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STAT4 Regulates Antiviral Gamma Interferon Responses and Recurrent Disease during Herpes Simplex Virus 2 Infection

Alexandra Svensson,[a] Petra Tunbäck,[b] Inger Nordström,[a] Andrey Shestakov,[a] Leonid Padyukov,[c] and Kristina Eriksson[a]

Department of Rheumatology & Inflammation Research, Sahlgrenska Academy, University of Gothenburg, Gothenburg,[a] Department of Dermatovenerology, Sahlgrenska University Hospital, Gothenburg,[b] and Rheumatology Unit, Department of Medicine, Karolinska Institutet, Stockholm,[c] Sweden

STAT4 is an important transcription factor that contributes to the incidence and severity of different autoimmune diseases and is implicated in the antiviral immune responses in mice. In this study, we evaluated the role of STAT4 in human and murine herpes simplex virus 2 (HSV-2) infections. We show that STAT4 regulates antiviral gamma interferon (IFN-γ) responses and disease severity during chronic HSV-2 infections in humans and vaccine-induced IFN-γ-mediated protection against HSV-2 infection in mice. In a cohort of 228 HSV-2-infected individuals, representing both patients with recurrent disease and asymptomatic HSV-2 carriers, we found that genetic variations in the *STAT4* gene were associated with asymptomatic HSV-2 infection, as well as with increased *in vitro* secretion of IFN-γ in response to the virus. Mice that lacked STAT4 had impaired HSV-2-specific IFN-γ production and delayed-type hypersensitivity responses following vaccination, which led to impaired viral clearance in the genital tract of vaccinated animals after a genital HSV-2 challenge. We conclude that STAT4 plays an important role in IFN-γ-mediated HSV-2-specific immunity, affecting the severity of genital HSV-2 infection.

Genital herpes infection caused by herpes simplex virus 2 (HSV-2) is one of the most common sexually transmitted infections in the world. Up to 25% of the Swedish population is seropositive for HSV-2 (5, 11, 31), while the prevalence in some sub-Saharan African countries is as high as 80% (47). The clinical effects of HSV-2 infection range from no symptoms (asymptomatic infection) to severe and recurrent episodes of genital lesions and ulcers. Although the mechanisms underlying these different disease outcomes are not known, strong gamma interferon (IFN-γ) responses (10, 37), previous HSV-1 infection (22), high levels of mannan-binding lectin (13), specific HLA alleles (23), and variants of both the *TLR2* gene (6) and the gene encoding mannose-binding lectin 2 (34) increase the likelihood of an asymptomatic infection.

Efficient HSV-2-specific acquired immunity requires $CD4^+$ T cells and IFN-γ production. Mice that lack $CD4^+$ T cells, IFN-γ, or the Th1-inducing transcription factor T-bet are unable to mount a protective immune response to HSV-2 following vaccination (14, 15, 40). However, the treatment of mice with IFN-γ circumvents the need for $CD4^+$ T cells, which implies that the main function of $CD4^+$ T cells in HSV-2-specific immunity is to produce IFN-γ (14). The situation is similar in humans, as HSV-2-infected individuals with recurrent disease have impaired virus-specific IFN-γ responses compared with asymptomatic HSV-2 carriers (10, 37).

IFN-γ secretion and Th1 differentiation are induced and maintained by two diverse and concurrent pathways that are characterized by the transcription factors T-bet and STAT4 (20, 42). Mice that lack either T-bet or STAT4 have impaired IFN-γ and Th1 responses but are still able to produce some IFN-γ, while mice that lack both T-bet and STAT4 have no Th1 responses, even under Th1-polarizing conditions (12, 20, 43). It is generally believed that Th1 differentiation is induced by T-cell receptor (TCR) activation, which leads to T-bet expression. In addition to IFN-γ secretion, T-bet induces the expression of the interleukin-12 (IL-12) receptor β2 on the T-cell surface (1), which allows the T cell to respond to IL-12 and to induce further IFN-γ production via STAT4 signaling (17, 18, 45). The secreted IFN-γ binds to the IFN-γ receptor and signals via Stat1 so as to amplify the expression of T-bet, thereby stabilizing the Th1 polarization (24).

Given that STAT4 is important for Th1 immunity, mice that are deficient for STAT4 are highly susceptible to infections (19). This is particularly true in the case of parasitic infections, whereby STAT4 deficiency is associated with increased parasitic burden and decreased IFN-γ responses to pathogens such as *Leishmania major* and *Toxoplasma gondii* (9, 38). Studies of viral infections, i.e., with lymphocytic choriomeningitis virus (LCMV), influenza virus, and HSV-1, have revealed diverse roles for STAT4 depending on the type of infection and the immune mediators that are involved in the antiviral response (2, 3, 7, 16, 29). As a consequence of the abrogated Th1 responses, STAT4-deficient mice have a decreased production of inflammatory cytokines (e.g., tumor necrosis factor alpha [TNF-α]) during autoimmune disease and are thereby resistant to the development of Th1-mediated autoimmune diseases, such as experimental autoimmune encephalomyelitis, arthritis, colitis, myocarditis, and diabetes (19).

Even though the role of STAT4 has been studied extensively in animal models of infectious diseases, little is known about its role in human infections. Most studies of human STAT4 have focused on correlations between variations in the *STAT4* gene and the incidence of autoimmune diseases. These diseases include rheumatoid arthritis, systemic lupus erythematosus (SLE), and primary Sjögrens syndrome, for which single nucleotide polymorphisms (SNPs) in the *STAT4* gene have been associated with the disease incidence in several different populations (21). Furthermore, genetic variations in *STAT4* may also be involved in disease

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Address correspondence to Kristina Eriksson, Kristina.Eriksson@microbio.gu.se.

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severity; a polymorphism in the *STAT4* gene has been associated with a more severe disease outcome in SLE patients (44).

The present study was undertaken to evaluate the role of STAT4 in immune-mediated control of HSV-2 infection. For this purpose, DNA samples from 143 patients with recurrent HSV-2 infection and 85 asymptomatic carriers of HSV-2 were screened for variations in 7 SNPs of the *STAT4* gene. The SNPs were subsequently correlated with HSV-2 disease severity and the levels of HSV-2-induced IFN-γ production by isolated peripheral blood mononuclear cells (PBMC). To study in greater detail the role of STAT4 during HSV-2 infection, mice that lack STAT4 were vaccinated and/or infected with HSV-2 and their abilities to mount a Th1 response and to resist the infection were monitored. We show that a variation in the human STAT4 gene is associated with increased HSV-2-specific IFN-γ production and asymptomatic disease. In addition, we show that STAT4 is required for adequate HSV-2-specific IFN-γ responses and for the induction of sterilizing immunity in mice.

MATERIALS AND METHODS

Human sample collection. (i) HSV-2-infected individuals. DNA samples were collected from 228 HSV-2-infected individuals (male, 57%; female, 43%) who were recruited from the sexually transmitted disease (STD) clinics at Sahlgrenska University Hospital, Borås Hospital, and Uddevalla Hospital, Sweden. The average age of the subjects was 39 years for men (range, 20 to 70 years) and 37 years for women (range, 20 to 68 years). Permission for this study was granted by the Ethics Committee of the University of Gothenburg, and all patients gave informed consent. HSV-2 infection was confirmed serologically by enzyme-linked immunosorbent assay (ELISA) (see below). The patients were divided into two groups based on clinical status.

The symptomatic HSV-2 infection group comprised 143 patients: 77 males (54%; average age, 38 years; range, 23 to 70 years) and 66 females (46%; average age, 38 years; range, 23 to 68 years) who had a typical history of recurrent genital herpes. Symptomatic HSV-2 infection was confirmed by PCR, and serology and history clinical recurrence were recorded. The aim was to recruit individuals with more than six relapses per year to ensure the presence of clinical disease. Overall, 113 of the 143 symptomatically infected individuals fulfilled this criterion, whereas 30 of the symptomatic individuals had fewer than six relapses per year. In total, 56 of the symptomatic individuals were on antiviral treatment at the time of sampling.

The asymptomatic HSV-2 infection group comprised 85 patients: 54 males (64%; average age, 40 years; range, 21 to 66 years) and 31 females (36%; average age, 34 years; range, 20 to 52 years) who were seropositive for HSV-2 without any signs of clinical disease. Asymptomatic patients were recruited from an ongoing screening study of HSV-2 infection in visitors to the STD clinics and among the partners of the HSV-2-infected patients. All the subjects were provided with detailed information about the clinical spectrum of herpes and interviewed about genital symptoms. Putatively asymptomatic HSV-2-seropositive patients who after receiving the above information admitted to having genital symptoms were excluded from the study.

Three individuals (all symptomatic) were on immunosuppressive treatment (for rheumatoid arthritis, Bechterew's disease, and CNS vasculitis, respectively) at the time of sampling. Other diseases that were present in the study population were hepatitis B (one symptomatic individual), diabetes (two symptomatic individuals), allergic asthma (one symptomatic individual), multiple sclerosis (one symptomatic individual), and lichen sclerosis (one asymptomatic individual).

(ii) Control subjects. For the control group, 162 healthy HSV-2-negative adult blood donors were recruited from the Blood Bank at Sahlgrenska University Hospital. This group consisted of 54% males and 46% females with an average age of 40 and 41 years, respectively (ranges, 20 to 63 and 20 to 65 years, respectively). All individuals were screened for HSV-2 infection by ELISA. All the individuals were routinely screened (and found to be negative) for blood-derived contaminating diseases, including hepatitis A and B, HIV-1 and -2, and human T-lymphotropic virus I (HTLV-I) and -II.

Genotyping. DNA for genotyping was extracted from heparinized venous blood using the salting-out method. Genotyping was performed on 228 HSV-2-infected individuals and 162 control subjects for seven *STAT4* SNPs [rs7574865 (T→G), rs4853543 (A→G), rs7572482 (A→G), rs13017460 (A→G), rs7601754 (G→A), rs3024896 (C→T), and rs6752770 (A→G)] using TaqMan allelic discrimination (premade or customized Applera assays) at the Core Facility of the Sahlgrenska Academy, Gothenburg University, Sweden. The seven SNPs were chosen based on the following criteria: (i) they are commonly detected in Caucasians (minor allelic frequency > 0.20, according to the Hap-Map-CEU northern and western European population data in the NCBI database), including the Swedish population (36); and (ii) they are spread across the entire *STAT4* gene. The genotyping rate was ≥94.4% (range, 94.4% to 100.0%).

ELISA for detection of HSV-2-specific antibodies. Plasma samples from HSV-2-uninfected individuals were screened for anti-HSV-2 glycoprotein G (gG) antibodies using an HSV-2 ELISA kit according to the manufacturer's manual (HerpesSelect2 ELISA IgG; Focus Technologies), and plasma samples from HSV-2-infected individuals were screened for mgG-2-specific antibodies using an ELISA, as previously described (46).

Virus and antigen preparation. HSV-2 strain 333 and the attenuated strain Lyons (35) were obtained as described previously (40). For antigen preparation, the virus was inactivated by exposure to UV light for 30 min.

IFN-γ responses of human PBMC. Freshly isolated PBMC (1×10^6 cells/ml) were cultured in 96-well plates in a total volume of 200 μl x-vivo medium (Lonza, Verviers, Belgium) supplemented with 1% L-glutamine, in the presence or absence of UV-inactivated HSV-2 (corresponding to 4×10^5 PFU/ml). Culture supernatants were collected after 48 h and stored at −20°C until assayed for IFN-γ using a human IFN-γ ELISA DuoSet kit (R&D Systems) according to the manufacturer's instructions.

Mice. For this study, 6-to-8-week-old female $STAT4^{-/-}$ and wild-type BALB/c mice were purchased from The Jackson Laboratory. All mice were maintained under standard conditions of temperature and light in the animal facilities at the Department of Rheumatology and Inflammation Research, University of Gothenburg. This study was approved by the Animals Ethics Committee in Gothenburg, Sweden.

Genital HSV-2 infection. Mice were pretreated with Depo-Provera (Pharmacia) and then vaccinated and infected as previously described (40). Mice were examined daily for vaginal inflammation, neurologic illness, and death. The severity of diseases was graded as follows: 0, healthy; 1, genital erythema (redness and swelling); 2, moderate genital inflammation (hair loss, small nonpurulent lesions); 3, severe and purulent genital lesions and/or generally bad condition; 4, hind-limb paralysis; and 5, death (26).

Virus quantification. Vaginal washings were collected 2 days postinfection and stored at −70°C until further use. Spinal cords were collected and homogenized 7 days (unvaccinated mice) or 14 days (vaccinated mice) after infection and stored at −70°C until further use. DNA was extracted in a MagNA Pure LC robot (Roche Diagnostics, Mannheim, Germany) using a MagNA Pure DNA Isolation kit according to the manufacturer's instructions. The number of HSV-2 DNA copies was determined by quantitative PCR, as described previously (28), and is expressed in terms of genome equivalents (geq).

DTH. Delayed-type hypersensitivity (DTH) reactions were performed as previously described (40).

Cytokine responses of mouse splenocytes. Assays of IFN-γ production from $CD4^+$ spleen cells and IL-12 production from spleen cells were performed using a cell ELISA that utilizes murine IFN-γ and IL-12 DuoSet ELISA kits (R&D Systems), as previously described (40). $CD4^+$ T-cell activation was performed with CD8-depleted splenocyte cultures in the presence of inactivated whole virus, as described previously (40). The 48-h

TABLE 1 Allele frequencies in symptomatic and asymptomatic HSV-2-infected individuals for seven SNPs of the *STAT4* gene[a]

SNP	Minor allele	% (n^a) allele distribution		P value (χ^2)
		Symptomatic group	Asymptomatic group	
rs7574865	T	21 (56)	29 (43)	0.0509
rs4853543	A	38 (103)	35 (52)	0.5138
rs7572482	A	41 (112)	29 (44)	0.0184
rs13017460	G	62 (172)	56 (84)	0.2034
rs7601754	G	18 (50)	14 (21)	0.2494
rs3024896	T	14 (38)	12 (18)	0.5260
rs6752770	G	26 (69)	26 (37)	0.9840

[a] *n*, number of chromosomes.

culture supernatants from HSV-2-activated spleen cells were also analyzed using a Mouse Th1/Th2 10plex Ready-to-Use FlowCytomix Multiplex kit (eBioscience) and a FACSCanto flow cytometer (BD).

Statistical analysis. For human samples, genotype and allele frequencies were compared using the chi-square test. Differences were considered significant at $P < 0.05$. The calculations were conducted using online software. Hardy-Weinberg tests, haplotype association tests, and permutation tests were performed using Haploview software. Bonferroni's correction for multiple testing was used when appropriate. All the allele and genotype frequencies were found to be in Hardy-Weinberg equilibrium. Statistical analyses of human IFN-γ secretion and the mouse experiments were performed using the nonparametric Mann-Whitney *U* test and GraphPad Prism ver. 5 software. Differences were considered significant at $P < 0.05$.

RESULTS

Genetic variations in *STAT4* are associated with HSV-2 disease severity. To assess the role of STAT4 in HSV-2 infection, we compared seven different SNPs (rs7574865, rs4853543, rs7572482, rs13017460, rs7601754, rs3024896, and rs6752770) of the human *STAT4* gene in asymptomatic and symptomatic HSV-2-infected individuals, as well as in healthy controls. We did not detect any correlations between the *STAT4* SNPs and the incidence of HSV-2 infection when comparing HSV-2-infected individuals and healthy controls (data not shown). However, for one of the SNPs, rs7572482, which is located in an intron close to the 5′ untranscribed region (UTR), the minor A allele variant correlated with the incidence of symptomatic infection (Table 1) ($P = 0.0184$).

TABLE 2 Haplotype frequencies in symptomatic and asymptomatic HSV-2-infected individuals for the seven SNPs of the *STAT4* gene[a]

Haplotype	% (n^b) frequency			P value (χ^2)
	Symptomatic group	Asymptomatic group	HSV-2-seronegative group	
CGAGAGG	35 (96)	31 (47)	33 (102)	0.4969
CGAGAAA	7.4 (20)	8.3 (12)	4.5 (14)	0.7243
CGAGAGA	8.2 (23)	5.4 (8)	8.9 (28)	0.2741
CAATAGG	4.1 (11)	12 (18)	4.2 (13)	0.0029
TAATGAG	3.9 (11)	6.1 (9)	4.5 (14)	0.3125
CAGGAAA	4.9 (13)	2.9 (4)	5.0 (15)	0.3264
CAGGAGG	3.4 (10)	4.5 (2)	4.7 (14)	0.5912

[a] Data were estimated using Haploview 4.2 software in the following order: rs3024896, rs13017460, rs7601754, rs7574865, rs6752770, rs4853543, and rs7572482. Only haplotypes with frequency > 4% are presented.
[b] *n*, number of chromosomes.

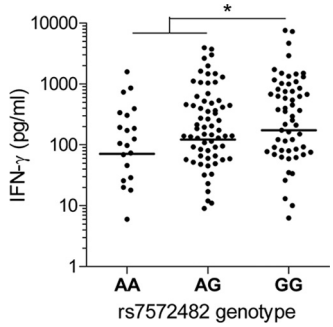

FIG 1 Increased HSV-2-specific IFN-γ secretion in individuals homozygous for the major allele variant of the *STAT4* SNP rs7572482. PBMC from HSV-2-infected individuals (66 asymptomatic and 111 symptomatic) were stimulated with UV-inactivated HSV-2 for 48 h and then analyzed for IFN-γ secretion. The data shown represent the individual levels (circles) and the median levels (lines) of secreted IFN-γ from HSV-2-infected individuals with the *AA* ($n = 26$), *AG* ($n = 84$), or *GG* ($n = 67$) genotype at rs7572482. *, $P < 0.05$ (Mann-Whitney test).

However, this difference was not significant after Bonferroni's correction for multiple testing. We also investigated whether the minor A allele of rs7572482 correlated with the need for antiviral treatment. The A allele distribution was 45% in HSV-2-infected individuals who required acyclovir treatment, compared with 33% in HSV-2-infected individuals who did not require antiviral treatment ($P < 0.02$).

To assess further the role of *STAT4* variations in HSV-2 infection, we analyzed the haplotype frequencies of the seven *STAT4* SNPs. Of the seven STAT4 haplotypes with >4% frequency in at least one of the groups, the CAATAGG combination was significantly more common in asymptomatically infected individuals than in individuals with symptomatic HSV-2 infection and in healthy controls. The CAATAGG haplotype frequencies were 4% and 12% in symptomatically and asymptomatically infected individuals, respectively ($P = 0.0029$), and remained significant after 100,000 permutations ($P = 0.012$) (Table 2).

Given that previous HSV-1 infection correlates with asymptomatic infection, we compared the frequencies of HSV-1 seropositivity in the asymptomatic and symptomatic HSV-2-infected individuals but found no statistically significant differences.

The *G* allele of *STAT4* rs7572482 is associated with stronger HSV-2-specific IFN-γ recall responses. Given the observed association of *STAT4* variations with HSV-2 infection and the necessity for STAT4 in Th1-mediated immunity (20), we examined whether variations in the *STAT4* gene affected IFN-γ production. For this purpose, we measured the levels of HSV-2-specific IFN-γ secretion in PBMC from HSV-2-infected individuals (both asymptomatic and symptomatic) who had different *STAT4* rs7572482 genotypes, representative for the CAATAGG haplotype. The levels of HSV-2-specific IFN-γ production were higher in the PBMC from individuals who were homozygous for the major *G* allele than in those from individuals who were heterozygous or homozygous for the minor *A* allele ($P = 0.04$; Fig. 1). Following HSV-2 stimulation, the median level of IFN-γ secretion was more than 2-fold higher in the cells from HSV-2-infected individuals with the *GG* genotype than in individuals with the *AA* genotype (Fig. 1). We and others have previously reported that asymptomatic HSV-2 infection is associated with higher T-cell recall IFN-γ responses, compared with symptomatic HSV-2 in-

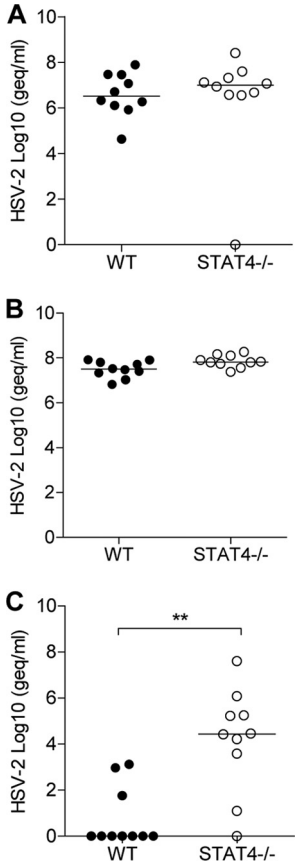

FIG 2 Vaccinated STAT4$^{-/-}$ mice do not develop sterilizing immunity. Naïve (A and B) and vaccinated (C) wild-type mice (filled circles) and STAT4$^{-/-}$ mice (empty circles) were challenged intravaginally with HSV-2. The levels of HSV-2 DNA were measured in vaginal fluids obtained 48 h after HSV-2 inoculation (A and C) and in spinal cords 7 days after HSV-2 inoculation (B). The data shown represent the $\log_{10}$ values for HSV-2 DNA copy numbers (geq; genome equivalents/milliliter) for each individual (circle) and the median for each group (line) obtained in two independent experiments with five mice/group. **, $P < 0.01$ (Mann-Whitney test).

fection (10, 37). However, we could not confirm this observation in the current patient cohort (data not shown).

Impaired viral clearance in the genital tracts of vaccinated STAT4$^{-/-}$ mice. To study the immune response to genital HSV-2 infection, naïve and vaccinated STAT4-deficient and wild-type mice were infected intravaginally with HSV-2 strain 333. The mice were examined daily for genital inflammation and neurologic illness, and the levels of viral DNA in vaginal washes and spinal cords were measured. Initially, we measured disease development during primary infection. No major differences between the STAT4$^{-/-}$ and wild-type mice were observed after genital inoculation of HSV-2. Neither disease development nor the survival rates were affected by STAT4 deficiency (data not shown). The STAT4$^{-/-}$ and wild-type mice had comparable levels of HSV-2 DNA in vaginal washes obtained 2 days postinfection (Fig. 2A) and in the central nervous system (CNS) on day 7 postinfection (Fig. 2B).

Subsequently, we challenged the vaccinated mice and assessed the viral loads and disease development. The viral load in the genital tract 2 days postinfection was significantly higher in the vaccinated STAT4$^{-/-}$ mice than in the wild-type mice (Fig. 2C). At this time point, we did not detect any viral DNA in the vaginal washes from wild-type mice whereas the median amount of viral DNA in the STAT4$^{-/-}$ mice was 4.4×10^6 ($\log_{10}$) copies of HSV-2 DNA/ml. Two weeks after the HSV-2 challenge, the vaccinated mice were sacrificed to measure the virus content in the CNS. None of the wild-type mice had any detectable levels of viral DNA, while 2 out of 10 STAT4$^{-/-}$ mice had measurable levels of HSV-2 DNA (data not shown). Nevertheless, the STAT4$^{-/-}$ mice were protected against terminal illness to the same extent as all the wild-type mice, and all the STAT4$^{-/-}$ mice survived the HSV-2 challenge.

Impaired HSV-2-specific Th1 responses in mice that lack STAT4. To assess the role of STAT4 in virus-specific CD4$^+$ T-cell responses, we measured the DTH and cytokine responses of the STAT4$^{-/-}$ and wild-type mice 4 weeks after HSV-2 vaccination. The mice that lacked STAT4 had impaired HSV-2-specific CD4$^+$ T-cell responses both *in vivo* and *in vitro*. The *in vivo* CD4$^+$ T-cell response was evaluated by measuring DTH footpad swelling 48 h after injection of inactivated HSV-2. The HSV-2-specific inflammatory responses were significantly lower in the STAT4$^{-/-}$ mice than in the wild-type mice (Fig. 3A). HSV-2-specific IFN-γ secretion in spleen cell cultures that were depleted of CD8$^+$ T cells was measured using a cell ELISA. We found that the levels of HSV-2-specific IFN-γ secretion from the CD4$^+$ T cells of the STAT4$^{-/-}$ mice were negligible compared to the high levels seen in the cells from the wild-type mice (Fig. 3B). In addition, we screened the 48-h culture supernatants of HSV-2-activated spleen cells for Th1, Th2, and Th17 cytokines. We confirmed that the IFN-γ levels were significantly reduced in the STAT4-deficient mice (Fig. 3C). However, there were no significant differences in the levels of the other Th1 cytokines (Fig. 3C) or Th2 cytokines (Fig. 3D) between the wild-type and STAT4$^{-/-}$ mice. We could not detect IL-17A in any of the culture supernatants (data not shown).

DISCUSSION

In the present report, we provide evidence to implicate STAT4 in HSV-2-specific immunity in both humans and mice. We show that a variation in the human *STAT4* gene is associated with asymptomatic HSV-2 infection and increased HSV-2-specific IFN-γ secretion. In addition, we show that HSV-2 does replicate in the genital tracts of vaccinated STAT4-deficient mice, most likely due to their impaired HSV-2-specific IFN-γ responses.

Genetic variations in the human *STAT4* gene have previously been associated with Th1-mediated inflammatory diseases, such as rheumatoid arthritis, SLE, and Sjögrens syndrome (21). The present study is, to the best of our knowledge, the first to show that *STAT4* variations are associated with an infectious disease. In our study population, which consisted of 143 symptomatic and 85 asymptomatic HSV-2-infected individuals, we found that one of the haplotypes of the human *STAT4* gene, consisting of seven SNPs from the same haplotype block, was associated with enhanced IFN-γ responses and asymptomatic HSV-2 infection. Therefore, *STAT4* represents, together with certain HLA alleles (23), TLR-2 (6), and mannan-binding lectin gene 2 (34), a human gene variation that might predispose individuals to asymptomatic HSV-2 infection. However, the effect size of these associations is very modest and can serve only as an indication that the gene is important for the expression of a specific phenotype. This also implies that more-profound sequence changes in this immune-

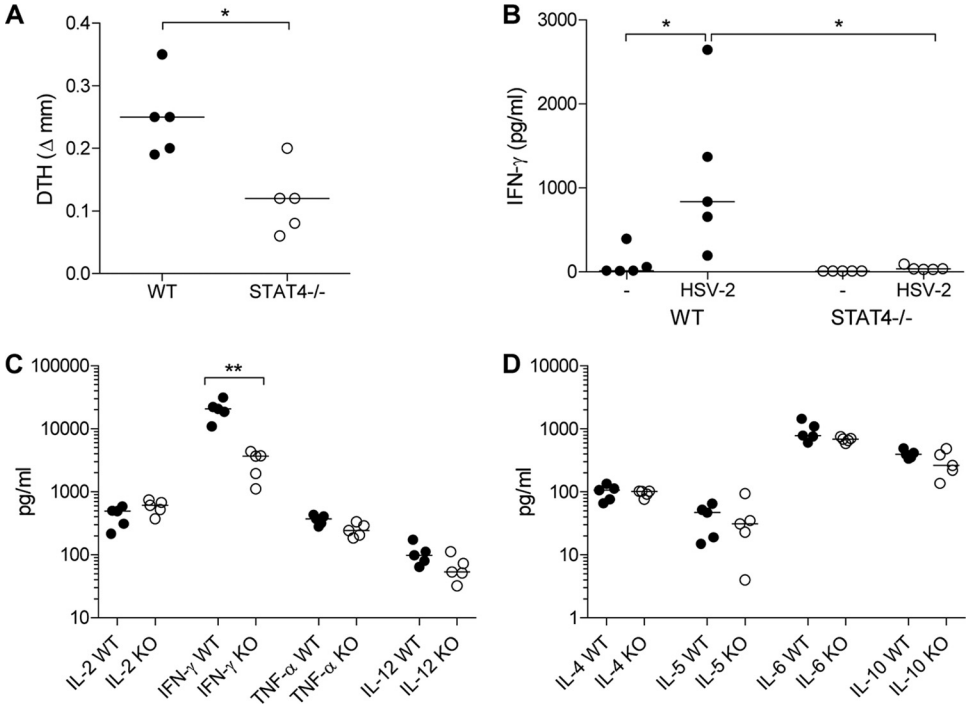

FIG 3 Impaired HSV-2-specific IFN-γ and DTH responses in STAT4$^{-/-}$ mice. Wild-type mice (filled circles) and STAT4$^{-/-}$ mice (open circles) were vaccinated intravaginally with HSV-2 Tk- and analyzed 4 weeks later for virus-specific T-cell responses. (A) DTH responses were elicited by injection of UV-inactivated HSV-2 in the left footpad and mock antigen in the right footpad. Specific footpad swelling is expressed as the difference between the increases in thickness of the experimental and control footpads after 48 h. (B) HSV-2-specific IFN-γ secretion from spleen cell populations depleted of CD8$^+$ T cells was assessed by cell ELISA. (C and D) Th1 cytokine responses (C) and Th2 cytokine responses (D) were determined in 48-h spleen cell cultures that were exposed to inactivated HSV-2, as described in Materials and Methods. The data shown represent the levels of (A) footpad swelling and (B to D) cytokine secretion (in picograms/milliliter) for individual animals (circles) and the median value (line) for experiments with five animals/group. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test).

regulating gene may cause severe impairments of the antiviral immune response.

We were not able to correlate genetic variations in the *STAT4* gene with susceptibility to HSV-2 infection. However, genetic variations in both innate and adaptive immune-regulating genes may affect susceptibility to HSV-2 infection; SNPs in the genes that encode the Th1-inducing transcription factor T-bet, TBX21 (39), and in the *TLR3* gene (41) are risk factors for HSV-2 acquisition. Thus, the host repertoire of several immune-regulating genes may influence both susceptibility to and the severity of HSV-2-mediated disease.

The roles of STAT4 in human cells and during human infections have not been extensively studied. Similar to murine cells, human CD4$^+$ T cells secrete IFN-γ upon IL-12 stimulation and STAT4 activation (4, 27). However, sufficient IFN-γ secretion is dependent upon IL-18 (27), as well as TCR stimulation and T-bet expression (32). In the present report, we show that a genetic variation in the human *STAT4* gene affects the *in vitro* levels of IFN-γ produced in response to HSV-2 recall activation. The major allele of *STAT4* rs7572482 is associated with increased HSV-2-specific IFN-γ secretion *in vitro*, as well as with asymptomatic HSV-2 infection. This supports the results of previous studies, in which increased IFN-γ responses were associated with symptom-free HSV-2 infection (8, 10, 37). Taken together, our data suggest that variations in the *STAT4* gene are involved in the regulation of HSV-2 disease severity, possibly by modulating virus-specific IFN-γ responses.

The significance of IFN-γ and CD4$^+$ T-cell responses in HSV-2 immunity has been well described in murine models of genital HSV-2 infection, in which IFN-γ (14, 25, 30), and T-bet expression (39) are critical for viral clearance and resistance to infection in the genital tract. Here, we show that vaccinated STAT4-deficient mice, in contrast to wild-type mice, do not develop sterilizing immunity to HSV-2 in the genital tract. The increased local viral load in STAT4$^{-/-}$ mice is associated with impaired CD4$^+$ T-cell responses, as evidenced by the impaired DTH responses *in vivo* and reduced IFN-γ secretion *in vitro*. However, even though we detected considerable amounts of viral DNA in the genital tracts of the vaccinated STAT4$^{-/-}$ mice, it appears that the virus did not reach the CNS. This implies that STAT4 is important for the vaccine-induced prevention of vaginal viral replication, whereas it plays a marginal role in preventing the virus from spreading to the CNS. This notion differs from the conclusions drawn from studies using the closely related HSV-1. Allen and colleagues have shown in a model of ocular HSV-1 infection that vaccinated STAT4-deficient mice resolve the infection to the same extent as wild-type mice (2). This discrepancy could be due to the different routes of infection. Moreover, the validity of our findings for STAT4-deficient mice is strengthened by the results of our human experiments, which show that STAT4 variations affect HSV-2-specific IFN-γ production. Therefore, we propose that STAT4 is important for HSV-2-specific IFN-γ responses in both humans and mice.

None of the analyzed polymorphisms of the *STAT4* gene were

associated with the incidence of HSV-2 infection in humans. Likewise, STAT4 deficiency did not affect either the viral load or disease progression in primary HSV-2 infection in mice. This is somewhat different from previous findings obtained using a model of ocular HSV-1 infection. Allen et al. found that STAT4-deficient animals did not differ from wild-type animals with respect to survival or disease development, despite having higher levels of viral replication in the eye (2). Banerjee et al. have reported that STAT4$^{-/-}$ mice are more susceptible to HSV-1-induced encephalitis, although in their hands the animals also succumbed earlier to the infection (3). It is interesting that the two Th1 transcription factors STAT4 and T-bet play different roles in HSV-2 infection in both humans and mice. T-bet appears to be more important during primary infection (39, 40), while STAT4 controls viral reactivation. This is probably linked to the functional differences between these two transcription factors. While T-bet expression is required during the early phase of Th1 differentiation, STAT4 is required later on to stabilize and maintain the Th1 phenotype by amplification of both T-bet and IFN-γ expression (33). Thus, the importance of STAT4 appears to be in the long-time maintenance of the HSV-2-specific IFN-γ responses that are required to control viral reactivation.

In summary, we show that STAT4 regulates HSV-2-specific CD4$^+$ IFN-γ responses in humans and mice. Genetic variations in the human *STAT4* gene are associated with increased IFN-γ synthesis and asymptomatic HSV-2 infection, while vaccinated mice deficient in STAT4 have impaired HSV-2-specific IFN-γ responses and do not develop sterilizing immunity. We suggest that certain genetic variations in *STAT4* affect HSV-2 replication and disease severity by altering the IFN-γ-mediated antiviral immune responses.

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