IAI Accepts, published online ahead of print on 29 September 2008
Infect. Immun. doi:10.1128/IAI.00552-08
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Title: **Regulatory T cells are locally induced during intravaginal infection of mice with *Neisseria gonorrhoeae***

Running Title: **T cell immune response against gonococcus**

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ABSTRACT

Neisseria gonorrhoeae is a gram-negative diplococcus that in human beings produces gonorrhea. Much clinical evidence has led to the conclusion that gonococcus has important mechanisms to evade host immune functions; however, these mechanisms are only now beginning to be elucidated. In this study, we determined that the Balb/c mouse is a good animal model to study gonococcus infection, and examined the immune response against the bacteria. We determined that after intravaginal inoculation of mice with *Neisseria gonorrhoeae*, the bacteria reached and invaded the upper female reproductive tissues and elicited a T cell specific immune response associated with a very weak humoral response, altogether resembling gonococcus infection and disease in women. Remarkably, in the draining lymph node of the genital tract of infected mice we found an increase of T regulatory lymphocytes, namely, TGF-$\beta 1^+$ $CD4^+$T cells and $CD4^+CD25^+Foxp3^+$ T cells. Altogether, results indicate that *N. gonorrhoeae* induces T regulatory cells, which might be related to the local survival of the pathogen and the establishment of a chronic asymptomatic infection.

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INTRODUCTION

Gonorrhoea is a sexually transmitted disease produced by the gram-negative diplococcus *Neisseria gonorrhoeae.* In women, infection affects the cervix and may spread to the uterus and oviduct, inducing endometritis and pelvic inflammatory disease. Strikingly, about 50% of the cases proceed without symptoms, inducing damage mainly in the fallopian tube, while in men infection occurs with distinctive clinical symptoms (11, 21). The hallmark of humoral immune response against *Neisseria gonorrhoeae* is the extremely low levels of anti-gonococcal antibodies found in serum and secretions of the human (male and female) during infection (19, 20). Antibodies are directed against several major membrane molecules, such as the Pili (Pil) and Opacity-associated outer membrane (Opa) proteins, Porin protein (Por), and against the Lipooligosaccharide (LOS) (5, 19, 20, 38, 39, 55). Although some of these have bactericidal activity, they are not protective and seem to be blocked by outer membrane protein 3 (RmP) specific IgGs (38).

The highly asymptomatic infection in women and the poor immune response related to gonococcus prime and multiple challenges might actually be related to mechanisms of immune evasion acquired by the bacteria to constrain immune response. Moreover, the presence of a significant, although weak humoral immune response during gonococcus infection (19) suggests that the bacteria might stimulate a regulatory type of immune response. One of these mechanisms might be the induction of non-inflammatory responses dominated by Th2 type cytokines and the activation of regulatory T cells (Tregs), which in turn would contribute to the suppression of most of the mechanisms of protection against intracellular pathogens (2, 40). Tregs are $CD4^+$ T lymphocytes involved in induction of suppressor responses; it has been experimentally determined that they show several phenotypes, while one of the major characteristics is related to the presence of TGF beta 1 (TGF-β1) (52), subgroups can be distinguished because of the expression of CD25 and the Foxp3 transcription factor. $CD4^+CD25^+$ T cells correspond to a subgroup expressing Foxp3, which originate in the thymus and are called natural regulatory T cells, while the $CD4^+CD25^-$ T cells are induced at the periphery after antigenic stimulation in the presence of a distinct cytokine environment (28, 31, 50, 52). TGF-β1 blocks T cell proliferation, inhibits Th1, Th2 and CTL differentiation and moreover induces Foxp3 expression in regulatory T cells (27).

Overall, primary cell and organ culture systems have been successfully developed to examine the initial phase of gonococcal pathogenesis (9, 10, 13, 29, 48) where several gonococcus membrane components, such as Pili and Opa, are highly relevant (30). However, the mechanisms explaining why female infection occurs in a high number of cases without inflammatory signs, with low levels of antibody induction and with no disease resolution responses, are not understood. Understanding pathogenesis at this level has been greatly hindered due to the ethical considerations associated with human research work and the lack of an animal model of experimental infection (9). Only recently, a murine model introduced by Ann Jerse has allowed studies on protection against gonococcus infection (23, 37).

In this study, we have further investigated the mouse experimental model of *Neisseria gonorrhoeae* infection and demonstrated that the bacteria reach and invade the upper female reproductive tissues (uterus and oviduct), resembling gonococcus infection and disease in women. The murine model allowed us to determine that infection elicits a T cell specific immune response associated with a weak humoral response. In addition, the local response includes the induction of

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regulatory TGF-β1^{+} T cells which, acting to suppress activation of the immune system, would support occurrence of infection. Altogether, results indicate that *N. gonorrhoeae* induces regulatory mechanisms of immunity which, in turn, might explain the local survival of the pathogen and the establishment of a chronic asymptomatic infection in women.

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MATERIALS AND METHODS

Bacteria and culture conditions. Three different variants of *N. gonorrhoeae* strain P9, kindly provided by Dr. Myron Christodoulides (University of Southampton, UK), were used in this study: P9-13 ($Pil^- Opa_a^+$), P9-16 ($Pil^- Opa_b^+$), and P9-17 ($Pil^+ Opa_b^+$) (8). Bacteria were routinely grown on agar GC (Becton Dickinson, Maryland, USA.) supplemented with 1% IsoVitaleX (Becton Dickinson) for 18 h at 37°C in 5% CO_2. Gonococcal variants containing the red-shift mutant GFP (rs-GFP) plasmid were grown in GC agar containing Ampicillin (5 μg/ml). Analysis of colony morphology under stereo-microscope and western blots for detection of Pili and Opa were routinely performed to discard phenotypic variability. Monoclonal antibodies against Pili and Opa were kindly provided by Professor Mumtaz Virji (University of Bristol, UK) and Dr. Mark Achtman (Max-Planck Institute, Berlin, Germany), respectively.

Animals. Balb/c mice were obtained from the Institute of Public Health (Santiago, Chile), housed under regulated light and temperature and sacrificed by cervical dislocation. Research was conducted in accordance to institutional guidelines and to the International Guiding Principles for Biomedical Research Involving Animals of the Society for the Study of Reproduction.

Mouse uterine cell cultures. Cultures were prepared from mouse uteri as previously described (17). Briefly, uterine horns were washed in RPMI 1640 medium (Gibco™ Invitrogen Co, Carlsbad, CA) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco™ Invitrogen Co). Organs were cut into small pieces and then transferred to a plate containing 0.25% Trypsin and 2.5% Pancreatin (Life Technologies, Grand Island, NY) and incubated for 60 min at 4 °C followed by 60 min at 20°C. Organs were transferred to a sterile tube containing 15 ml of cold Hank's salt balanced solution (HBSS; Gibco™ Invitrogen Co). Digested uteri were then vortexed and the released epithelial cells were recovered and transferred to a clean tube. The procedure was repeated three times and cells in suspension were collected, centrifuged for 8 min at 180 *g* and resuspended in RPMI-1640, supplemented with 10% FBS (HyClone Laboratories, Inc., Logan, UT), 1 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin. Uterine cells were cultured at 37 °C in 5% CO_2 until 90% confluence. Positive immunostaining with a mouse anti-cytokeratin monoclonal antibody (Chemicon International, Temecula, CA) and negative staining with anti-vimentin antibody (Chemicon International) confirmed the epithelial origin of these cells. Typically, cultures had more than 95% epithelial cells.

Infection of primary uterine cell cultures. Gonococcal isolates were taken from frozen stocks and cultured on GC agar plates at 37 °C in a 5% CO_2-air atmosphere. Bacteria were then scraped from confluent culture plates and resuspended in 1 ml of Dulbecco's Modification of Eagle's Medium (DMEM) without phenol red (Gibco™ Invitrogen Co). The concentration was estimated by comparison as one optical density unit at 600 nm corresponding to 3.2×10^9 CFU/ml. Cells were infected with *N. gonorrhoeae* fluorescence variants at a multiplicity of infection (M.O.I) of 10 and cultured at 37 °C in 5% CO_2 until 90% confluence. To determine uptake of bacteria, epithelial cells were treated with 100 μg/ml of gentamicin for 60 min at 37 °C to kill extracellular bacteria. Cells were then diluted in PBS containing 1% saponin and incubated for 15 min at 37 °C. A tenfold dilution series was prepared and 100 μl were spread on GC plates. The internalization of bacteria in epithelial cells was evident by the recovery of colonies composed of gram-negative, oxidase-positive diplococci after 18 h growth at 37°C.

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Mouse experimental infection. Experimental infection of Balb/c mice was performed as previously described (23) with some modifications. Briefly, seven days before bacteria inoculation, 4 groups of 5 female Balb/c mice (6 to 8 weeks old) were daily injected with 3 µg of Cetrorelix acetate (s.c. administration). On day 4, half of the groups were additionally inoculated daily with 300 ng of estradiol (estra-1, 3, 5(10)-triene-3,17-beta-diol, Sigma) until the end of the experiment. On day 7, one estradiol-treated group and one control group were intravaginally inoculated with 10^8 CFU *N. gonorrhoeae* fluorescence variants suspended in DMEM without phenol red (Gibco™ Invitrogen Co). The other two groups were treated with medium. The procedure was performed using a sterile syringe connected to flexible tubing which allowed instillation. Tubing was inserted into the vagina, first dorsally and then cranially until the cervix was reached. Fifty µl of bacterial suspension were inoculated in the mice. At one, three and five days post inoculation, the genital tracts of infected and control mice were removed. Uteri were weighed to assess the effectiveness of estradiol treatment.

Microscopy. For microscopic analysis, cells were cultured on coverslips and infected as described above. After indicated times, cells were fixed with 1 % paraformaldehyde in PBS. In the case of vagina, uteri and oviducts, tissues were fixed in 4% paraformaldehyde in PBS for 1 h, before sequential transfer to 10% sucrose in PBS for 1 h, 20% sucrose in PBS for 1 h, and 30% sucrose in PBS overnight. Organs were mounted in embedding compound (Cryo-M-Bed, Bright Instrument Co. Ltd, Huntingdon, UK) and frozen at –20°C. Slices of 5–10 µm were cut using a Bright Starlet Cryostat at –20 °C. Fixed cultured cells and tissue sections were counterstained using a solution of 1 µg/ml propidium iodide in PBS and mounted in a solution of PBS containing 10% (v/v) 1,4-diazobicyclo [2.2.2] octane (DABCO; Sigma) and 90% (v/v) glycerol. Analysis of interactions of *Neisseria gonorrhoeae* with uterine cells was performed by Confocal Microscopy (Zeiss LSM510). Briefly, Confocal Z-slices were obtained and orthogonal views and 3-D images were generated from isolated cells and tissues. For Transmission Electron Microscopy analysis, cells were fixed in 1% glutaraldehyde in PBS (pH 7.4) and dehydrated for embedding in epoxy resin. Ultra-thin sections were stained with Uranyl acetate and Lead citrate. Control and infected samples were viewed with a Transmission Electron Microscope Philips EM-200.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed for serum titration. Briefly, whole protein extracts were prepared by freeze-thawing *N. gonorrhoeae* strain P9-17 in water and protein levels were determined by Bradford assay. Extracts prepared from Escherichia coli JM109 were used as a negative control. Microtiter plates (Falcon, Becton Dickinson Co, Santiago, Chile) were activated with 10 µg of protein per well and incubated overnight at 4°C for 24 h. After washing, serum collected from of infected (n=4) and control (n=4) mice were added to plates (1:20, 1:50, 1:100, 1:200 y 1:400). Specific antibodies to gonococcus were detected with goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) and p-nitrophenyl phosphate (pNPP) (Sigma). Each mouse serum was assayed in duplicate.

T-cell antigen proliferation assays. To determine specific T cell response, mice were intravaginally infected as described above and then boosted intraperitoneally on day 15. Seven days after boost (day 22), mice were sacrificed and the spleen and lymph nodes (renal, iliac and caudal) were removed (34). $CD4^+$ T cells were isolated by negative selection using antibody-coated magnetic beads (Dynal; Invitrogen). Isolated T-cells ($1{\times}10^5$ cells/well) from treated and

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control mice were cultured with mitomycin-treated autologous splenocytes (2×10^4 cells/well) previously pulsed with 1 or 10 µg of whole protein extract of *N. gonorrhoeae*. Experiments were carried out in 96-well plates in triplicates. As positive control, T cells were stimulated with Phytohemaglutinin (PHA) (10 µl/ml; Gibco™ Invitrogen Co). Plates were incubated at 37°C and 5% CO_2 for 72 h and then pulsed with 1 µCi Methyl-^{3}H Thymidine (Amersham Biosciences, Buckinghamshire, UK) for the final 18 h before harvesting. The amount of incorporated radioactivity was measured using a liquid scintillation counter (Tri-Carb 2100 TR; Packard). Data were expressed as experimental minus control counts per minute (cpm).

Leukocyte isolation. A previously described protocol was followed (24). Briefly, uteri dissected from mice (4 animals/experiment) were placed in sterile ice-cold Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, N.Y.), weighed, and then transferred to a mixture of Pancreatin (Gibco), Trypsin (Sigma, St. Louis, Mo.) and DNase (Worthington, Lakewood, N.J.). Under sterile conditions, uterine tissues in the enzyme mixture were cut into small pieces, transferred to 6-well culture plates, and incubated, first for 1 h at 4 °C, and then for an additional hour at room temperature with gentle shaking. Cells were recovered from the supernatant and were pooled for each experiment.

Immunofluorescence staining and analysis. For TGF-β1 detection, leukocytes (5×10^5 cells/ml) were incubated with anti-TGF-β1 antibody (1:100, Santa Cruz Biotechnology) for 1 h at 4 ºC. After washing with PBS, cells were incubated with a FITC-conjugated polyclonal anti-rabbit IgG (1:200, Santa Cruz Biotechnology) for 1 h at 4 ºC. For CD11b detection, cell were incubated 30 min on ice with 2 ml of RPMI-10% FBS with 10 % heat-inactivated normal mouse serum to reduce the non-specific binding of antibodies. After incubation, 10^6 cells/ml were labeled with PE-conjugated anti-mouse CD11b (Santa Cruz Biotechnology, inc.) for 1 h at 4°C. For analysis of $CD4^+CD25^+Foxp3^+$ T lymphocytes, 1×10^6 cells were labeled using Pecy5.5-conjugated anti-CD4 antibody (clon GK1.5, e-bioscience, San Diego, CA, USA) and FITC-conjugated anti-CD25 antibody (clon PC61.5; e-bioscience). After washing, cells were resuspended in 200 µL of Fix/Perm buffer and left at 4°C for 30 minutes. After washing, cells were incubated with PE conjugated anti-mouse Foxp3 (clon FJK-16s; e-bioscience, CA, USA) for 30 min. Isotypic controls were routinely included in all experiments. Labeled cells were analyzed on a FACScan flow cytometer (Becton Dickinson) with CellQuest software.

MLR. Fresh T cells isolated from the lymph nodes and spleens of control and infected Balb/c mice (H-2^d) were first labeled with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE). T cell responders (10^5) were incubated with spleen cells (5×10^5) isolated from C57BL/6 (H-2^b) mice in 200 µl of RPMI at 37 ºC. After 5 days of culture in 96-well round-bottom plates, cells were washed and fixed in 1% paraformaldehyde and CFSE dilution was assessed by flow cytometry.

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RESULTS

Neisseria gonorrhoeae infects isolated murine uterine cells. Previously, it had been extensively described that *N. gonorrhoeae* is a strictly human pathogen; nevertheless, recent reports indicated that the bacteria are also able to colonize the mouse genital female tract. Before initiating studies of infection *in vivo*, our first aim was to demonstrate that the bacteria attach, bind and invade murine genital tract cells. Isolated mouse uterine epithelial cells were chosen as targets for *in vitro* studies. Cells were infected with *N. gonorrhoeae* fluorescent (GFP) strain P9, variant P9-17 ($Pili^+$, Opa_b^+) with a multiplicity of infection (m.o.i.) of 10 bacteria per cell. Simultaneous detection of green GFP-gonococcus and the red stained nucleus on 0.4-μm-thick cross-sectional images by confocal microscopy showed that, after 24 h incubation, gonococci were found associated with the epithelial cells with a significant number inside the cells surrounding the nucleus (Figure 1 A). Similar results were obtained for the variants P9-16 and P9-13. Intracellular localization of the bacteria was confirmed by transmission electron microscopy as micrographs exhibit *N. gonorrhoeae* P9-16 enclosed in membrane-bound vesicles of the mouse uterine cell (Figure 1 C-D). Similar structures were not found in control cells (not shown). In addition, an average of 3.5 x 10^4 intracellular gonococci per well (24-well plate) was recovered from infected uterine epithelial cells after selective antibiotic killing of extracellular bacteria. Colonies had the expected morphology, i.e variants exhibit small opaque colonies with sharp edges which correspond to pili+ opa+ gonococci. Much higher number of bacteria were observed under fluorescent microscopy indicating that gonococci were predominantly adhered to the infected uterine cell, exactly as previously described for infection of human endometrial and oviductal cells (8). Results demonstrated that *N. gonorrhoea* is able to attach and invade the uterine epithelial thus unequivocally identifying murine cells as target of gonococcus infection.

Neisseria gonorrhoeae invades the upper female reproductive tract of the mouse. Once it was determined that *N. gonorrhoeae* is able to colonize isolated uterine epithelial cells, the next step was to corroborate that the bacteria colonize the upper reproductive tract in mice, as seen in women. Because estradiol seems to be crucial for gonococcus infection in mice (23), we inoculated the bacteria strain P9-17 expressing GFP (10^8 CFU) into the vagina of estradiol-treated Balb/c mice. Uteri were removed after several days post inoculation, processed as described in Methods and analysed by Confocal microscopy. Fluorescent bacteria were found in the uterus of all inoculated mice (n=25), particularly in the epithelial and subepithelial tissues of the organs. On day 1 after inoculation, gonococci were mostly observed in the epithelium and occasionally in the stroma, while on day 3, most of the bacteria were present in the stroma (Figure 2A). On day 5, the bacteria were distributed throughout the stroma, but also in the most external tissues of the uterus (Figure 2B). An orthogonal view of the infected uterus (midplane Z-section, height 1.3 µm,) confirmed the bacteria localization within the depths of tissue (Figure 2C). As the bacteria were also found in the epithelial and subepithelial tissues of the vagina and the oviducts, overall these results indicate that intravaginal inoculation of *N. gonorrhoeae* allows the bacteria to reach, attach and invade the mucosal tissues of lower and upper organs of the mouse genital tract. Further experiments were performed to evaluate the contribution of estradiol to allow infection. The experiments were repeated several times in different mice at random stages of the reproductive cycle and surprisingly, infection was also observed in all mice tested. Moreover, infection seems to be persistent because the bacteria were detected as late as 22 days post inoculation, however, as in women, no clinical signs of the disease were apparent in the mice.

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Immune response. After corroborating that gonococcus infects the mouse genital tract, as it does in humans**,** we wanted to establish whether the bacteria also induce an antigen-specific response in mice. To evaluate humoral immune response, sera from infected and control animals obtained at day 22 after treatment were tested using ELISA against whole protein extract of P9-17 bacterial variant. Very low-antibody titres in response to *N. gonorrhoeae* were detected in two out of the four infected mice (Figure 3) while, as expected, no detectable antibody was observed in serum samples of control mice. To examine T cell response, CD4$^+$ T lymphocytes isolated from uterus draining lymph nodes from infected and control mice were examined for the ability to proliferate in response to whole protein extract of the bacteria (variant P9-17). The results show that T cells from infected Balb/c mice exhibited a dose-dependent T cell proliferative response to gonococcus protein extract (Figure 4). These findings indicate that *N. gonorrhoeae* induces a local antigen-specific T cell response associated with a low or no antibody response during experimental infection in the mouse.

Regulatory T cells increase in infected animals. As mentioned previously, specific stimulation of regulatory T cells might explain the weak anti-gonococcal humoral immune response and the absence of protective immunity. Thus, we evaluated whether the Balb/c T cell response to gonococcus also involves the stimulation of Treg cells. As the major phenotype of Tregs is the synthesis of TGF-β1, we first quantified the percentage of TGF-β1 producing T cells locally induced 22 days after infection. In six independent experiments, the percentage of CD4$^+$TGF-β1$^+$ T cells isolated from the regional lymph nodes of infected mice (Figure 5C, upper right panel; Figure 5F) were two-fold higher than those cells of the control group of mice (Figure 5B, upper right panel; Figure 5F). No changes in the CD4$^+$TGF-β1$^+$ T lymphocytes were observed in T cells isolated from spleens of the same animals (Figure 5D and 5E, upper right panels; Figure 5F). Interestingly, a strong shift in CD4$^-$ TGF-β1$^+$ splenocytes from infected (Fig 5E) mice versus non-infected mice (Fig. 5D) occurred. This effect was not observed in the regional lymph nodes and cells might correspond to a different type of regulatory cells. In addition, we examined the stimulated T cells in four independent experiments to determine the presence of CD25 and Foxp3, also molecular markers of Treg cells. Results revealed that the percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells detected in the local lymph nodes of the group of infected animals was higher than that of the control group (p=0.005) (Figure 6A and 6B, upper right panel; Figure 6E). Once again, no statistically significant differences were found when cells were quantified in the spleen of the animals (Figure 6C and 6D, upper right panel; Figure 6E). Then, we tested the suppressive function of Tregs in an allogeneic MLR. Only cells from the lymph nodes of infected Balb/c mice, where Tregs have been expanded by gonococcus infection, showed suppression to the MLR stimulated by C57BL/6 (B6) spleens cells while cells from the lymph nodes of control mice and from the spleens of infected mice, where no T regulatory expansion was observed, showed a regular response to allogeneic B6 stimulation (Fig. 7). Overall, these results indicate that gonococcus infection of the murine genital tract induces a significant stimulation of regulatory T cells as part of the local immune response.

TGF-β1$^+$ cells infiltrate the uterine tissues. We further investigated whether local immune response was also accompanied by uterine infiltration of TGF-β1$^+$ cells. Because *in vitro, N. gonorrhoeae* induces expression of TGF-β1 in macrophages (unpublished data), cells isolated from the uteri of infected and control animals were labeled with anti TGF-β1 and anti CD11b (a macrophage marker) antibodies. Data from a flow cytometric analysis show that infected animals have a

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moderate but significant increase of $CD11b^{+}TGF\text{-}\beta 1^{+}$ cells in the uterus (Figure 8 C and D, upper right panel), which indicates that gonococcus induces mucosal infiltration of macrophages whose phenotype would favor the differentiation of T regulatory cells. Interestingly, in addition to macrophages, infection is also associated to infiltration of a great number of $CD11b^{-}$ $TGF\text{-}\beta 1^{+}$ cells (Figure 8 E), which might correspond to T regulatory cells.

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DISCUSSION

In this study, we established that *Neisseria gonorrhoeae* invades the murine mucosa of the upper genital tract, results that confirms and extends the extensive mouse work developed by Ann Jerse and collaborators (23, 37, 43). Moreover, we demonstrated that infection induces a weak humoral immune response associated with a local increase of regulatory TGF-β1$^+$ CD4$^+$ T cell.

Until now, 'it has been widely accepted that *Neisseria gonorrhoeae* is a strictly human pathogen, questioning the validity of the use of mice as an animal model of experimental infection. This is reinforced by the fact that most of human receptors for attachment and invasion do not seem to be expressed in the mouse (30). However, we here demonstrated that *N. gonorrhoeae* is able to infect murine epithelial cells isolated from the uterus; the uptake of gonococcus by murine uterine epithelial cells was demonstrated by means of three independent assays, a gentamicin resistance test, confocal multiple scan analysis and TEM. Moreover, *in vivo*, we showed GFP-gonococci are able to reach the upper genital organs and to invade uterine tissue in all infected animals. Because in the experiments there were no other source of green fluorescence than that of the bacteria, the uterus is normally sterile, and colonies of gonococcus were recovered from uterine cells of infected mice, we are confident that the 1 to 2 μm fluorescent spots seen in tissues by confocal microscopy correspond to the bacteria. Therefore, although mice are not natural hosts for *N. gonorrhoeae*, results confirmed that gonococcus infects the lower genital tract of Balb/c mice (23) and demonstrated for the first time that the bacteria not only reach the upper genital tract but also invade the upper murine mucosa, as occurs in humans.

Analyses performed to characterize the immune response in Balb/c showed that once gonococcus colonizes the female genital tract, bacterial antigens are detected by the immune system as revealed by the ability of CD4$^+$ T lymphocytes to respond to the bacterial extract and by the presence of specific antibodies in serum. However, as has been observed in humans, antibodies that recognize *N. gonorrhoeae* were detected at low levels in the infected mice. Gonococcal activation of CD4+ T cells has also been described in humans as gonococcal pili interaction with CD4+ T cells induces the activation and proliferation of lymphocytes and stimulates the secretion of IL-10 (36). In contrast, it has also been shown that *N. gonorrhoeae* Opa proteins mediate binding to CEACAM-1 expressed by CD4+ T cells and suppress activation and proliferation of naive lymphocytes (4, 25). Although not fully comparable, this does not seem to occur in mice, since PHA-stimulated naive T cells from control mice showed proliferation (not shown).

The analysis of the nature of the CD4+ T cells stimulated by gonococci during the experimental infection leads us to observe that infection induces TGF-β1$^+$ CD4$^+$ T cell responders in the mucosal lymph nodes including a subset of CD25$^+$Foxp3$^+$ regulatory T cells. In particular, these Treg cells showed a small but significant increase in number which is similar to that observed after infection with parasites (15, 47, 53). Interestingly, induction of this type of immunity did not occur at the systemic level. Even more, the regulatory activity was confirmed *in vitro* in an allogeneic MLR, indicating that *Neisseria gonorrhoeae* might induce this type of response to avoid the host mechanisms of protection. Results support the idea that suppression is induced at least in part by TGF-β1, which either in a cell-surface bound or a secreted form, inhibits immune response at a variety of levels, i.e., inhibits IL-2 production, IL-12 dependent cell activation, Th1 development, among others (27, 46). The source of TGF-β1 was not demonstrated in this study, nevertheless,

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epithelia and stromal cells of the reproductive organs of the mouse and human, which are targets of gonococcus infection, express high levels of TGF-β1 and other molecules involved in conditioning immune privilege sites (6, 17, 22, 51). Because TGF-β1 also has a role in the induction of Treg cells (54) and moreover, $CD4^+CD25^+$ peripheral T cells can be converted to $Foxp3^+$ T regulatory cells by stimulation via the TCR in the presence of TGF-β1 (7, 12), we believe that the cytokine milieu found in the reproductive tract subsidises the induction of Tregs by *N. gonorrhoeae*. Moreover, antigen presenting cells, such as macrophages and dendritic cells, regularly present in the reproductive tissues (16, 44) might also contribute to Treg differentiation as they may produce TGF-β1 after infection. Actually, we detected an increase of $CD11b^+$ macrophages infiltrating the reproductive mucosa of infected mice, which is consistent with such a role. Moreover, a high number of $CD11b^-TGF\text{-}\beta1^+$ cells, which seem to be T cells by morphology criteria, also infiltrated the mucosal reproductive tissues during infection, suggesting that regulatory T cells also play an important role at the mucosal level.

If extrapolated to humans, the results would indicate that gonococcus infection in women might also be related to a similar Treg induction, explaining in part the lack of protective immune response. In fact, there are various examples of evasion through pathogen-induced modulation of the immune response, one of these has been reported in the human filarial infection, where the parasite also induces an imbalance towards Th2 response, which is at the same time accompanied by a diminished production of inflammatory factors and an increase of anti inflammatory components, including regulatory T cells (1). In human beings, *N. gonorrhoeae* can induce other highly efficient manners to overcome immune defence mechanisms resulting in disease or chronic infection (14). Previous studies have focussed attention on the antigenic variation (49), epitopes mimicry (18, 33) and phagosome subversion (3) as molecular mechanisms of immune evasion. Moreover, recent studies have explored a putative role of T cells as a determinant of successful gonococcus infection. As mentioned above, Opa proteins from gonococci have the ability to inhibit CD4+ T cell proliferation what will prevent adaptive immune response. What is more interesting is the gonococci dependant induction of IL-10 (36), a cytokine that it is also involved in the differentiation of Treg cells (Tr1 type) (26, 41). A study on infected patients will shed lights on the mechanism of pathogenesis and the presumed role of Tregs.

Altogether, results showed that infection of mice with *Neisseria gonorrhoeae* induces a tolerant type of response which may correspond to a form of immune evasion that has not been previously studied for gonorrhea. Most studies describing these evasion mechanisms have been reported in parasite infection and chronic diseases caused by viruses (14, 32, 35, 42, 45). In those cases, immune regulation seems to favour persistence of infection which becomes evident when Treg cells are depleted and the disease is soon controlled by the immune system and the pathogen cleared. We are currently investigating the effect that depletion of Treg may have on the development of gonorrhea and moreover, studies on human subjects are currently in progress to investigate the role of these regulatory cells during infection in women.

ACKNOWLEDGEMENTS

This work was supported by grant 1020354 from the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT), 020743IB and 020540AC from the Dirección de Investigaciones Científicas y Tecnológicas (DICYT) and IPA06 from the Programa Bicentenario en Ciencia y Tecnología (PBCT).

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FIGURE LEGENDS

Figure 1. (A) Confocal photomicrograph of the serial sections (0.4 μm) shows the presence of *Neisseria gonorrhoeae* (P9-17) around the nucleus of uterine epithelial cells. Bacteria appear green (arrows) and the cell nuclei are red. (B) Uterine cells, control cultures stained with propidium iodide. (C-D) Transmission electron microscopy images of isolated uterine epithelial cells infected with *N. gonorrhoeae* (variant P9-16) show multiple cytoplasmic bacteria enclosed in membrane-bound vesicles (arrows). Bars: (B) 20 μm; (C) 1μm; (D) 0.1 μm.

Figure 2. Confocal images of uterine sections of mice intravaginally infected with GFP-expressing *N. gonorrhoeae* (variant P9-17). The cell nuclei are stained with propidium iodide. (A) 3 days after inoculation; scale bar: 50 μm. (B) 5 day after inoculation; scale bar: 20 μm. (C) Orthogonal views of a midplane Z-section, height 1.3 μm. (D) Negative control. Arrows denote fluorescent bacteria.

Figure 3. Analysis of serum response to *N. gonorrhoeae* by ELISA. Mouse sera were assayed on whole protein extract of P9-17 bacterial variant in a reciprocal dilution series starting at 1:50 and binding detected with an alkaline phosphatase anti-mouse conjugate. Black symbols are from infected mice (n=4) and open symbols from control mice (n=4).

Figure 4. Proliferative response of isolated $CD4^+$ T cells (LT) from infected mice to a protein extract of *N. gonorrhoeae*. T cells were stimulated with PHA or added to Balb/c splenocytes (APC) pretreated with a 1 or 10 μg of whole protein extract of *N. gonorrhoeae* P9-17 (Ngo). Proliferation was measured by $[^3H]$ thymidine incorporation. Each bar represents the mean ± SEM of triplicates. Data were expressed as experimental minus control counts per minute. Two independent experiments were done with similar results. $^*p < 0.05$; $^{**}p < 0.0001$ vs. APC+T by ANOVA.

Figure 5. Flow cytometric analysis of TGF-β1 producing CD4+T cells. A representative experiment is shown in the upper part of the figure. (A) Control of autofluorescence. (B) Cells isolated from the regional lymph nodes of control mice. (C) Cells isolated from the regional lymph nodes of infected mice. (D) Splenocytes of control mice. (E) Splenocytes of infected mice. The percentage of TGF-$\beta1^+$ $CD4^+$T cells is shown in the upper right panel of each figure. (F) Percentage of TGF-$\beta1^+$ $CD4^+$T cells in lymph nodes and spleens of infected (white bars) and control (black bars) Balb/c mice. Bars represent mean ± SEM of six independent experiments. Lymph nodes of infected mice showed a significantly higher percentage of TGF-β1 producing CD4+T cells than the lymph nodes of the control group. No differences were found in spleens. (*) $p<0.02$ Mann Whitney test.

Figure 6. Flow cytometric detection of regulatory $CD4^+$T cells. A representative experiment is shown in the upper part of the figure where results are expressed as the percentage of CD25+ or CD25− T cells expressing FoxP3 in the gated CD4+ T-cell population of (A) Local lymph nodes of control Balb/c mice. (B) Local lymph nodes of infected Balb/c mice. (C) Spleens of control mice and (D) spleens of infected mice. (E) Percentage of $CD4^+CD25^+Foxp3^+$ T cells in lymph nodes and spleens of infected (white bars) and control (black bars) mice. Bars represent mean ± SEM of four independent experiments. Lymph nodes of infected mice showed a significantly

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higher percentage of $CD4^+CD25^+Foxp3^+$ T cells than the lymph nodes of the control group. No differences were found in spleens. (*) $p<0.05$ Mann Whitney test.

Figure 7. Geometric mean fluorescence of CFSE-labeled T. Responder cells were isolated from the lymph nodes and spleens of control (white bars) and infected (black bars) Balb/c mice. For MLR, stimulators were obtained from C57BL/6 spleens. CSFE-labeled responder cells were incubated with stimulator cells (ratio of 1:5) for 5 days and CFSE dilution was assessed by flow cytometry at the end of the experiment. Bars represent geometric mean ± SEM of nine independent experiments. Only cells from the lymph nodes, where Tregs have been expanded by gonococcus infection, showed suppression to the MLR. (*) $p<0.0001$ Kruskal-Wallis test.

Figure 8. Flow cytometry analysis of $CD11b^+TGF\text{-}\beta1^+$ cells infiltrating genital tract tissues following infection with *N. gonorrhoeae*. (A) Autofluorescence control, (B) Secondary antibody control, (C) $CD11b^+TGF\text{-}\beta1^+$ cells from control and (D) infected tissues. (E) White bars: $TGF\text{-}\beta1^+$ cells from control groups. Black bars: $TGF\text{-}\beta1^+$ cells from infected mice.

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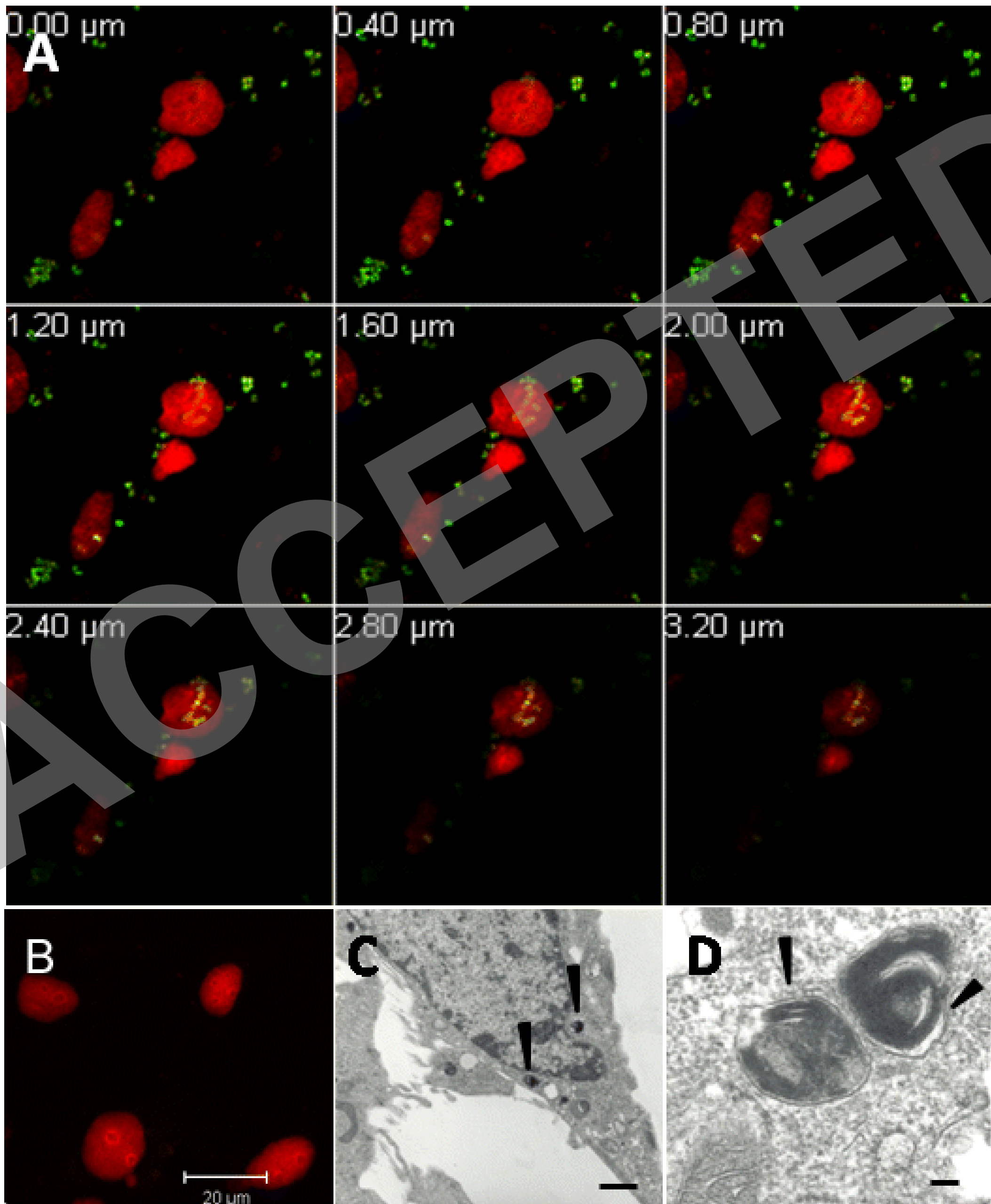

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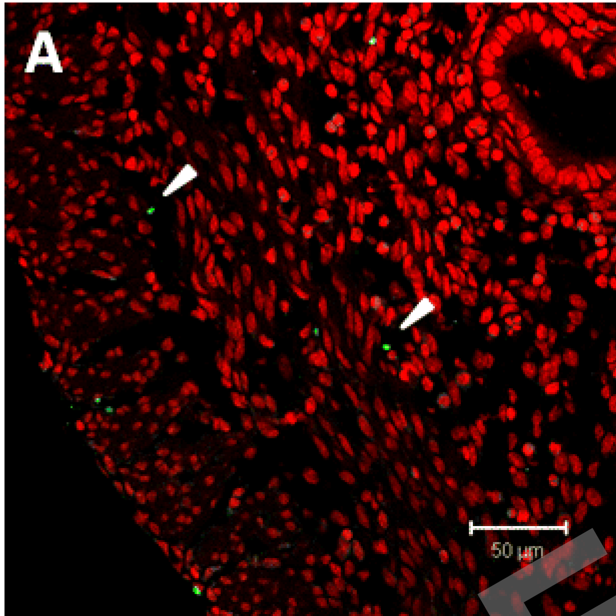

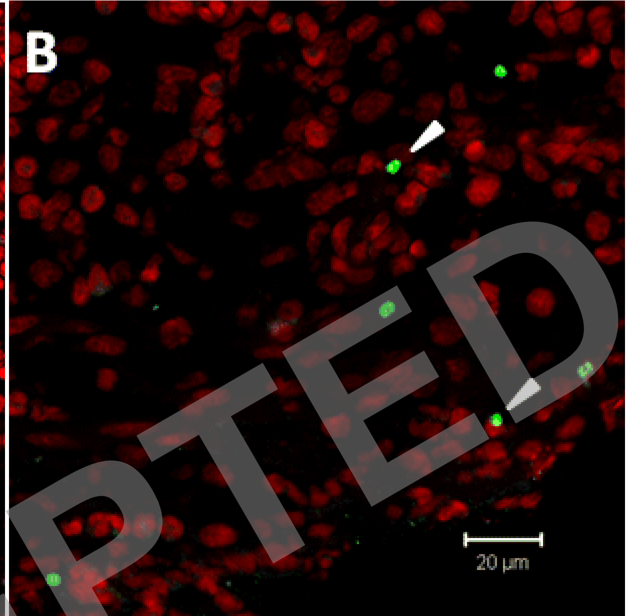

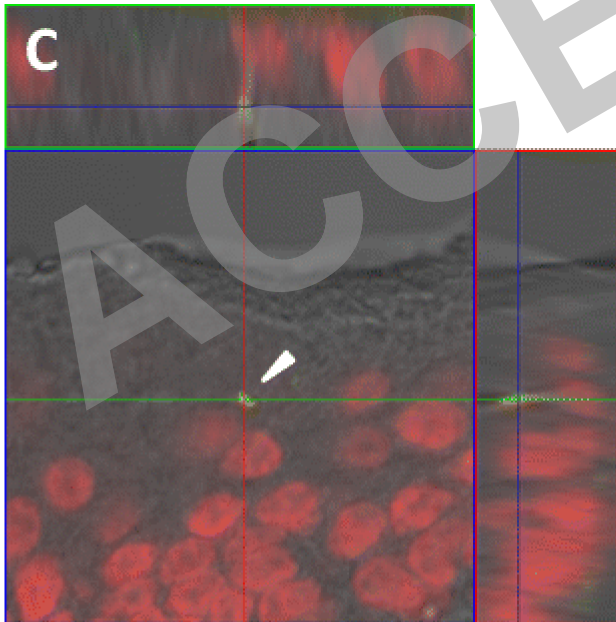

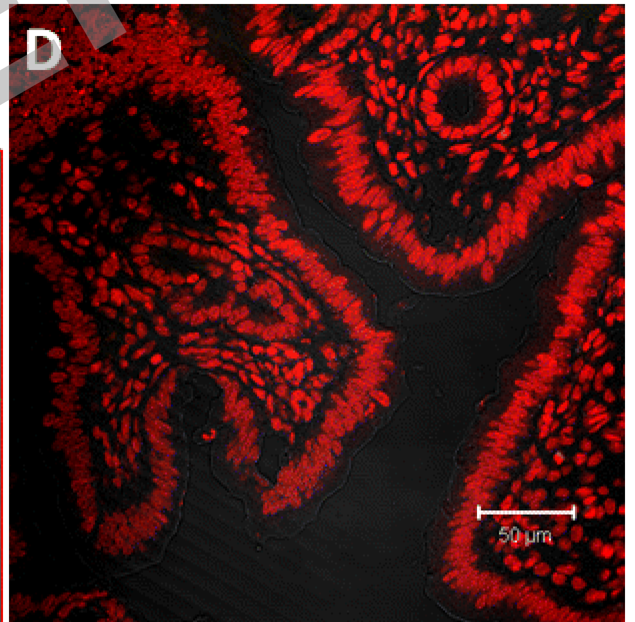

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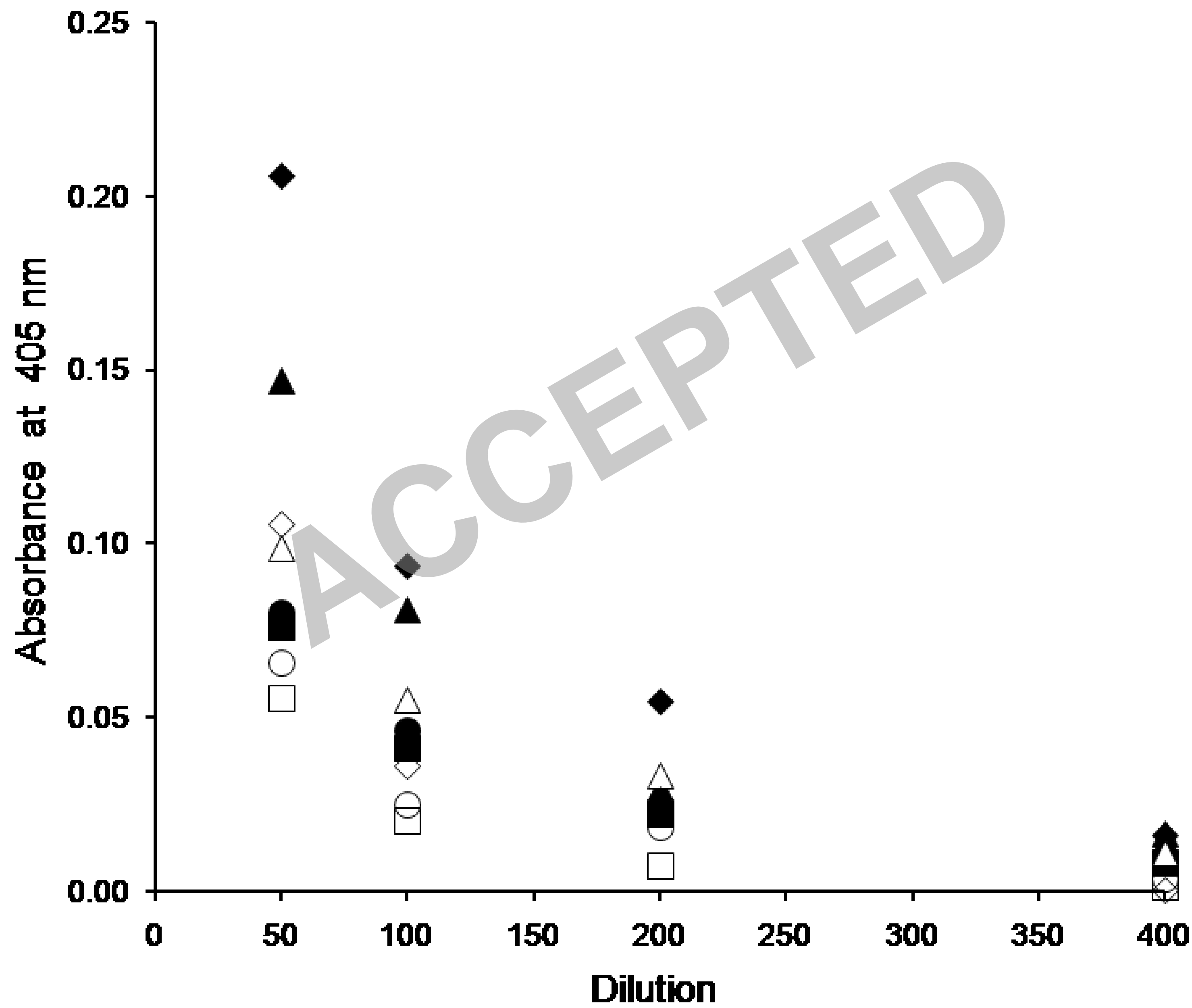

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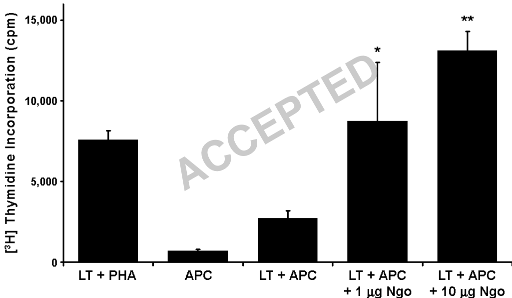

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A

0.0% 0.0%

99.0% 0.0%

B

43.4% 5.7%

27.4% 23.4%

C

38.1% 12.2%

23.6% 26.1%

D

21.0% 5.9%

33.2% 39.9%

E

19.7% 6.3%

14.9% 59.2%

CD4

TGF-β1

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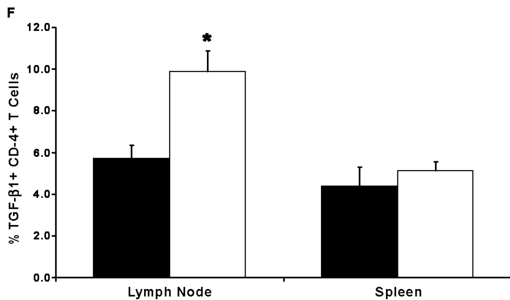
F



A

1.2%	6.9%
87.7%	4.2%

B

2.9%	12.7%
81.0%	3.4%

C

3.7%	13.8%
77.1%	5.4%

D

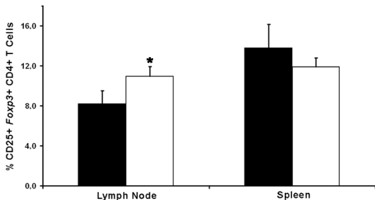
2.4%	12.8%
75.8%	9.0%

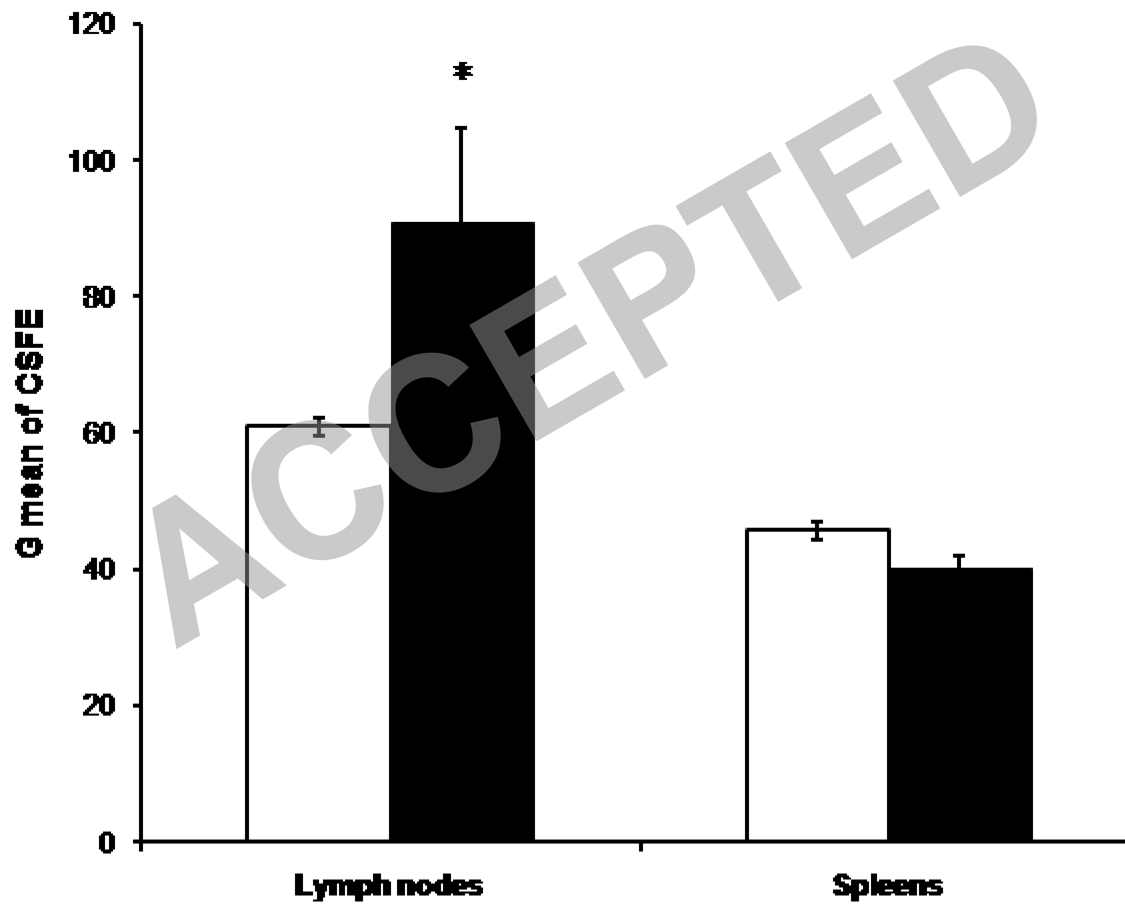
Foxp3

CD25

ACCEPTED

E





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CD11b

A 1.4% 3.3% 0.3%

B 0.4% 3.0% 18.5%

C 0.4% 5.2% 20.1%

D 0.9% 5.6% 47.5%

TGF-β1