santa Cruz, CA).

Microscopic analysis of bacterial binding and uptake by primary $CD4^+$ T cells. Lymphocytes were purified and either left unstimulated or stimulated with IL-2 as described above. The cells were then infected (MOI=10) with gonococcal strains that had been prelabeled with Texas red-X, succinimidyl ester (Molecular Probes), according to the manufacturers' specifications. Extracellular bacteria were then labeled with the polyclonal anti–gonococcal serum (UTR01), which was raised against *N. gonorrhoeae* N302 (Opa^-) with the use of standard procedures. These bacteria were labeled with a BODIPY-FL–conjugated secondary antibody (Molecular Probes). Intracellular *versus* extracellular bacteria were then distinguished by visualization with a Leica DM-IRBE inverted fluorescence microscope (Leica Microsystems Inc., Toronto, Ontario).

Acknowledgments

We thank E. M. Bertram, J. Cannons, A. Cochrane, H. Ginzberg, K. Ireton, M. Luscher, E. Liao, S. E. McCaw, K. McDonald, M. Ostrowski and T. Watts for critical comments on and/or technical assistance. Supported by the Canadian Institutes for Health Research Operating Grant # MT-15499.

Competing interests statement

The authors declare competing financial interests: see the *Nature Immunology* website (http://immunology.nature.com) for details.

Received 27 September 2001; accepted 4 February 2002.

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