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Epstein-Barr Virus Negativity among Individuals Older than 60 Years Is Associated with HLA-C and HLA-Bw4 Variants and Tonsillectomy

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Epstein-Barr virus (EBV) infects ~95% of the adult population. The factors that confer protection in the remaining ~5% remain unknown. In an exploratory study, we assessed immunogenetic factors and tonsillectomy in a cohort of 17 EBV-negative and 39 EBV-positive healthy individuals aged >60 years. Analyses of HLA genotypes revealed an association between EBV negativity and the presence of HLA-C-35T/T and/or HLA-Bw4 alleles. In addition, EBV-negative donors presented with a history of tonsillectomy more often than EBV-positive donors.

For most, primary Epstein-Barr virus (EBV) infection occurs during childhood and is asymptomatic or causes an acute self-limiting lymphoproliferative disease (infectious mononucleosis). After acute infection, EBV enters life-long latency, which leaves the infected individual at risk for viral reactivation and, in rare cases, the development of EBV-associated malignancy (1). Why ~5% of the adult population remain EBV-seronegative throughout their lives is not known, yet understanding natural resistance to EBV infection might provide fundamental insight into the host-pathogen interaction and pinpoint targets for novel preventive and/or therapeutic strategies.

Here, after Institutional Review Board (IRB) approval and written informed consent, 515 consecutive healthy blood donors aged >60 years who were routinely presenting at the Blood Transfusion Center Basel were serologically tested for EBV by multiplex microparticle technology (Luminex 200 Technology, Luminex, Austin, TX, USA). Seventeen of 515 donors were EBV seronegative (median age, 64 years; range, 62 to 70 years; 3 female, 14 male). The seropositive control cohort consisted of 39 individuals (median age, 64 years; range, 63 to 70 years; 5 female, 34 male). In the EBV-seronegative cohort, we (i) tested whether EBV seronegativity reflects the absence of viral genome and of EBV-specific cellular memory and (ii) searched for specific HLA-B and HLA-C polymorphisms associated with EBV negativity.

To relate serostatus and the presence of EBV DNA, a sensitive PCR was performed on DNA extracted from B cells (the primary target cells of EBV) (2), using a published real-time PCR protocol (3). The EBV genome was not detected in any of the 17 EBV-seronegative donors. In contrast, 22/25 EBV-seropositive donors tested positive [mean, 97 genome equivalents (geq)/(1×10^6) B cells; range, <3 to 1,072 geq/(1×10^6) B cells] (Fig. 1A). To assess whether a negative EBV serostatus also indicates the absence of EBV-specific cellular immunity, we applied a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay as previously described (4, 5; also data not shown). IFN-γ secretion in bulk peripheral blood mononuclear cells (PBMC) was measured in response to a pool of peptides consisting of 91 major histocompatibility complex (MHC) class I-restricted and 33 MHC class II-restricted optimal EBV epitopes, testing for $CD8^+$- and $CD4^+$-specific reactivity (4, 5). We did not detect responses to the peptide pools in any of the 17 EBV-seronegative individuals, whereas PBMC from 13/24 and 19/23 EBV-seropositive donors secreted IFN-γ in response to one or multiple MHC class I- and class II-restricted EBV peptide pools (Fig. 1B and C). Together, these data established that neither direct (EBV DNA) nor indirect (T cell reactivity) evidence for latent EBV infection was present in any of the 17 EBV-seronegative individuals. The expression of the EBV receptor CD21 and the coreceptor HLA-DR did not differ between the cohorts as assessed with fluorescence-activated cell sorter (FACS) staining. And, importantly, B cells from EBV-seronegative donors could readily be infected and transformed *in vitro*, excluding host resistance to EBV infection at the target cell level (data not shown).

Control of established EBV infection depends on functional cytotoxic $CD8^+$ T cells, and yet NK cells (i.e., innate immune cells) play an important role in shaping the clinical phenotype of EBV infection as well (6–9). NK cell functionality is largely determined by a family of receptors that interact with HLA I molecules, the killer cell immunoglobulin-like receptors (KIRs) (10–12). HLA and HLA-KIR compound genotypes have been shown to influence resistance to HIV infection among highly exposed seronegative individuals (13–16). In chronic HIV infection, HLA-Bw4 alleles, which interact with the inhibitory NK cell receptor KIR3DL1, have been collectively attributed a protective role, particularly the subset of Bw4 allotypes containing isoleucine at position 80 (Bw4 80Ile) as opposed to threonine (Bw4 80Thr) (17). Also of note is an allele variant 35 kb upstream from HLA-C (−35 C), known to be related to higher HLA-C mRNA expression levels, which has recently been associated with slower progression to AIDS (18). In EBV infection, no consistent roles for specific HLA class I and KIR variants have been reported.

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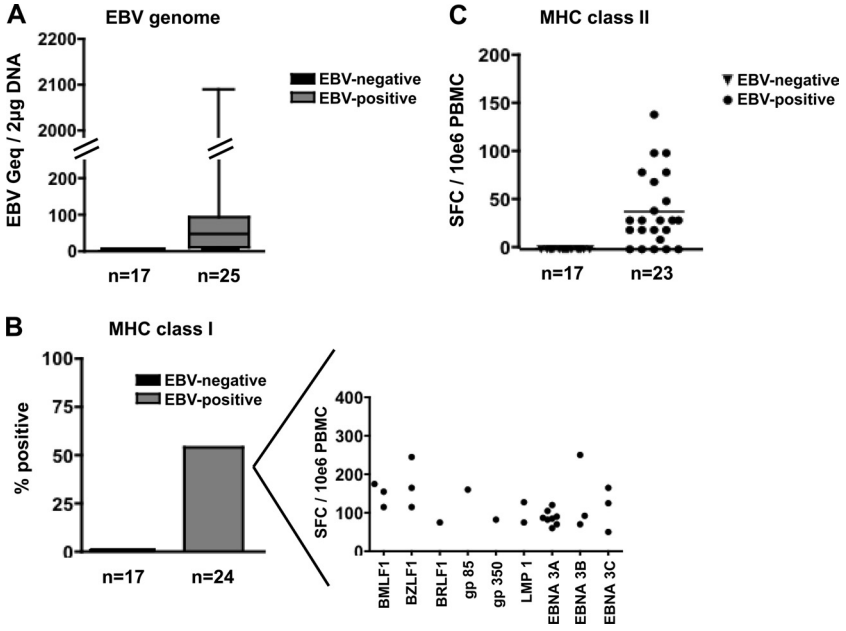

FIG 1 EBV genome content of B cells and EBV-specific T cell reactivity. (A) The number of viral genome equivalents in 17 EBV-seronegative and 25 EBV-seropositive individuals was assessed by BALF5 (DNA polymerase catalytic subunit) reverse transcription (RT)-PCR using DNA extracted from 1×10^6 B cells (2 μg). For each donor, the RT-PCR was run twice from the same DNA stock. (B) *Ex vivo* $CD8^+$ T cell reactivity to EBV-derived latent and lytic epitopes was assessed by IFN-γ ELISpot. Whereas 13/24 (54%) EBV-positive donors displayed IFN-γ secretion in response to at least one HLA-restricted EBV-derived epitope, no IFN-γ secretion could be detected in EBV-negative donors. The inset on the right shows the magnitude of $CD8^+$ T cell responses of the 13 reactive study subjects to the individual peptide (some responding to more than one peptide). (C) IFN-γ secretion in response to MHC class II-restricted EBV peptides as measured by ELISpot. None of the 17 EBV-seronegative donors reacted in response to a pool of 33 MHC class II-restricted EBV peptides. In contrast, 19/23 EBV-seropositive donors displayed IFN-γ secretion when tested with the same peptide pool [mean, 39 spot-forming cells (SFC)/(1×10^6) PBMC; standard deviation, ±9 SFC/(1×10^6) PBMC; $P < 0.001$].

Against this background, we specifically analyzed −35 C allele variants (by genotyping the HLA-C rs9264942 single-nucleotide polymorphism [SNP] using a commercial ABI TaqMan kit [Applied Biosystems, Branchburg, NJ, USA]) and HLA-Bw4 epitopes (by sequence-based genotyping [Histogenetics, New York, NY, USA]). HLA-B alleles were assigned to the Bw4 and HLA-Bw4 80Ile groups according to http://hla.alleles.org/wmda/index.html. KIR3DL1 and KIR3DS1 genotypes were determined by multiplex PCR, followed by a reverse sequence-specific oligonucleotide (rSSO) method according to the manufacturer's instructions (One Lambda, Inc., Canoga Park, CA, USA).

The HLA-C variant with TT at position −35, which is associated with lower HLA-C levels, was significantly underrepresented in the EBV-negative cohort (15/35 EBV-positive donors were homozygous for the −35 T allele, compared to only 2/17 EBV-negative donors [$P = 0.03$]) (Fig. 2A). In contrast, the frequency of HLA-Bw4 epitopes was significantly higher among the EBV-negative group, with 16/17 donors carrying at least one HLA-Bw4 epitope, versus 26/39 among the EBV-positive control cohort ($P = 0.04$) (Fig. 2B). Eight of 17 EBV-negative donors were homozygous for HLA-Bw4, compared to only 4/39 EBV-positive donors ($P = 0.004$), and individuals homozygous for HLA-Bw4 80Ile were significantly overrepresented among the EBV-negative cohort ($P = 0.003$) (Fig. 2B). In chronic HIV infection, the protective effect of HLA-Bw4 is thought to be mediated through interaction with KIR3DL1 and/or KIR3DS1 (19, 20). In our hands, the combined genotype of HLA-Bw4 homozygous and KIR3DL1 homozygous (KIR3DL1 homozygous equals the absence of KIR3DS1) was present significantly more often in the EBV-negative cohort (4/17 [24%]) than in the EBV-positive cohort (2/38 [5%]; $P = 0.045$). No significant association was found between HLA-Bw4 alleles and KIR3DS1 (data not shown).

The oropharyngeal lymphatic tissue represents the entry site for EBV (1). Reduction of oropharyngeal lymphoid tissue, thereby removing substrate that can be infected by EBV, may affect an individual's risk to become infected. In addition to the genetic makeup, we therefore assessed how tonsillectomy, ethnicity, and socioeconomic status influence susceptibility to infection. No differences were found in ethnicity and socioeconomic status (data not shown). Intriguingly, 17/39 (44%) EBV-positive versus 13/17 (76%) EBV-negative individuals had a history of tonsillectomy ($P = 0.023$) (Fig. 2C). No significant difference in median age at tonsillectomy was found between the two groups (EBV negative, age 7 at tonsillectomy, versus EBV positive, age 9.5; $P = 0.75$). Of note, tonsillectomy and protective genetic variants in HLA-B alleles did not cocluster among long-term EBV-negative individuals (data not shown).

In summary, our preliminary study for the first time provides a genetic/anatomic signature capturing long-term protection from EBV. Genetic studies suggest a model whereby inhibition of NK cells is weaker by specific KIR-HLA combinations than by others, with weaker inhibition resulting in a more pronounced activation of NK cells and, therefore, better control of viral infections (21). However, more analyses of KIR/HLA combined genotypes are required to interpret the between-group differences in HLA-Bw4

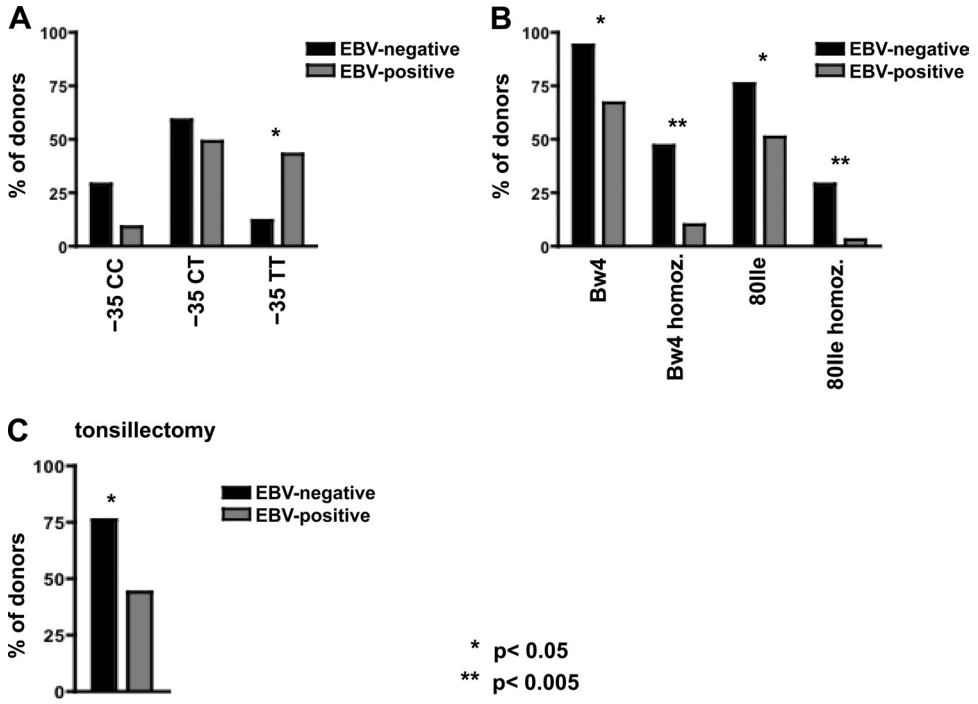

FIG 2 HLA I genotype and tonsillectomy in long-term EBV-protected individuals and EBV-positive controls. (A) Individuals ($n = 52$) were grouped according to −35 genotype into −35 CC, CT, and TT HLA allele variants. Within each group, EBV-negative individuals ($n = 17$ total, filled bars) were compared to EBV-positive individuals ($n = 35$ total, gray bars). A significant difference was found within the group of −35 T homozygous donors, with only 2/17 (12%) within the EBV-negative and 15/35 (43%) within the EBV-positive group. (B) HLA-Bw4-positive, HLA-Bw4-homozygous, HLA-Bw4 80Ile-positive, and HLA-Bw4 80Ile-homozygous donors were compared according to EBV status. In EBV-negative individuals, HLA-Bw4 and HLA-Bw4 80Ile alleles were overrepresented compared to their frequency in EBV-positive controls. (C) A significant difference was found in tonsillectomy rates between EBV-negative individuals (filled bars) and EBV-positive individuals (gray bars). *, $P < 0.05$; **, $P < 0.005$.

and HLA-Bw4 80Ile-homozygous genotypes in the context of EBV infection observed here.

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