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Transplantation Immunology:
Aspects of allo- and EBV-specific reactivity

The EBV non-infected elderly – factors of resistance

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Bojana Durovic

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auf Antrag der Professoren:

Christoph Hess, Daniela Finke und Antonius Rolink

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Prof. Dr. Martin Spiess

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General Summary

Defining the relevant components of cellular alloimmunity remains an important unresolved issue in clinical transplantation. The most promising marker of cellular alloreactivity available to date is donor-specific secretion of interferon-gamma (IFN-γ) as captured in Enzyme Linked Immuno-Spot (ELISPOT) assays (1, 2). How pre-transplantation allo-specific secretion of acute phase cytokines relates to allograft damage and to classic measures of adaptive allo-specific immune function has not been defined. We longitudinally assessed allo-specific secretion of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) in a prospective observational cohort of 38 renal transplant recipients. Allo-specific secretion of IL-6 pre-transplantation was more often detected in individuals with subsequent rejection. Levels of IL-6 correlated with the frequency of IFN-γ secreting cells, which themselves identified a subset of rejecting individuals with high specificity. These data for the first time link allo-inducible secretion of the acute phase signature cytokine IL-6 with subsequent allograft rejection and the frequency of IFN-γ secreting cells, suggesting a link between innate/acute phase and adaptive immunity in the pathogenesis of transplant injury. (Durovic, et al - submitted)

Post-transplant Non-Hodgkin lymphoma is a life-threatening complication after solid organ and hematopoietic stem cell transplantation (HSCT). While pharmacologically suppressed adaptive immunity plays a major role in its development, the precise circumstances leading to tumor growth remain unclear (4). We explored the possibility that factors intrinsic to EBV-transformed B cells may induce EBV-specific T cell immunodeficiency as observed in individuals developing post-transplant lymphoproliferative disease (PTLD). Expression of T cell co-stimulatory molecules, MHC or maturation markers on EBV-transformed B cell clones did not impact their immunogenicity vis-à-vis T cells. By contrast, proliferation rates of B cell clones positively correlated with their capacity to induce IFN-γ secretion in EBV-specific $CD8^+$ T cells, whereas they were associated inversely with $CD8^+$ T cell mediated cytotoxicity. Induction of IFN-γ secreting, yet poorly cytotoxic, T cells represents an unexpected potential

of EBV to induce $CD8^+$ T cell responses skewed towards inefficiency. Knowledge of this viral capacity has implications with regards to lymphomagenesis, and may help directing future screening and therapeutic strategies. (Durovic, et al – submitted)

Epstein-Barr virus (EBV) is ubiquitous among the world's population with greater than 90% of adults being infected. Why 5-10% of the adult population remain EBV-seronegative throughout their lives is not known. Insights into mechanisms that confer protection from EBV infection might help in understanding EBV-associated disease and disclose targets for therapeutic interventions. We screened 515 healthy donors aged >60 years and identified 17 EBV-negative individuals with no evidence of viral infection in terms of humoral and cellular immunity and absence of viral genome within peripheral B cells. In these 17 EBV-negative individuals and 39 EBV-positive age- and sex-matched controls, medical history, immunological profiles and immunogenetic factors were assessed. EBV-negative donors presented significantly more often with a history of tonsillectomy than EBV-positive controls. At the molecular level, detailed analysis of MHC class I / killer cell immunoglobulin receptor compound genotypes, associated an HLA-Bw4 / KIR3DL1 compound genotype – representing an inhibitory interaction– with EBV-negativity. These data identify anatomical and immunogenetic factors likely to be involved in protection from becoming latently infected with EBV. (Durovic, et al – in preparation)

General Introduction

Two major obstacles in transplant immunology are immunologic injury of the allograft on the one hand, and side effects of the immunosuppressive medication on the other. Allograft rejection can be mediated by cellular and humoral components. Hyperacute rejection usually occurs as a consequence of pre-formed antibodies directed against MHC antigens or other alloantigens such as the ABO blood group. Acute cellular rejection is mediated by recipient T cells recognizing donor MHC on antigen-presenting cells (APC) of donor origin (direct allorecognition) or donor-derived peptides presented on autologous APC (indirect allorecognition). Pathologically it is manifested by the accumulation of mononuclear cells in the interstitium, accompanied by inflammation of the tubules and sometimes arteries (3). With current therapeutic strategies, the incidence of acute rejection is approximately 5-10% in the first year in unsensitized patients (4). Chronic rejection may occur by both cellular and/or humoral mechanisms and is characterized by progressive graft dysfunction.

Adaptive immune mechanisms have been firmly linked to graft injury. By contrast, the role of innate immune mechanisms remains poorly defined. One basic principle linking innate and adaptive immune responses is the activation of pattern recognition receptors such as the toll-like family of receptors (TLR). Engagement of TLR is associated with cytokine release, serving to recruit and activate cells of the innate arm, which in turn can promote adaptive immune responses. Acute-phase cytokines, such as IL-6, TNF-α and IL-1β are released predominantly by innate immune cells as so called acute-phase cytokines (5). In a mouse model, animals subjected to burn injury had increased TLR reactivity, leading to higher IL-6, TNF-α and IL-1β production in macrophages and dendritic cells (DC) (6). All three cytokines have been proposed to promote antigen-dependent alloimmunity by increasing the production of other cytokines and up-regulating co-stimulatory molecules (7, 8). In **Section 1**, a new link between the secretion of the acute-phase cytokine IL-6 pre-transplant and subsequent allograft rejection is described.

While immunosuppressive treatment effectively reduces graft rejection, it increases considerably the risk for bacterial, viral and fungal infections. Beyond the first months after transplantation, viral pathogens emerge as the most important group of infections in solid organ transplant recipients. EBV-associated post-transplant lymphoproliferative disorders (PTLD) are a heterogeneous group of mostly EBV-associated B cell proliferations in immunocompromised individuals (9). The PTLD spectrum ranges from EBV-driven polyclonal outgrowth of B cells, that can spontaneously regress, to aggressive lymphoma (9). The World Health Organization (WHO) suggests four main categories: a) reactive, plasmacytic hyperplasia or infectious mononucleosis-like lymphoid hyperplasia, b) polymorphic PTLD, c) monomorphic PTLD corresponding to B and T cell Non-Hodgkin Lymphoma, and d) Hodgkin Lymphoma and Hodgkin-like PTLD. The incidence of PTLD is directly related to the amount and duration of immunosuppression, and insufficient EBV-specific adaptive immunity has been firmly established as a key factor in the pathogenesis of PTLD (10, 11). As a consequence, treatment of PTLD has largely focused on strategies to boost the immune response by either withdrawing immunosuppression or by transfer of EBV-specific cytotoxic $CD8^+$ T cells (9).

High proliferation rates of lymphomas have been associated with a poor prognosis (12-14). *In vivo* the overall growth rate of lymphoma reflects the balance between lymphoma growth (largely determined by the proliferation rate of malignant cells) and the rate at which host mechanisms eliminate/kill malignant cells. Current concepts suggest that immunosuppression permits outgrowth of EBV-transformed B cells due to suppressed lymphocyte function. In **Section 2**, the influence of EBV-transformed B cells on the ensuing T cell response was explored.

In **Section 3,** a summary of anatomical and immunogenetic factors, likely to be involved in protection from EBV infection, is given. In the vast majority of cases, EBV infection occurs during childhood. The virus is then thought to persist as a lifelong infection. If delayed until adolescence, EBV infection can cause infectious mononucleosis (IM), an acute self-limiting lymphoproliferative disease accompanied by fever, sore throat, and lymphadenopathy.

Although EBV infection is benign in the majority of individuals, the virus has been implicated in the etiology of several lymphoid and epithelial malignancies and in the pathogenesis of multiple autoimmune diseases, such as systemic lupus erythematodes, multiple sclerosis, rheumatoid arthritis, Sjogren's syndrome, and dermatomyositis (15). The onogenic potential of EBV is unique with more than a dozen of lymphoid and solid malignancies in both immunocompetent and immunocompromised individuals that have been associated with the virus.

EBV enters the body through the oropharynx to subsequently infect resting, naïve B cells of the local lymphoid tissue. Most EBV-infected individuals, and especially those with IM, shed infectious virus into saliva, which ensures transmission of virus from one individual to another. Viral replication within oropharyngeal epithelial cells was described as an important early step in primary infection, providing virus to infect sub-epithelial B cells. EBV infects B cells via binding of the major viral structure protein gp350 to CD21 (complement receptor for the C3d component) and the binding of a second viral glycoprotein, gp42, to human MHC class II molecules (16, 17). Infection of other cell types (mainly epithelial cells) is much less efficient and occurs through separate, CD21-independent, mechanisms (17, 18). Once EBV adsorption takes place, CD21 aggregates within the membrane and the virus is internalized into cytoplasmic vesicles (19). Infectious virus is produced only during the lytic phase of viral life cycle. A switch to the latent phase is accompanied by absence of virus production and highly restricted numbers of viral genes expressed. Despite the emergence of EBV-specific T cells and humoral immunity, EBV eventually establishes a lifelong latency in memory B cells.

EBV has the unique capacity to transform resting B cells into permanently growing lymphoblastoid cell lines (LCL), providing an in vitro system to study latent EBV infection and lymphomagenesis. LCL express six nuclear antigens (EBNA 1, 2, 3A, 3B, 3C and –LP), three latent membrane proteins (LMP 1, 2A, and 2B), two small non-coding RNAs (EBER1 and EBER2), and transcripts from the *Bam*HI A region (BARTs). This pattern of latent EBV gene expression is referred to as latency III and is characteristic of acute EBV infection and some PTLD.

Primary EBV infection elicits strong $CD8^+$ T cell responses (20, 21). In infectious mononucleosis, an unusually large number of oligoclonal EBV-specific $CD8^+$ T cells is seen with individual epitope responses accounting for 1-40% of the total $CD8^+$ T cell population (22). After recovery, virus-specific T cells contract, yet still constitute up to 5% of the total circulating T cell pool (23), predominantly targeting epitopes from the EBNA-3A, -3B, and -3C subset of latent proteins and the lytic protein BZLF1 (22).

Natural Killer cells (NK) cells comprise 10-15% of all circulating lymphocytes and are key components of the early innate immune response to many microbes. There is evidence of an important role for NK cells in the early control of herpes virus infections in mice and humans (24, 25). Expansion of activated NK cells has been described in blood of acute IM patients (26), and studies on patients with isolated NK cell deficiencies indicate an important role for these lymphocytes in control of EBV infection (27). Data from in vitro infection models have demonstrated a protective effect of NK cells if added within a few days after EBV infection; in part mediated through release of IFN-γ by NK cells (28). The Killer cell immunoglobulin-like receptors (KIR) are a group of currently 15 known genes encoding inhibitory and activating receptors found on NK cells, NK/T cells and a subset of $\gamma\delta$-T cells. They interact with MHC class I or MHC class I-related molecules and have been shown to influence resistance to infection, susceptibility to autoimmune disease and the outcome after hematopoietic stem-cell transplantation. Based on the number of activating KIR receptors, two KIR haplotypes have been proposed. Haplotype A is characterized by a single activating receptor KIR2DS4 and has been proved disadvantageous for CMV control in immunocompromised individuals (29-31). Haplotype B encompasses all other combinations of activating KIR.

Several genetic studies suggest a model whereby inhibition of NK cells by some KIR-HLA combinations is weaker than others, with weaker inhibition resulting in more pronounced activation of NK cells and therefore better control of virus infection (32). HLA C1 and C2 alleles encode ligands for the inhibitory KIR2DL2/3 and KIR2DL1, respectively. The interaction of C2 with KIR2DL1 is stronger and more specific than that of C1 with KIR2DL2/3. For HLA-B, a similar sequence dimorphism defines two determinants, HLA-Bw4 and HLA-

Bw6, with different ligand-binding specificities. Only one of the two forms, HLA-Bw4, functions as a KIR ligand engaging the inhibitory KIR3DL1 (33, 34). The strength of this inhibitory interaction varies among different Bw4 epitopes, with allotypes having isoleucine at position 80 (HLA-Bw4 80Ile) being the strongest inhibitors (33). The HLA-Bw4 80Ile and KIR3DS1 compound genotype has been attributed a protective role in HIV infection by delaying progression to AIDS (35). In EBV infection, no consistent role for KIR and MHC class I variants has been identified so far.

Section 1: Pre-transplant allo-specific secretion of IL-6 identifies a subset of individuals at risk for allograft injury

Introduction

Humoral and cellular elements can both mediate allograft rejection. Several routine diagnostic tests with well-established clinical significance are available to assess humoral alloimmunity (36-38). By contrast, defining the relevant components of cellular alloimmunity remains an important unresolved issue in clinical transplantation. Given the complexity of the immunological processes mediating graft injury –a network of interrelated events occurring over time and at distinct anatomical sites– it seems unlikely that a single parameter accurately reflects or predicts all cellular graft rejection episodes. The most promising marker of cellular alloreactivity available to date is donor-specific secretion of IFN-γ as captured in ELISPOT assays. IFN-γ ELISPOT analyses have been used in risk stratification and immune monitoring protocols (2, 39-42). From experimental models there is evidence that triggering of innate immune mechanisms can create a pro-inflammatory milieu that promotes ensuing adaptive responses, such as expansion of IFN-γ producing alloreactive cells (7). Here we report prospective human data assessing for the first time the interrelation between allo-specific secretion of innate/acute-phase cytokines pre-transplantation, and subsequent allograft damage and donor-specific secretion of IFN-γ.

Patients and Methods

Patient population

The study was performed after written informed consent and IRB approval at the University Hospital Basel, Switzerland. Among 78 consecutive patients who received a kidney transplant from a living donor, all 20 transplant recipients that within the one-year follow-up suffered from one or more biopsy-proven rejection episode(s), and of whom enough samples were available, were included in the study. Eighteen randomly chosen patients with normal allograft histology in protocol biopsies at 3 and 6 months post-transplantation and stable graft function served as control population.

Patient's material

EDTA blood was obtained from all study participants before, and at 3, 6 and 12 months after transplantation. Blood from organ donors was obtained prior to organ explantation. Peripheral blood mononuclear cells (PBMC) were isolated using standard density gradient protocols (Lymphoprep™, Fresenius Kabi, Oslo, Norway). Protocol transplant biopsies were performed 3 and 6 months after transplantation. Additional biopsies were carried out as clinically indicated.

HLA genotyping

HLA typing was performed both serologically (Lymphotype plates™, Biotest, Dreieich, Germany) and by reverse PCR-SSP (sequence-specific primer) hybridization (SSP Kits, Protrans, Hockenheim, Germany) using genomic DNA extracted from PBMC.

Mixed lymphocyte reaction with fixed target cells

Target cells were prepared/fixed as previously described (43). Effector cells from organ recipients (1x10^5 PBMC) were incubated with fixed target PBMC from organ donors at a ratio of 1:5 (=optimal ratio [data not shown]). Mixed lymphocyte reactions (MLR) were

performed overnight in R10 (RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 U/mL penicillin, 50 μg/mL streptomycin) (all from GIBCO™) at 37°C, 5% CO_2.

Quantification of cytokines

Supernatant from autologous and allogeneic overnight MLR were transferred to 96 well MULTI-SPOT plates (Meso Scale Discovery [MSD], Gaithersburg, Maryland, USA) and cytokines quantified according to the manufacturer's protocol. Plates were analyzed with the SECTOR Imager 2400 (MSD).

ELISPOT assay

MultiScreen HTS™ IP 96 well plates (MSIPS4510, Millipore, Billerica, MA, USA) were coated with anti-human IFN-γ mAb (1-D1K) and anti-human interleukin-10 (IL-10) mAb (9D7) (both Mabtech AB, Stockholm, Sweden) diluted 1:2000 in PBS. Plates were developed with anti-human IFN-γ mAb coupled with alkaline phosphatase (7-B6-1-ALP) (1:200 in PBS 1% bovine serum albumin [BSA], Sigma-Aldrich, Steinheim, Germany) and biotinylated anti-human IL-10 mAb (1:2000 in PBS 1% BSA) (12G8-Biotin) (both from Mabtech) for 2 h, followed by incubation with streptavidin-horseradish peroxidase (1:2000 in PBS 1% BSA) (Mabtech) for 45 minutes. Spots were revealed using the HistoMark® RED™ phosphatase system and HistoMark® TrueBlue™ peroxidase system (KPL, Gaithersburg, Maryland, USA), and counted by visualization with the AID CytoSpot Reader System (CSR01, Autoimmun Diagnostika [AID] GmbH, Strassberg, Germany).

FACScan analyses

Antibodies recognizing CD3 (SK7), CD4 (SK3), CD8 (LT8) and appropriate isotype controls all from R&D systems (R&D systems Europe, United Kingdom) were used. Data were acquired on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, California, USA) and analyzed with the FlowJo 8.8.6 software (Tree Star, Inc., Ashland, Oregon, USA).

Caspase-based cytotoxic activity assay

Induction of caspase activity in target cells was assessed using the CyToxiLux®PLUS! assay (OncoImmun, Inc., Gaithersburg, Maryland, USA). Briefly, 5x10^4 target PBMC were fluorescently labeled before being co-incubated with 5x10^5 effector cells for 30 min. at 37°C in the presence of a fluorogenic caspase 6 substrate. Caspase activity was measured using the spectrofluorimeter Spectramax Gemini XS (Molecular Devices, Sunnyvale, California, USA).

Proliferation assay

1x10^5 CFSE-labeled PBMC were stimulated with 1x10^5 irradiated stimulator PBMC in LCM-10 and 100 U/ml IL-2 in 96 well plates. On day 3 fresh medium and 100 U/mL IL-2 were added. Cells were analyzed by flow cytometry on day 7.

Statistical analyses

Normally distributed data were analyzed by Student's t-test, non-normally distributed data by Mann-Whitney U and Kruskal-Wallis test, respectively. For categorical data Fisher's exact test was used. Correlations of parametric and nonparametric data were calculated using Pearson and Spearman Correlation. All tests were performed with Prism4 software (GraphPad Software, Inc. San Diego, California, USA). P values <0.05 were considered statistically significant.

Results

Cellular alloresponses in kidney transplant recipients

Donor-specific alloreactivity was tested in participants of a well-characterized prospective cohort of renal transplant recipients enrolled in the living donor program at the University Hospital Basel. Alloreactivity was assessed by testing recipient PBMC obtained before, and at 3, 6 and 12 months after transplantation against organ donor PBMC obtained before organ explantation. Thirty-eight kidney transplant recipients were tested. Twenty/38 suffered from a biopsy-proven rejection, 18/38 remained rejection free throughout the predefined one-year follow-up. Clinical data comparing patients with and without rejection are summarized in **Table 1**.

Table 1 Patient characteristics.

Patient characteristics	IFN-γ positive	IFN-γ negative	No rejection
Nr. of patients	6	14	18
Recipient age, median (range)	59 (34-67)	49 (32-70)	55 (38-67)
Recipient sex, nr. females (%)	2 (33)	4 (29)	7 (39)
Donor age, median (range)	47 (35-60)	54 (35-66)	55 (28-64)
Pre-transplantation risk assessment			
HLA-A, -B and –DR, mismatches/patient	3.5	3.5	3.5
Donor-specific antibodies, nr. (%)	0	1 (7)	2 (11)
Allograft function (mean creatinine, mmol/L)			
At month 1	112	148	123
At month 3	108	145	117
At month 6	115	138	110
At month 12	160	134	114
Type of rejection[a], no (%)			
Clinical rejection	3 (50)	5 (36)	
Tubulitis	1 (17)	1 (20)	
Endothelialitis	0	2 (40)	
AMR	2 (33)	2 (40)	
Subclinical rejection	3 (50)	9 (64)	
Tubulitis	5 (36)	7 (50)	
Endothelialitis	1 (17)	2 (14)	
AMR	2 (33)	0	
Median time to rejection (days)	63	115	
Graft loss	1 (17)	1 (7)	0
Medication at time of rejection, no (%)			
Triple	5 (83)	6 (43)	
Dual	1 (17)	8 (57)	

[a] Classified according to Banff 2005/08 criteria (14)

Recipient-derived inflammatory cytokines released in response to fixed donor cells

In ex vivo overnight mixed lymphocyte reactions (MLR) we measured levels of the inflammatory cytokines IL-6, TNF-α, and IL1-β released by recipient cells in response to fixed donor target cells before and at 3, 6, and 12 months after transplantation. All 20 recipients who had been diagnosed with a rejection episode, and 13/18 patients without evidence of rejection were available for testing. Cytokine secretion in MLR using autologous target cells served as control. For all cytokines a threshold of 50 pg/mL (after subtraction of the autologous reaction) was defined as cut-off for positivity. Results are given as number of patients exceeding this value, stratified according to rejection. Prior to transplantation more 'cytokine-positive' patients were among the rejection group. A statistically significant discriminating role between rejecting and non-rejecting study participants was observed for IL-6, which was positive in 9/20 pre-transplant MLR of individuals with subsequent rejection, versus 1/13 being positive in the group not suffering from rejection (**Figure 1**).

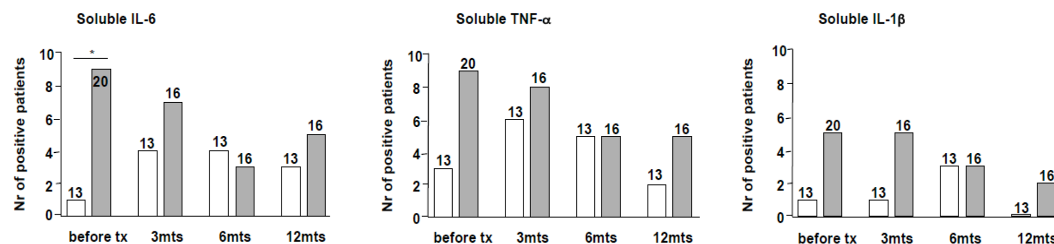

Figure 1 Number of individuals secreting IL-6, TNF-α or IL-1β in overnight MLR using fixed donor PBMC as stimulator cells. Gray bars indicate patients with at least one biopsy-proven rejection episode; white bars indicate patients without rejection throughout the one-year follow-up. Throughout the figures numbers above columns indicate individuals tested for the given time-point. Before transplantation, more subsequently rejecting individuals released IL-6 in an allo-specific manner. * indicates $p<0.05$

Frequencies of donor-specific IFN-γ and IL-10 producing cells

Having established an association of the acute phase cytokine IL-6 with rejection we next tested for the presence of IFN-γ positive cells, an established marker for allo-specific cellular reactivity. In overnight MLR we assessed the frequency of IFN-γ producing cells present before, and at 3, 6, and 12 months after transplantation. On each occasion and for all patients autologous reactions were performed as an internal control, and reactivity subtracted from those detected in allogeneic MLR.

Consistent with our data obtained among 45 healthy individuals (data not shown), in patients with no clinical and/or histological signs of rejection, no donor-specific secretion of IFN-γ was detected in pre-transplant samples (n=18). Importantly, in none of these non-rejecting kidney transplant recipients donor-specific IFN-γ secretion became detectable in overnight MLR at any time point tested after transplantation. By contrast, in 6/20 patients with biopsy-proven rejection (clinical or subclinical), donor-specific secretion of IFN-γ was detected at least once (rejection [6/20] vs. no rejection [0/18], p=0.02) (**Figure 2,** upper left panel). In four of the six patients with rejection, IFN-γ producing cells were detected in overnight MLR already before transplantation (**Figure 2,** upper right panel). Importantly, within the group of patients with rejection, the frequency of cells producing IFN-γ positively correlated with pre-transplant levels of IL-6 induced in overnight MLR (r=0.59, p=0.006).

Interestingly, in none of the 6 patients in whom allo-specific IFN-γ secreting cells were detected, donor-specific antibodies were observed pre-transplantation. PBMC from 5/6 patients with alloreactive cells detectable in overnight MLR were available for testing also against HLA-unrelated third-party target cells (n=2 each). In only 1/10 third-party experiments secretion of IFN-γ was induced (data not shown).

Simultaneously with IFN-γ, we also quantified the frequency of cells secreting the regulatory cytokine IL-10. Donor-specific IL-10 producing cells, while readily detectable before transplantation and throughout the one-year follow-up, showed no association with the number of IFN-γ producing cells or levels of IL-6. While the number of patients with allo-

specific IL-10 producing cells was higher in rejecting vs. non-rejecting individuals, this difference was not statistically significant (**Figure 2,** lower left panel). The frequency of IL-10 producing cells before and at the pre-defined time-points after transplantation among rejecting and non-rejecting individuals is shown in **Figure 2,** lower right panel.

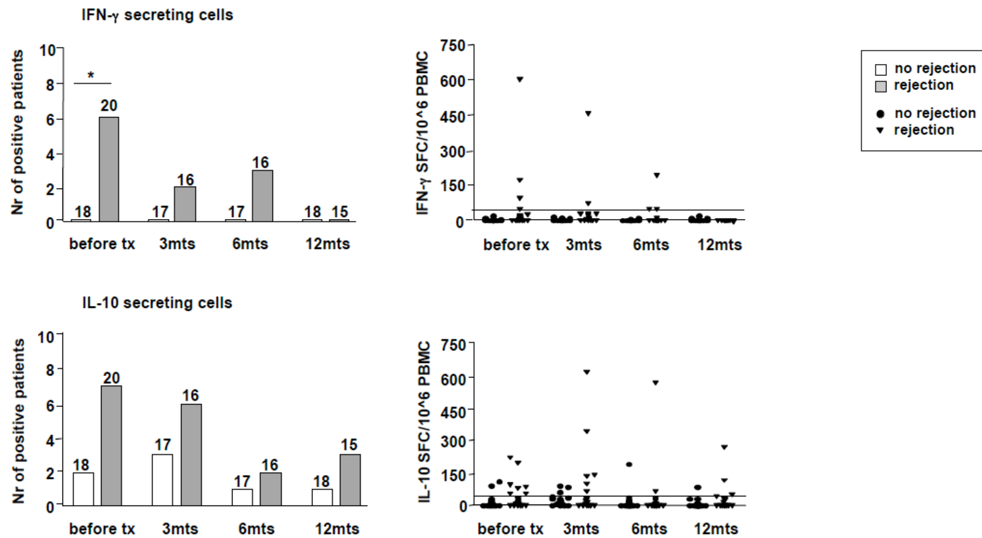

Figure 2 Number of individuals with detectable allo-specific IFN-γ secretion (upper left panel), and frequency of allo-reactive IFN-γ secreting cells (upper right panel) in rejecting vs non-rejecting individuals. The number of individuals with detectable allo-specific IFN-γ secretion before transplantation was significantly higher in rejecting vs. non-rejecting individuals. Lower left panel: number of individuals with detectable allo-specific IL-10 secretion; lower right panel: frequency of allo-reactive IL-10 secreting cells, in rejecting vs non-rejecting individuals, respectively.

Donor-specific cytotoxic activity

Nine patients, of whom 6 suffered from a rejection and 3 remained rejection free, were available for testing allo-specific cytotoxic activity vis-à-vis donor cells, each before, and at 3, 6, and 12 months after transplantation. In 7/9 patients cytotoxic activity was very low for any of the time-points tested, with caspase activity induced in only 0-5% of target cells. Defining 5% caspase activity as an arbitrary positive cut-off, only 1/9 patients displayed donor-specific

killing before transplantation (**Figure 3A**), with 23% caspase-activity induced in target cells. This particular patient suffered from a subclinical rejection at 3 and 6 months after transplantation. Thus, in contrast to in vitro inducible allo-specific cytotoxic activity that can be readily induced (data not shown), relevant cytotoxic activity against organ donor-derived allogeneic PBMC thus was an infrequent event.

Proliferation of $CD4^+$ and $CD8^+$ T cells in response to donor stimulator cells

The same 9 transplant recipients that were assessed for donor-specific cytotoxicity were also analyzed for proliferation of $CD4^+$ and $CD8^+$ T cells after one week of stimulation with donor PBMC. Proliferation rates were calculated as the percentage of cells undergoing ≥1 cell division in response to the allogeneic target minus the percentage of cells that have divided in response to IL-2 only. No significant differences in proliferation rates of rejecting vs. non-rejecting individuals were observed (**Figure 3B**).

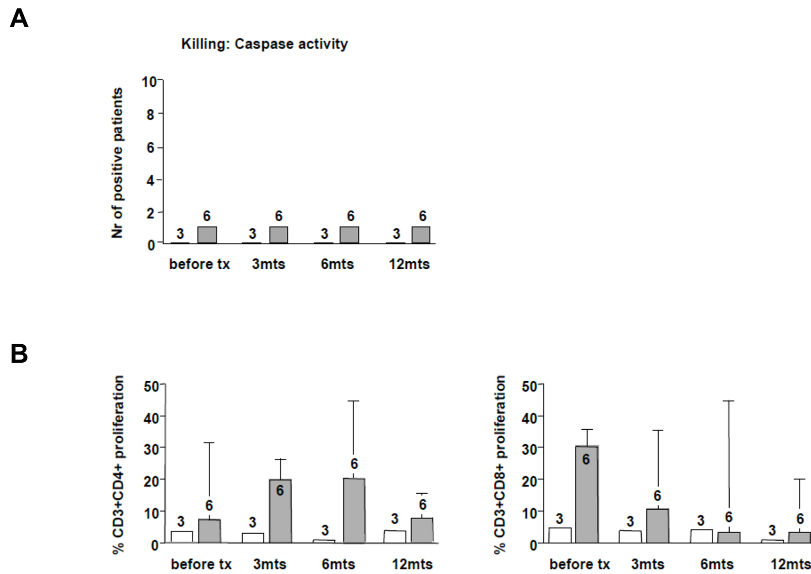

Figure 3 (**A**) Donor-specific cytotoxic activity in non-rejecting (white bars) vs rejecting individuals (gray bars). (**B**) Percentage of donor-specific proliferation of $CD4^+$ and $CD8^+$ T cells (median and interquartile range).

Discussion

IL-6, TNF-α and IL-1β are released predominantly by innate immune cells as acute-phase cytokines (5). Whether allo-specific secretion of these cytokines can be detected before transplantation, and how their pre-transplant levels relate to the frequency of IFN-γ secreting cells and the risk for subsequent transplant injury/rejection has not been assessed. The key findings of our study were that (i) allo-specific secretion of IL-6 can be detected and was more often observed in pre-transplant MLR from individuals subsequently suffering from a rejection episode and (ii) the pre-transplantation frequency of IFN-γ secreting cells correlated with levels of allo-specific IL-6 released at these same time-points.

Adaptive immune mechanisms have been firmly linked to graft injury. By contrast, the role of innate immune mechanisms remains poorly defined. Acute-phase cytokines, such as IL-6, TNF-α and IL-1β, have all been proposed to promote antigen-dependent alloimmunity by increasing the production of other cytokines and up-regulating co-stimulatory molecules –thus indirectly contributing to allograft damage (7, 8). Furthermore, genetically determined IL-6 production has been linked to the development of renal allograft injury (44). Supporting a role for acute phase cytokines in alloimmunity we here found that donor-specific secretion of IL-6 as detected before transplantation was linked with an increased risk for graft rejection. The fact that secretion of IL-6 was induced in a donor-specific manner was surprising, suggesting that distinct allo-constellations might favor induction of this cytokine. To date donor-specific secretion of IFN-γ represents the most valuable clinical marker of cellular (adaptive) alloreactivity (2, 39-42). Here we also found that secretion of this cytokine identifies a subset of rejecting individuals with high specificity. Importantly, we further observed that the pre-transplant frequency of cells secreting IFN-γ and the corresponding levels of allo-inducible IL-6 correlated significantly. The biological basis of this correlation remains to be determined. However, the correlation supports a model in which innate and adaptive factors contribute in an interlinked manner to the clinical end-point of allograft injury.

In conclusion, assessing allo-inducible secretion of the acute phase cytokine IL-6 prior to kidney transplantation may identify patients at risk of allograft injury. The observed link between secretion of IL-6 and the frequency of allo-specific IFN-γ secreting cells should facilitate future research aiming at defining the interplay between innate/acute-phase and adaptive immunity in the pathogenesis of allograft damage.

Section 2: Rapidly proliferating EBV-transformed B cells skew CD8$^+$ T cells towards inefficiency

Introduction

Epstein-Barr virus (EBV), a B cell tropic γ-herpes virus, is ubiquitous among the human population, with greater than 90% of adults being infected. After acute, lytic infection the virus enters a state of latency. Subsequent intermittent viral reactivation, as well as EBV-driven growth transformation of infected B cells, is controlled by host defense mechanisms. In vivo, CD8+ T cells have been demonstrated to be key to maintaining the balance between EBV and host, and when exposed to autologous EBV-transformed B cells in vitro, virus-specific CD8+ T cells are induced to secrete IFN-γ and can kill infected cells (45, 46).

Post-transplant lymphoproliferative disorders (PTLD) are a heterogeneous group of mostly EBV-associated diseases occurring in immunosuppressed individuals (9). The PTLD spectrum ranges from EBV-driven polyclonal outgrowth of B cells to aggressive lymphoma (9). Insufficient EBV-specific adaptive immunity –resulting from iatrogenic immunosuppression– has been firmly established as a key factor in the pathogenesis of PTLD (10, 11). As a consequence, treatment of PTLD has largely focused on strategies to boost the immune response to EBV by either withdrawing immunosuppression or transfer of EBV-specific cytotoxic CD8+ T cells (9).

High proliferation rates of lymphomas have been associated with a poor prognosis (12-14). In vivo the overall growth rate of lymphoma reflects the balance between lymphoma growth (largely determined by the proliferation rate of malignant cells) and the rate at which host mechanisms eliminate/kill malignant cells. Current concepts suggest that immunosuppression permits outgrowth of EBV-transformed B cells due to suppressed lymphocyte function. Here we explored the –not mutually exclusive– possibility that EBV-transformed B cells intrinsically dictate the nature of the ensuing T cell response. To that end we characterized the EBV-

specific T cell response induced by phenotypically and functionally defined EBV-transformed B cells.

Materials and Methods

Generation of EBV-transformed B cell clones

After written informed consent and IRB approval, bulk PBMC of healthy EBV positive donors were used to generate EBV-transformed B cell clones. Briefly, 1 mL of supernatant from the EBV-producing marmoset cell line B95.8 was used to infect 1x10^6 PBMC. After 1 hour of incubation at 37°C, an equal volume of LCM-10 (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 50 U/mL penicillin, 50 mg/mL streptomycin, 2% L-glutamin, 1% sodium pyruvate and 1% non-essential amino acids (MEM NEAA) [all from GIBCO™, LuBioScience GmbH, Basel, Switzerland]) supplemented with 2 μg/mL Cyclosporin A (Sandimmune®, Novartis, Basel, Switzerland) was added and cells were cultured at 1x10^6 PBMC/well in 24 well plates at 37°C 5% CO_2. After outgrowth cells were kept in LCM-10 at a density of 5x10^5 cells/mL. Applying this method, cell lines were previously demonstrated to evolve to monoclonal cultures within 8 weeks (47). We assessed and confirmed clonality of our EBV-transformed B cells by dual staining of surface kappa/lambda light chains (data not shown).

Expansion of EBV-reactive T cells with autologous EBV-transformed B cell clones

1x10^5 bulk PBMC, or (as indicated) $CD4^+$ and $CD8^+$ T cells isolated with MACS bead technology (Miltenyi Biotec, Bergisch Gladbach, Germany), were cultured with 5x10^4 paraformaldehyde-fixed EBV-transformed B cells in 96 well plates in LCM-10 medium supplemented with 200 U/mL IL-2 (Roche, Basel, Switzerland). Purity of $CD8^+$ and $CD4^+$ T cells after positive selection was ≥98% (data not shown). On day 3 fresh medium supplemented with 200 U/mL IL-2 was added. By day 7 cultures contained no B cells as assessed by CD19 staining and FACScan analysis (data not shown).

ELISPOT assay

MultiScreen HTSTM IP 96 well plates (Millipore, Zug, Switzerland) were coated with anti-human IFN-γ mAb (1-D1K) (Mabtech AB, Stockholm, Sweden) diluted 1:2000 in PBS.

100,000 cells/well were used to assess EBV-specific responses. EBV-positive and EBV-negative human B cell extracts were added at 2.26 μg/mL (Virusys Corporation, www.virusys.com), EBV peptide pools at 14 μg/mL. PHA at 1.8 μg/mL served as positive control. After overnight incubation at 37°C plates were developed with anti-human IFN-γ mAb (1:200) coupled with alkaline phosphatase (7-B6-1-ALP) (both from Mabtech). Spots were revealed using the HistoMark® RED phosphatase system (KPL, Gaithersburg, Maryland, USA), and counted by direct visualization with the AID CytoSpot Reader System (CSR01, Autoimmun Diagnostika [AID] GmbH, Strassberg, Germany) using the ELISPOT 3.5 software (AID GmbH). Results are given as spot forming cells (SFC) per million input cells.

FACScan analysis

Antibodies directed against CD3 (SK7), CD4 (SK3), CD8 (LT8), CD19 (HIB19), CD23 (M-L233), CD27 (M-T271), CD38 (HB7), CD40 (HM40-3), CD80 (B7-1), CD86 (2331), IgM (145-8), kappa (TB28-2), lambda (1-155-2), and appropriate isotype controls were used (all from BD Biosciences, Allschwil, Switzerland). Staining was performed in PBS 1% BSA (Sigma-Aldrich, Steinheim, Germany) for 20 min. at 4°C. For intracellular staining of Ki67 (F07-88, Dako, Baar, Switzerland) cells were fixed and permeabilized with the Cytofix/Cytoperm buffer (BD) before incubation with the antibody. Data were acquired on a FACS Calibur flow cytometer (BD) and analyzed with the FlowJo 8.8.6.1 Software (Tree Star, Inc., Ashland, Oregon, USA).

Proliferation analyses

To assess proliferation, 1x10^6 EBV-transformed B cells were stained with CFSE and cultured at 5x10^5 cells/mL in LCM-10 at 37°C 5% CO_2. A proliferation index was calculated for each clone by dividing CFSE fluorescence intensity before incubation by CFSE fluorescence intensity after 4 hours of culture. This short (4 hour) incubation period was chosen based on the rapid proliferation rates of EBV-transformed B cells, and allowed distinction of all B cell clones according to their proliferation rate. CFSE ratios were related to the following parameters of proliferation: cell numbers after 24 hours of culture; intracellular

expression of Ki67 after 4 hours of culture; percentage of cells actively synthesizing DNA as assessed by BrdU incorporation after 4 hours of culture.

Cell cycle analysis

To assess cell cycle kinetics a BrdU Flow Kit was used (BD Pharmingen). Briefly, EBV-transformed B cells were pulsed with BrdU at a final concentration of 10 μM. Cells were fixed and permeabilized twice with the Cytofix/Cytoperm buffer, treated with 300 μg/mL DNase to expose incorporated BrdU, then stained with an anti-BrdU-FITC antibody. Total DNA was stained with 7-amino-actinomycin D (7-AAD). Cell cycle positions were analyzed quantitatively on a FACS Calibur flow cytometer (BD) by comparing expression of total DNA and incorporated BrdU.

Calcein release cytotoxicity assay

Target cells were resuspended in Hanks Balanced Salt Solution (GIBCO^{TM}) at a final concentration of 2x10^6/mL and incubated with 5 μM calcein-AM (Invitrogen, Basel, Switzerland) for 30 min. at 37°C. After thorough washing, 1x10^4 target cells were incubated with 2x10^5 PBMC for 2h at 37°C 5% CO_2. Calcein release was measured using the spectrofluorimeter Spectramax Gemini XS (Molecular Devices, Sunnyvale, California, USA).

Statistical analyses

Normally distributed data were analyzed by Student's t-test, non-normally distributed data by Mann-Whitney U and Kruskal-Wallis test. Paired analyses were performed as appropriate. Correlations of parametric and nonparametric data were calculated using Pearson and Spearman Correlation, respectively. All tests were performed using Prism4 software (GraphPad Software, Inc. San Diego, California, USA). P values <0.05 were considered statistically significant.

Results

Expansion of IFN-γ secreting cells triggered by autologous EBV-transformed B cell clones

Our goal was to define how cell surface characteristics of EBV transformed B cells shape the ensuing immune response. Experiments were hence performed using stimulating B cells after mild fixation with paraformaldehyde. Paraformaldehyde-fixation of target cells did not change their cell surface phenotype as assessed by FACS analysis (data nor shown). Cytokine secretion of target cells was completely abrogated after fixation, permitting analysis of effector cell-derived cytokines only. Using 16 autologous EBV-transformed B cell clones from 5 individuals, the number of PBMC induced by such clones to secrete IFN-γ expectedly increased from a mean of only 5 spot forming cells (SFC) per 10^6 PBMC directly *ex vivo*, to a mean of 116 SFC/10^6 PBMC after one week of stimulation (**Figure 4A**). Reactivity was documented to be EBV-specific by using EBV-infected and non-infected B cell lysate, and an EBV peptide library (data not shown). Intriguing was the fact that the frequency of IFN-γ secreting cells after stimulation was distinct for a given clone, though highly variable between clones from different donors, as well as between separate EBV-transformed B cell clones from a single donor (**Figure 4B/C**).

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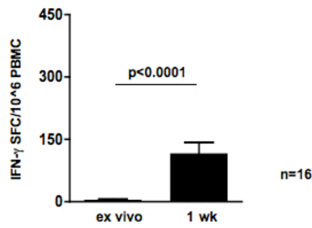

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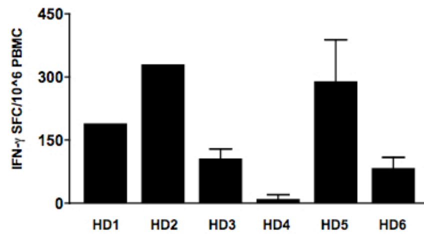

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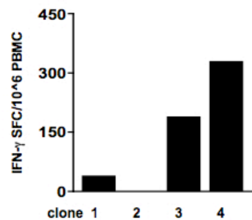


Figure 4 Expansion of IFN-γ secreting cells by autologous EBV-transformed B cell clones**.** PBMC were stimulated with fixed autologous EBV-transformed B cell clones, and frequencies of IFN-γ secreting cells quantified by ELISPOT before and after stimulation. **A)** Overall, the number of IFN-γ secreting cells significantly increased after one week of stimulation. **B)** Large inter-individual differences were observed when separately assessing the frequency of IFN-γ secreting cells induced by one autologous B cell clone each in PBMC from 6 healthy donors (HD 1-6). Individual clones tested several times (done here 4-6 times for HD 3-6), however, always induced similar frequencies of IFN-γ secreting cells. **C)** Individual clones derived from a single individual (clone 1-4) also induced differing frequencies of IFN-γ secreting cells.

Expression of MHC, T cell co-stimulatory molecules and differentiation markers on EBV-transformed B cell clones, and their capacity to induce IFN-γ

Aiming to uncover the factor(s) linked to the clone-specific capacity to trigger IFN-γ secretion, we first performed cell surface phenotyping of EBV-transformed B cells. A total of eight clones from 4 donors (2 clones/donor) were assessed. Only little inter- and intra-individual variation in the expression of the T cell co-stimulatory molecules CD27, CD40, CD80, and CD86 was observed. Expression of MHC molecules on B cell clones was more heterogeneous, both inter- and intra-individually (**Figure 5A**). No correlation, however, was detected between the mean fluorescence intensity (mfi) of MHC class I and MHC class II molecules on EBV-transformed B cells and the frequency of IFN-γ secreting cells triggered by the respective cell clone (**Figure 5B**). Expression of CD23 and CD38 has been used to

define subsets of EBV-transformed B cells with differing proliferative potential and antibody secretion capacity (48). *In vitro*, 60% of B cell clones expressed intermediate levels of CD23 and CD38 ('lymphoblastoid' phenotype), 40% had a CD23 (low) CD38 (high) phenotype ('plasmocytoid' phenotype) (n=25 clones from 10 individuals). Yet, no association was observed between 'lymphoblastoid' or 'plasmocytoid' EBV-transformed B cell clones and the frequency of IFN-γ secreting cells triggered by these cells (**Figure 5C**).

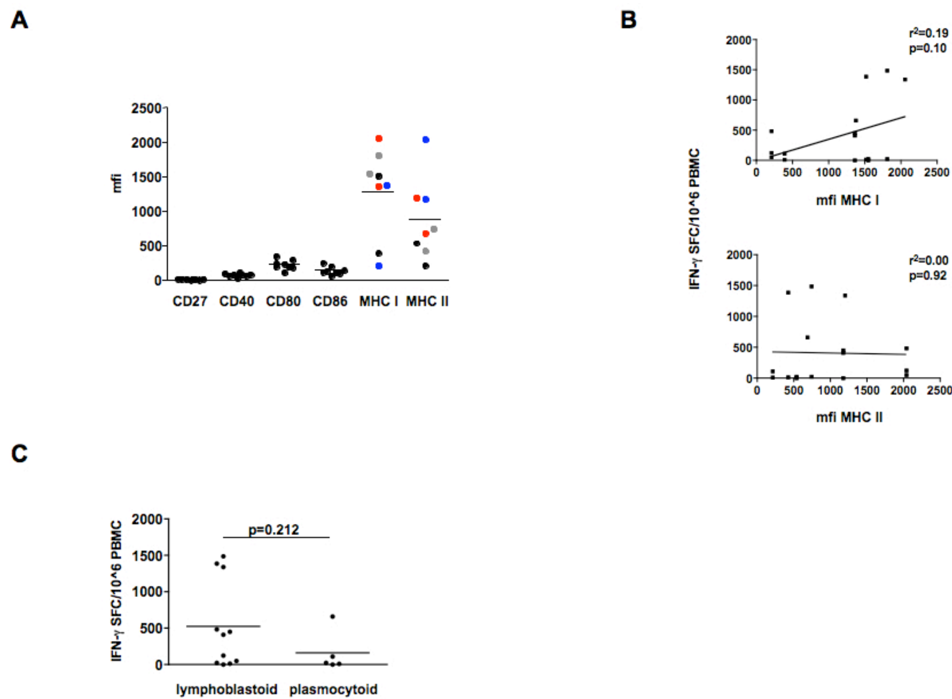


Figure 5 Expression of T cell co-stimulatory molecules, MHC class I and class II, and differentiation markers on EBV-transformed B cell clones, and induction of IFN-γ secretion in autologous cells. **A**) B cell clones displayed only minor differences in cell surface expression of T cell co-stimulatory molecules, whereas inter- and intra-individual expression of MHC molecules was more variable. **B**) No significant correlation between B cell-expressed MHC class I or class II, and the capacity of clones to induce IFN-γ secretion was observed. **C**) Also no association was found comparing 'lymphoblastoid' and 'plasmocytoid' B cell clones –defined by the differentiation markers CD23 and CD38– with regards to their capacity to induce IFN-γ secretion in autologous PBMC.

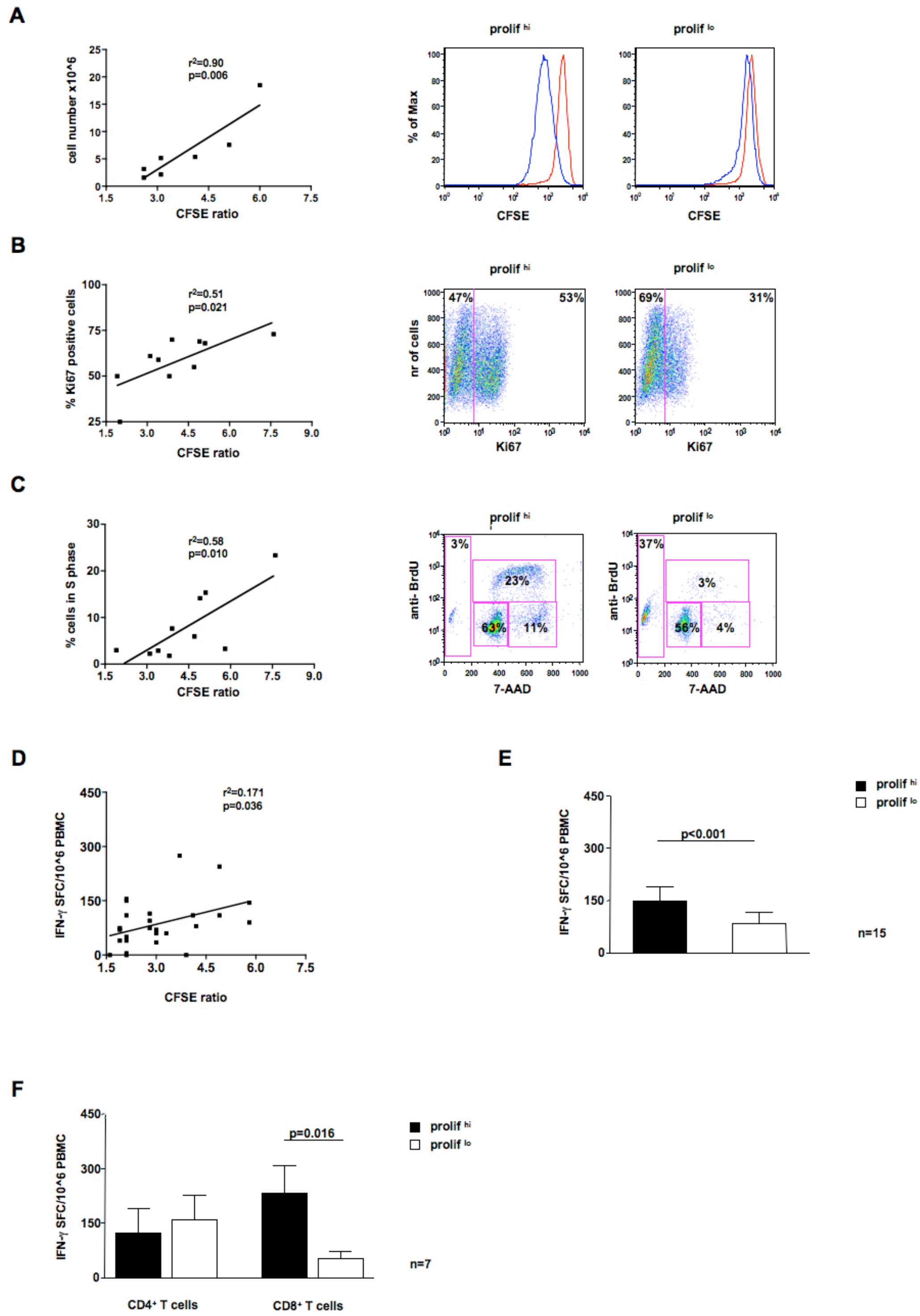
Proliferative capacity of EBV-transformed B cell clones and expansion of IFN-γ producing cells

In absence of an obvious link between cell surface expression of key molecules governing B cell function and their capacity to induce IFN-γ producing cells, we next tested whether cell surface characteristics related to the B cell proliferative capacity might shape the ensuing immune response. Proliferation of EBV-transformed B cell clones was assessed by CFSE dilution assays and cell counting, Ki67 staining and BrdU incorporation studies. CFSE dilution rates –defined by the ratio 'CFSE fluorescence intensity pre-culture' divided by 'CFSE fluorescence intensity after 4 hours of culture' (CFSE ratio)– were paralleled by the increase in B cell numbers after 24h of cell culture (**Figure 6A**). Also, CFSE dilution rates correlated with the percentage of cells expressing the proliferation marker Ki67 (**Figure 6B**), and with the number of cells in S phase as measured by BrdU incorporation (**Figure 6C**). EBV-transformed B cell clones were documented to establish stable proliferation rates within a few weeks of cell culture (as determined by CFSE ratios; data not shown).

Importantly, CFSE ratios during steady-state proliferation *significantly differed between distinct clones* despite identical cell culture conditions. We thus used fixed EBV-transformed B cell clones of defined and differing CFSE ratios to stimulate autologous PBMC. By doing so, and plotting CFSE ratios as a continuous variable against the frequency of cells induced to secrete IFN-γ, a significant positive correlation between the two was uncovered (**Figure 6D**). Analogous data were obtained comparing fast and more slowly proliferating *pairs of EBV-transformed B cell clones derived from single individuals* (n=15 pairs from 5 different donors) (**Figure 6E**). These data indicate that the proliferative capacity of EBV-transformed B cell clones relates to their immunogenicity, in the sense that clones that replicated faster are more efficient in inducing IFN-γ secreting cells. To define the T cell subset(s) triggered to secrete IFN-γ, analogous experiments were performed exposing isolated $CD4^+$ and $CD8^+$ T cells to fixed EBV-transformed B cells with a high vs. low proliferative capacity. While in $CD4^+$ T cell cultures comparable numbers of IFN-γ producing

cells were induced, $CD8^+$ T cell cultures contained significantly more IFN-γ producing cells after being stimulated with fixed EBV-transformed B cells of high proliferative capacity (**Figure 6F**).

Figure 6 Proliferation of EBV-transformed B cell clones and induction of IFN-γ secretion in autologous cells. The proliferation rate of individual B cell clones was captured by the ratio between their mean CFSE fluorescence intensity before and after 4 hours of cell culture. This CFSE ratio strongly correlated with **A**) cell numbers as enumerated after 24 hours of cell culture, **B**) intracellular expression of Ki67 after 4 hours of cell culture, and **C**) the number of cells in S phase after 4 hours of cell culture. **D**) Relating the proliferation rate of stimulating clones from various donors to the frequency of cells induced to secret IFN-γ by these clones, a significant positive correlation was detected. **E**) Likewise, when comparing rapidly proliferating B cell clones with slow ones derived from the same donor, the frequency of cells secreting IFN-γ was significantly higher in PBMC cultures stimulated with clones characterized by a high proliferative capacity. **F**) Separately testing the capacity of B cell clones to induce IFN-γ secretion in cultures of $CD4^+$ T and $CD8^+$ T cells, rapidly proliferating B cell clones selectively induced more IFN-γ secreting cells within the $CD8^+$ T cell subset.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CFSE ratio
r²=0.90
p=0.006
prolif hi
prolif lo
% of Max
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B
% Ki67 positive cells
r²=0.51
p=0.021
nr of cells
Ki67
47%
53%
69%
31%
C
% cells in S phase
r²=0.58
p=0.010
anti- BrdU
7-AAD
3%
23%
63%
11%
37%
3%
56%
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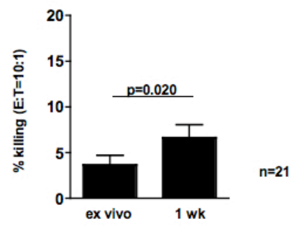
D
IFN-γ SFC/10^6 PBMC
r²=0.171
p=0.036
E
p<0.001
n=15
F
p=0.016
n=7
CD4⁺ T cells
CD8⁺ T cells

Proliferative capacity of EBV-transformed B cell clones and induction of cytotoxicity

Having established a positive correlation between the proliferation rate of autologous EBV-transformed B cell clones and their capacity to induce EBV-specific secretion of IFN-γ among $CD8^+$ effector T cells, we analogously tested for cytotoxic activity. Fixed EBV-transformed B cells reliably induced cytotoxic activity among autologous PBMC within 7 days of co-culture (**Figure 7A**). Similarly to the induction of IFN-γ secreting cells, cytotoxic activity of bulk PBMC was distinct for a given B cell clone, yet highly variable between clones from different donors and between EBV-transformed B cell clones from a single person (**Figure 7B/C**). In sharp contrast to the induction of IFN-γ secreting cells, however, no association of target cell proliferative capacity and induction of cytotoxic capacity in effector PBMC was observed (**Figure 7D/E**). Even more striking, when analyzing T cell subsets, a significant *inverse association* between the proliferative capacity of stimulating EBV-transformed B cell clones and $CD8^+$ T cell-mediated cytotoxicity was uncovered (**Figure 7F**).

Figure 7 Proliferation of EBV-transformed B cell clones and induction of cytotoxic activity in autologous cells. Induction of cytotoxic activity was assessed after stimulation with autologous EBV-transformed B cell clones of defined and varying proliferation rates. **A**) Overall, cytotoxic activity of bulk PBMC increased significantly from 3% pre-stimulation to 7% after one week of stimulation. **B)** Large inter-individual differences were observed when separately assessing cytotoxic activity induced by one autologous B cell clone each in PBMC from 5 healthy donors (HD 1-5). Individual clones tested several times (done here 5-12 times for HD 1-4), however, always induced similar cytotoxic activity. **C)** Individual clones derived from a single individual (clone 1-4) also induced differing cytotoxic activity. **D**) Contrasting the induction of IFN-γ secretion, relating the proliferation rate of stimulating B cell clones from various donors to the cytotoxic activity induced by these clones, no significant correlation was detected. **E**) Likewise, when comparing rapidly proliferating B cell clones with slow ones derived from the same donor, cytotoxic activity was not significantly different. **F**) Separately testing the capacity of B cell clones to induce cytotoxic activity in cultures of $CD4^+$ T and $CD8^+$ T cells, *slowly* proliferating B cell clones selectively induced *more* cytotoxic activity within the $CD8^+$ T cell subset.

A



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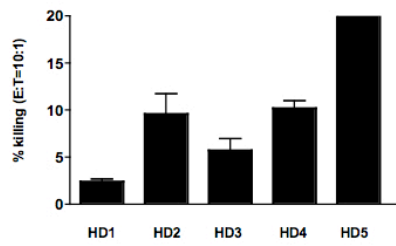

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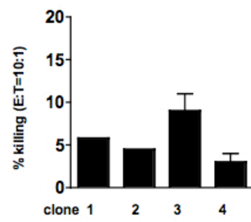

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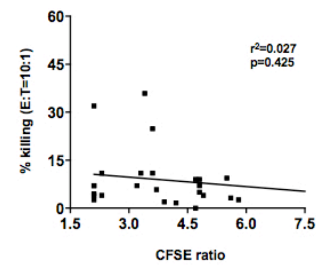

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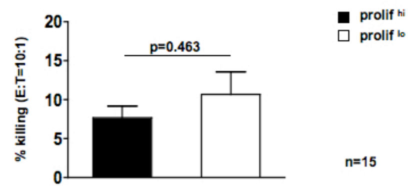

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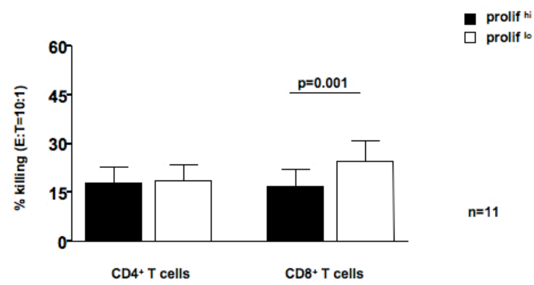


Discussion

In vivo, EBV infects and activates B cells, exploiting the mechanisms that produce and maintain long-term B cell memory (49). Defective T cell function can result in uncontrolled lymphoproliferation and formation of malignancy (50). However, frank lymphoma remains the exception even in cohorts of severely immunosuppressed individuals. Based on this observation we hypothesized that individuals developing lymphoma might differ with regard to factors intrinsic to EBV-transformed B cells that in turn impact the tumor-specific T cell response. To approach this hypothesis we chose to 'freeze' EBV-transformed B cell clones of defined characteristics using mild paraformaldehyde fixation. By doing so we were able to investigate how defined and non-changing cell surface characteristics of EBV-transformed B cell clones impact on the quality of the ensuing T cell response. This strategy uncovered that the replication capacity of EBV-transformed B cell clones relates to the quality of the induced CD8+ T cell response. Specifically we show that B cell clones characterized by a high replication capacity –indicating significant malignant potential– are highly immunogenic to induce IFN-g secreting CD8+ T cells, yet they hamper the development of CD8+ T cells with cytotoxic activity. Important in this context is that cytotoxic CD8+ T cells are crucial for the control of EBV infection, whereas secretion of IFN-g per se by no means reflects efficient anti-viral immunity (51-53). Induction of IFN-γ secreting, yet poorly cytotoxic T cells represents a tumor escape mechanism that is opposite in nature to EBV's widely assumed strategy to evade recognition by cytotoxic T cells via down-regulating cell surface MHC and co-stimulatory molecules (49, 50). In line with our data, EBV-specific CD8+ T cells with little or no cytotoxic activity towards autologous EBV-transformed B cells have previously been identified both in vitro and in vivo (54-56). Looking ourselves for in vivo evidence of skewed cytotoxicity in tumor-infiltrating CD8+ T cells we analyzed five EBV-positive post-transplant B cell lymphomas. Areas with an inverse relation between Ki67 expressing malignant B cells and Granzyme B negative CD8+ T cells (i.e. cells with little cytotoxic capacity) could be readily found. The overall picture, however, was heterogeneous –both within and between

tumors– and the number of available cases too small to permit any firm conclusions (data not shown). If, however, the here established property of EBV is confirmed to contribute to lymphomagenesis in vivo, screening for EBV-transformed B cells able to proliferate rapidly – and hence with potential to skew CD8+ T cell immunity towards inefficiency– may represent a novel strategy to recognize individuals at risk to develop EBV-associated malignancy. Inversely, slowing proliferation of EBV-transformed B cells in tumor patients may restore the property of malignant cells to induce CD8+ T cell cytotoxicity, and hence be of value in treating lymphoproliferative disease.

In summary, we show that the proliferative capacity of EBV-transformed B cells reflects their immunogenic potential, and uncover a novel immune evasion mechanism of EBV, progressively evolving in a growth rate dependent manner and diametrically opposed to the virus's classically ascribed strategy of hiding from immune recognition.

Section 3: The EBV non-infected elderly – defining factors of resistance

Introduction

For most, primary EBV infection occurs during childhood and is usually asymptomatic. If delayed until adolescence the virus can cause infectious mononucleosis (IM), an acute self-limiting lymphoproliferative disease. After acute infection, EBV enters life-long latency and leaves the individual at risk for viral reactivation and in rare cases development of malignancy especially in immunocompromised hosts. Why 5-10% of the adult population remain EBV-seronegative throughout their lives is not known.

In contrast to the knowledge derived from in vitro studies, the understanding of EBV infection in vivo is still rudimentary. EBV enters the body through the oropharyngeal mucosa to subsequently infect resting, naïve B cells of the local lymphoid tissue. Given the initial absence of host immunity upon primo infection, EBV-infected B cells proliferate and expand to eventually reach germinal centers enabling infected B cells to down-regulate expression of viral proteins (57). Despite the emergence of EBV-specific T cells, the virus eventually establishes a life-long latency in memory B cells with only limited expression of viral genes. A balance between occasional viral reactivation and host immune surveillance is eventually established (49).

EBV infects B cells via binding of the major viral structure protein gp350 to the CD21 receptor and the binding of a second viral glycoprotein, gp42, to human MHC class II molecules (16, 17). Infection of other cell types (mainly epithelial cells) is much less efficient and occurs through separate, CD21-independent, mechanisms (17, 18). A soluble form of CD21 (sCD21), consisting of the entire extracellular domain of the complement receptor, is released upon B cell activation and can be found in human serum (58). In vitro, the protein was shown to retain ligand-binding capacity and to block EBV infection (59). The in vivo role of sCD21 in EBV infection has not been addressed so far.

CD8^{+} T cells play a major role in protection from viral infection. They recognize viral antigens presented as short peptides by MHC class I molecules on infected cells and are thought to restrict outgrowth of EBV-transformed B cells. Primary EBV infection elicits strong CD8^{+} T cell responses (20, 21). After recovery, virus-specific cells contract but still constitute up to 5% of the total circulating T cell pool (23). Given this central role of CD8+ T cells in EBV infection, it is conceivable that interindividual differences in T cell function could facilitate or hinder clearance of virus.

Studies on patients with isolated lymphocyte deficiencies indicate a role for NK cells in control of EBV infection (27). The Killer cell immunoglobulin-like receptors (KIR) are a group of currently 15 genes encoding inhibitory and activating receptors found on NK cells, NKT cells and a subset of γδ-T cells. They interact with MHC class I molecules and have been shown to influence resistance to infection, susceptibility to autoimmune disease and the outcome after hematopoietic stem-cell transplantation. Based on the number of activating KIR receptors, two KIR haplotypes have been proposed. Haplotype A is characterized by a single activating receptor KIR2DS4 and has been proved disadvantageous for CMV control in immunocompromised individuals (29-31). Haplotype B encompasses all other combinations of activating KIR. Several genetic studies suggest a model whereby inhibition of NK cells by certain KIR-HLA combinations is weaker than others, with weaker inhibition resulting in better activation of NK cells and therefore better control of virus infection (32). HLA-Bw4 alleles, which interact with the inhibitory receptor KIR3DL1, have been attributed a protective role in HIV infection by delaying progression to AIDS (35). In EBV infection, no consistent role for KIR and HLA class I variants has been reported so far.

Here, we screened 515 healthy blood donors aged >60 years and established a cohort of 17 EBV-negative individuals. In this cohort we tested i) whether EBV-seronegativity reflects absence of EBV-specific cellular memory and absence of viral genome, and ii) assessed clinical, immunological and immunogenetic parameters characteristic of these EBV-negative individuals.

Materials and Methods

Assessment of EBV and CMV serostatus and blood sample collection

515 (as by 07/05/2010) healthy blood donors aged >60 years presenting at the Blood Transfusion Centre of both Basel, Switzerland were assessed for their serological EBV status after giving written informed consent. Multiplex microparticle technology (Luminex 200 Technology, Luminex, Austin, TX, USA) was used to test for IgG antibodies specific for the EBV antigens VCA, EBNA-1 and EA (EBV-IgG Plus Test, AtheNA Multi-Lyte, Inverness Medical, Princeton, NJ, USA). From a total of 17 EBV-negative and 39 EBV-positive age- and sex-matched controls buffy-coat as well as serum and plasma were obtained after blood donation. Again written informed consent was obtained. At blood donation, EBV serology was repeated for all EBV-negative donors with multplex microparticle technology. CMV serostatus was recorded from previous donations (if CMV-positive) or assessed in serum or plasma at time of donation (if previously not tested or CMV-negative) utilizing CMV lysate-coated microparticels (strain AD169) for the capture of human anti-CMV IgG (ARCHITECT CMV IgG Assay, Abbott, Baar, Switzerland). Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-Paque centrifugation and stored in liquid nitrogen until use. Serum and plasma (EDTA potassium) samples were stored at -80°C.

BALF5 real-time PCR

A quantitative analysis of EBV DNA was performed using a real-time PCR protocol adapted from Kimura et al. (60), targeting the BALF5 gene encoding the viral DNA polymerase. DNA extracted from 1x10^6 B cells was tested in quadruplicates using 5 μl of DNA extract or reference plasmid dilutions. One replicate was spiked with 1000 copies of the reference plasmid to monitor for PCR inhibition. The 25 μl PCR reaction contained 300 nM of each primer, 200 nM FAM-labeled probe and 12.5 μl of 2-fold concentrated amplification master mix (Eurogentec, Seraing, Belgium) containing polymerase, 10 mM MgCl2, dNTP (including dUTP) and uracil-N-glycosylase. A standard curve with three concentrations (10^2, 10^4 and 10^6 copies/PCR) of the reference plasmid pEBV containing the target sequence was

generated. The temperature profile consisted of a preincubation step at 50°C, 2 min to allow for enzymatic decontamination of potential uracyl-containing amplicons, followed by 95°C; 10 min for activation of the polymerase and 45 cycles of 95°C; 15 sec; 60°C; 60 sec. The sensitivity of the assay was 3.13 copies/PCR reaction.

Generation of lymphoblastoid cell lines (LCL)

1x10^7 bulk PBMC from randomly chosen EBV-positive and EBV-negative donors (n=9 each) were used to generate EBV-transformed B cell clones. Briefly, 1 mL of supernatant from the EBV-producing marmoset cell line B95.8 was used to infect 1x10^6 PBMC. After 1 hour of incubation at 37°C, an equal volume of LCM-10 (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 50 U/mL penicillin, 50 mg/mL streptomycin, 2% L-glutamin, 1% sodium pyruvate and 1% non-essential amino acids (MEM NEAA) [all from GIBCO™, LuBioScience GmbH, Basel, Switzerland]) supplemented with 2 μg/mL Cyclosporin A (Sandimmune®, Novartis, Basel, Switzerland) was added and cells were cultured at 1x10^6 PBMC/well in 24 well plates at 37°C 5% CO_2. After outgrowth cells were kept in LCM-10 at a density of 5x10^5 cells/mL.

EBV-specific IFN-γ ELISPOT assay

MultiScreen HTSTM IP 96 well plates from Millipore (Billerica, MA, USA) were coated with anti-human IFN-γ mAb (1-D1K) (Mabtech, Stockholm, Sweden) at 2 μg/ml in PBS. 100,000 viable PBMC/well were used to assess EBV-specific responses. EBV-positive and EBV-negative cell lysate were added at 2.26 μg/mL (Virusys, Taneytown, MD, USA) and a pool of peptides consisting of 91 HLA I-restricted optimal EBV epitopes (61, 62) at 14 μg/mL. PHA at 1.8 μg/mL served as positive control. After overnight incubation at 37°C plates were developed with anti-human IFN-γ mAb coupled with alkaline phosphatase at 0.5 μg/ml (7-B6-1-ALP) (Mabtech). Spots were revealed using the HistoMark® RED phosphatase system (KPL, Gaithersburg, MD, USA), and counted by direct visualization with the AID CytoSpot Reader System (CSR01, Autoimmun Diagnostika ([AID] GmbH, Strassberg, Germany) using

the ELISPOT 3.5 software (AID GmbH). Results are given as spot forming cells (SFC) per million input cells. 50 SFC/10^6 PBMC were used as an arbitrary cut-off for positive results.

FACScan analyses

Antibodies directed against CD3 (SK7), CD4 (SK3), CD8 (LT8), CD23 (M-L233), CD38 (HB7), CCR7 (FAB197P), CD45RA (HI100), CD16 (NKP15), CD56 (NCAM 16.2), CD19 (HIB19), CD21 (1048), and appropriate isotype controls were used (all from BD Biosciences, Allschwil, Switzerland). Staining was performed in phosphate buffered saline (PBS) 1% bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany) for 20 min. at 4°C. Data were acquired on a FACS Calibur flow cytometer (BD) and analyzed with the FlowJo 8.8.6.1 Software (Tree Star, Inc., Ashland, Oregon, USA).

Soluble CD21 ELISA

Soluble CD21 was measured in serum with the sCD21 ELISA Kit from Abcam (Cambrdige, UK) according to manufacturer's protocol.

KIR and HLA Genotyping

KIR genotypes were determined by multiplex PCR, followed by a reverse sequence-specific oligonucleotide method according to the manufacturer's instructions (Onelambda Inc., Canoga Park, California, USA). Briefly, three separate PCR reactions (exons 3, 5, and 7-9) were conducted for each sample using biotinylated KIR exon-specific primer sets. Each PCR product was denaturated, hybridized to cDNA probes coupled to fluorescently coded microspheres, and stained with phycoerythrin-conjugated streptavidin. Binding of PCR product to the microspheres was then assessed using a LABScan 100 flow analyzer (Luminex Corporation, Austin, TX, USA). KIR genes analyzed included 2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1. Presence of KIR2DL2, KIR2DS2, KIR2DS3, and KIR3DS5 was confirmed by SSP PCR according to a published protocol (63). HLA genes were analyzed by high-resolution (four digit) sequence based genotyping (Histogenetics, New York, NY, USA). HLA-C alleles with an asparagine at position 80 (e.g.

HLA-Cw1, HLA-Cw3, HLA-Cw7, HLA-Cw8) were assigned to group 1 and HLA-C alleles with a lysine residue at position 80 (e.g. HLA-Cw2, HLA-Cw4, HLA-Cw5, HLA-Cw6) to group 2. HLA-C specificities were confirmed by SSP PCR as described elsewhere (64). HLA-B alleles assigned to the Bw4 group included B5, B13, B17, B27, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B59, B63, B77, and A9, A23, A24, A25, A32 and to the Bw6 group B7, B8, B14, B18, B22, B27, B35, B39, B40, B41, B42, B45, B46, B48, B50, B54, B55, B56, B60, B61, B62, B64, B65, B67, B70, B71, B72, B73, B75, B76, B78, B81, B82. Bw4 epitopes with an isoleucine at position 80 were assigned to the 80Ile group and those with a threonine to 80The (www.hla.alleles.org).

Statistical analyses

Normally distributed data were analyzed by Student's t-test, non-normally distributed data by Mann-Whitney U and Kruskal-Wallis test. Paired analyses were performed as appropriate. Correlations of parametric and nonparametric data were calculated using Pearson and Spearman Correlation, respectively. Tests were performed using Prism4 software (GraphPad Software, Inc. San Diego, California, USA). For analyses of single frequencies of HLA and KIR alleles and HLA-Bw4-KIR3DL1 and HLA-Bw4-KIR3DS1 compound genotypes Pearson's Chi Square test was applied using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). P values <0.05 were considered statistically significant.

Results

Characteristics of the study cohort

17 EBV-seronegative healthy donors aged >60 years (median 64, range 62-70 years) and 39 age- and sex-matched EBV-seropositive individuals (median age 64, range 63-70 years) were included. Mean lymphocyte count was 2082/μl peripheral blood for the EBV-negative and 2033/μl for the EBV-positive group (p=0.711). The rate of CMV infection was 6/17 in EBV-negative vs 14/39 in EBV-positive donors (both 35%, p=0.978).

EBV-seronegative individuals have no detectable EBV genome in peripheral B cells

The frequency of EBV-infected B cells in healthy seropositive individuals was reported to range from 1-50/10^6 B cells and the viral content was estimated at 335 genome copies per 10^6 PBMC (65). To assess the possibility that EBV-seronegative individuals have no detectable EBV-specific antibodies despite a previous infection with EBV, we tested for the presence of EBV genome in DNA extracts from peripheral B cells of all 17 EBV-negative donors and compared them to 25 of the 39 EBV-positive controls (randomly chosen). A quantitative analysis of EBV genome copies was performed by RT-PCR for the viral polymerase BALF5 with a sensitivity of 3 copies/reaction. EBV-seropositive donors displayed genome copies ranging from 3 to 1072 genome equivalents (Geq)/2 μg of B cell DNA with a mean of 97 Geq/2 μg (2 μg of B cell DNA correspond 1x10^6 B cells). In 3/25 EBV-positive donors tested, no EBV DNA could be found despite the presence of EBV-specific antibodies. For all 17 EBV-seronegative donors EBV genome detection was invariably negative (**Figure 8**).

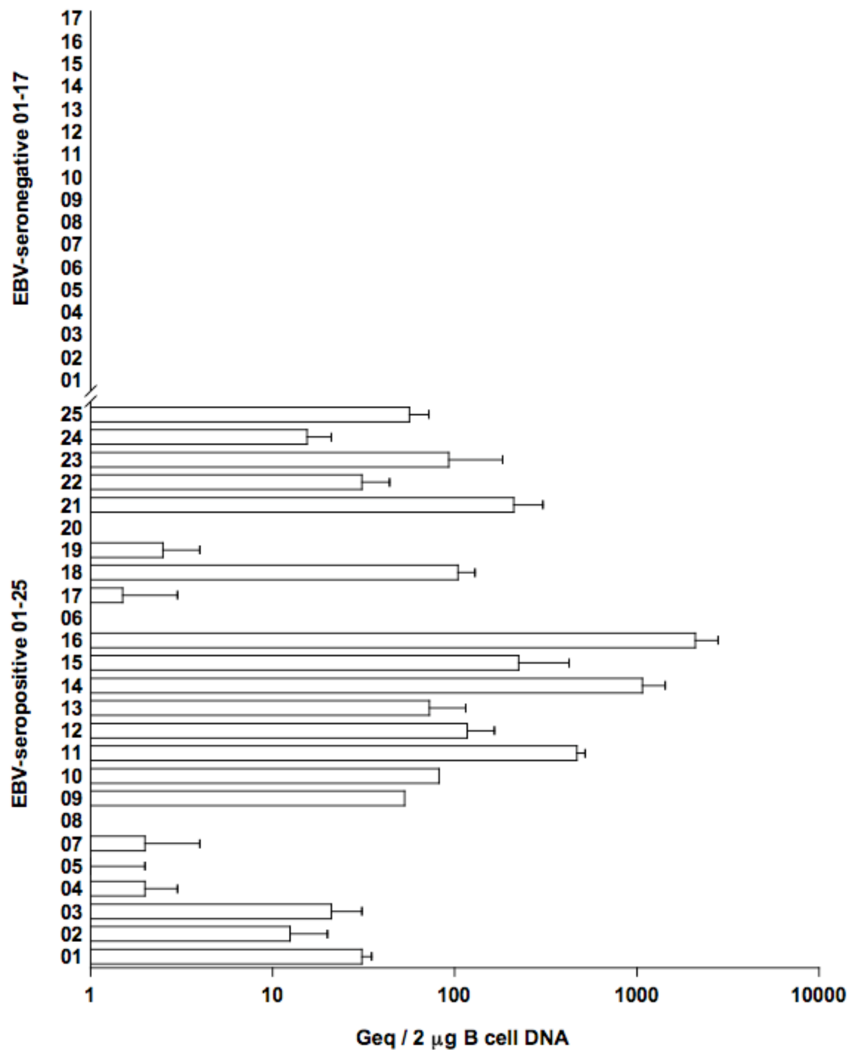


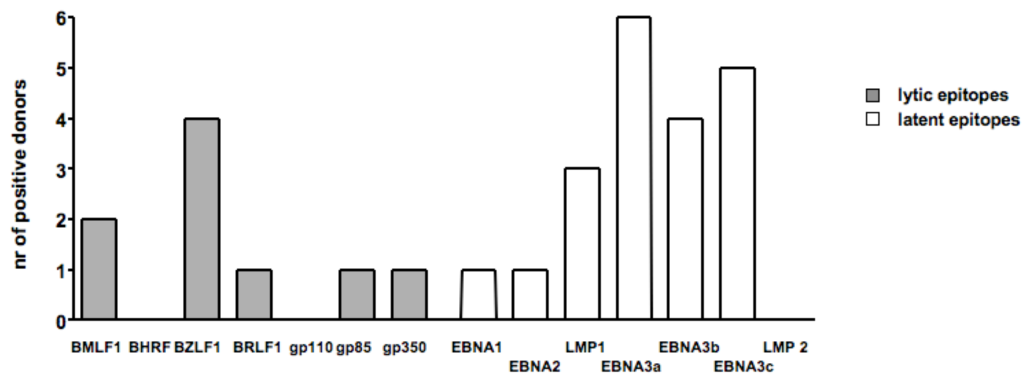
Figure 8 EBV gonome content of peripheral B cells: number of viral genome equivalents (Geq) shown in a logarithmic scale, as assessed in 17 EBV-seronegative and 25 EBV-seropositive (white bars) individuals by BALF5 RT-PCR. For each donor the RT-PCR was run twice from the same DNA stock. All 17 EBV-seronegative donors were negative for EBV DNA. In EBV-positive donors EBV genome copies ranged from 3 to 1072 genome equivalents (Geq) / 2 μg of B cell DNA with a mean of 97 Geq /2 μg DNA, which is equivalent to 1x10^6 B cells.

EBV-seronegative individuals have no detectable EBV-specific cellular immunity

To test whether a negative EBV-serostatus is associated with absence of EBV-specific cellular immunity, we measured IFN-γ secretion in bulk PBMC in response to a pool of peptides consisting of 91 HLA I-restricted optimal EBV epitopes (61, 62). This analysis was performed in the same 25/39 randomly chosen EBV-positive controls. In all 17 EBV-seronegative individuals no reactivity could be detected to any of the pools tested. In 15/25 EBV-seropositive donors (60%) IFN-γ secretion was detected in response to multiple EBV peptide pools. Detailed analysis of EBV peptide specificity in EBV-seropositive donors is shown in (**Figure 9a)**.

Having confirmed the absence of EBV-specific $CD8^+$ T cell memory, $CD4^+$ T cell reactivity was assessed using EBV-positive cell lysate loaded onto autologous PBMC in an IFN-γ ELISPOT assay. 57% (13/23) of EBV-seropositive donors and none of the EBV-negative donors (0/17) displayed IFN-γ secretion in response to EBV-positive lysate. No reactivity to EBV-negative control lysate was detected in either group (**Figure 9b**). Thus, EBV-seronegative individuals had no detectable EBV-specific $CD8^+$ or $CD4^+$ T cell memory in peripheral blood.

A



B

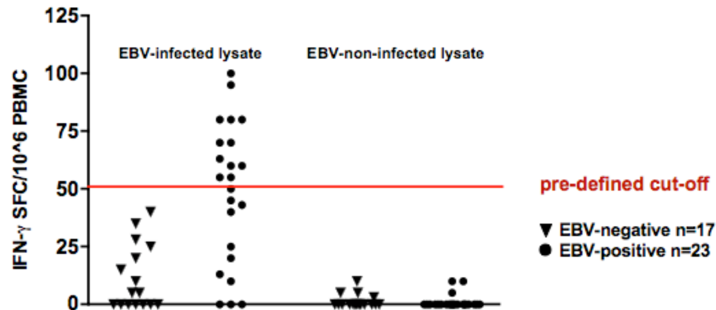


Figure 9 EBV-specific cellular responses: reactivity to EBV was assessed by IFN-γ ELISPOT using an HLA class I restricted peptide library to assess CD8$^+$ T cell responses and EBV-positive lysate for the CD4+ T cell response. **A**) EBV-specific CD8$^+$ T cell reactivity of 25 EBV-positive individuals to lytic (gray bars) and latent (white bars) EBV peptides depicted as number of individuals tested positive for a given peptide. **B**) For CD4+ T cell responses 50 SFC/10^6 PBMC were defined as positive cut-off. 13/23 EBV-positive and none of the EBV-negative donors reacted to EBV-positive lysate. No IFN-γ secretion was detected to EBV-non-infected control lysate.

Peripheral blood cell count and memory T cell phenotype

Large clonal expansions of peripheral $CD8^+$ T cells have been described in elderly CMV-positive individuals reflecting a life-long exposure of the host immune system to the virus (66). Within our cohort, we tested for changes in the composition of peripheral blood lymphocytes characteristic of EBV-infected individuals. We compared absolute numbers of T, B, and NK cells ($CD56^{bright}$ and $CD56^{dim}$), as well as memory T cell subsets defined by cell surface expression of CCR7 and CD45RA. EBV-positive donors displayed significantly lower numbers of NK cells (p=0.033) and higher numbers of T cells (p=0.072) compared to EBV-negative individuals. The higher percentage of T cells in EBV-infected donors was mostly attributable to the CD8+ T cell subset (p=0.073) whereas CD4+ T cell numbers remained largely unchanged (p=0.234). For both the $CD4^+$ and $CD8^+$ T cell compartment, the number of memory T cells was higher in EBV-positive donors, although these differences were not statistically significant (**Figure 10**). The most pronounced differences were observed for the $CD8^+$ effector memory subset ($CCR7^-CD45RA^-$) (p=0.075). Since the prevalence of CMV infection was similar among both groups (p=0.978), a significant impact of CMV infection on our results seems unlikely.

When segregating the EBV-negative and EBV-positive cohorts for CMV, CMV-positive donors (n=20) displayed higher numbers of so-called terminally differentiated $CD8^+$ memory T cells ($CCR7^-CD45RA^+$) (p=0.015) and of $CD4^+$ T cells with a central memory phenotype ($CCR7^+CD45RA^+$) than CMV-negative donors (n=37, p=0.021). EBV and CMV infection thus have differential effects on the composition of the peripheral memory T cell pool. The reduction in NK cell numbers seen in EBV-positive donors was not present in CMV-infected individuals.

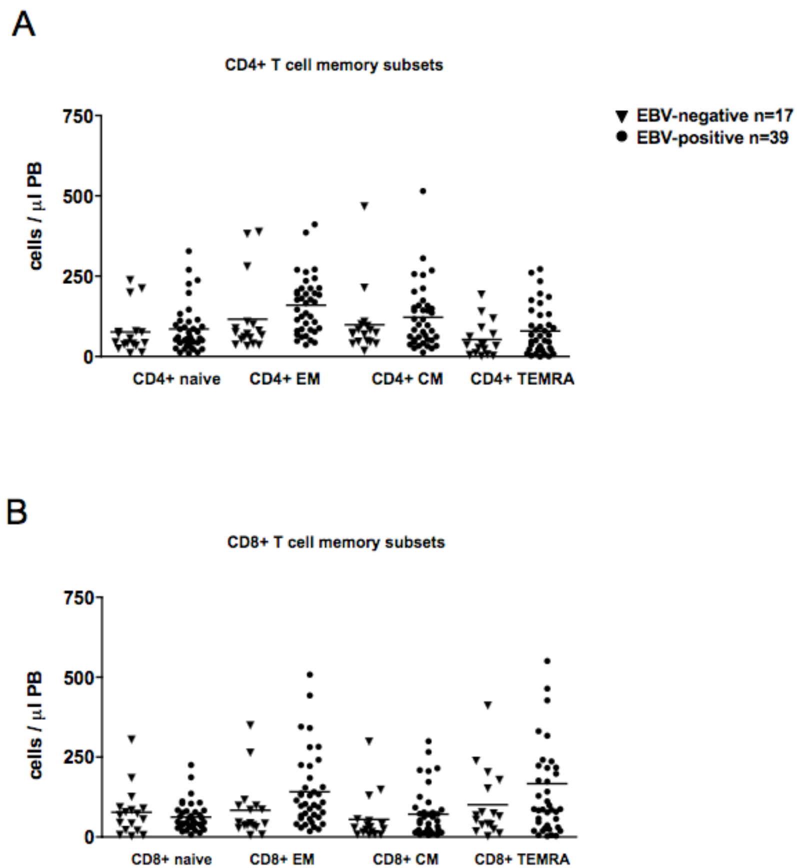


Figure 10 Peripheral memory T cell subsets. T cell subsets as identified by lymph node homing chemokine receptor CCR7 and phosphatase isoform CD45RA: $CCR7^{+}CD45RA^{+}$ naïve, $CCR7^{-}CD45RA^{-}$ effector memory, $CCR7^{+}CD45RA^{-}$ central memory, $CCR7^{-}CD45RA^{+}$ terminally-differentiated effector-memory (TEMRA) T cells. Number of cells/µl peripheral blood within the $CD4^{+}$ **A)** and $CD8^{+}$ T cells subset **B**) do not show significant differences between EBV-negative (triangles, n=17) and EBV-positive (dots, n=39) individuals. The difference between EBV-negative and EBV-positive donors was most pronounced for the $CD8^{+}$ effector memory T cell subset (p=0.075)

Peripheral B cells of EBV-seronegative individuals are susceptible to EBV infection in vitro

Next, PBMC of EBV-negative and EBV-positive donors (n=9 each, randomly chosen samples of the cohorts) were infected with either EBV-containing supernatant alone or EBV-containing supernatant and Cyclosporin A (CSA). In all 9 EBV-negative individuals outgrowth of lymphoblastoid cell lines (LCL) was demonstrated under both conditions, whereas in EBV-positive donors LCL could be only generated by addition of CSA in 5/9 individuals. No phenotypic difference in terms of cell surface expression of the B cell activation markers CD23 and CD38 could be detected between LCL of EBV-negative (n=8) and EBV-positive (n=7) donors (p=0.955).

EBV-seronegativity is positively associated with tonsillectomy

A history of tonsillectomy was reported by 46% (18/39) of EBV-positive and 76% (4/17) of EBV-negative individuals (p=0.002). No significant difference in median age at tonsillectomy was found between the two groups (EBV-negative at age 7 vs EBV-positive at age 9.5, p=0.746). EBV-negative donors with no prior history of tonsillectomy (n=4) displayed lymphocyte counts resembling those of the EBV-positive group with lower NK cell counts compared to EBV-negative donors with a history of tonsillectomy (p=0.027). Grouping donors with and without tonsillectomy regardless of their EBV status, revealed even a stronger association of tonsillectomy with higher NK cell numbers in peripheral blood (p=0.018). NK cell subsets with regard to CD56 expression remained unchanged (p=0.869).

Cell surface expression and serum levels of the EBV target receptor CD21

Binding of the structural EBV protein gp350 to the CD21 receptor mediates internalization of the virus into the target cell (16). Conflicting data on the lack of EBV receptors on B cells of long-term EBV-seronegative individuals have been reported (67, 68). We analyzed CD21 expression on peripheral blood B cells of 17 EBV-negative individuals and compared CD21 expression in these individuals to 39 EBV-positive controls. No significant differences were found in the number of CD21 expressing B cells (p=0.140) and absolute B cell counts

(p=0.225) between groups (**Figure 11a**). The majority of donors from both groups displayed CD21 expression on ≥95% of peripheral B cells (**Figure 11b**).

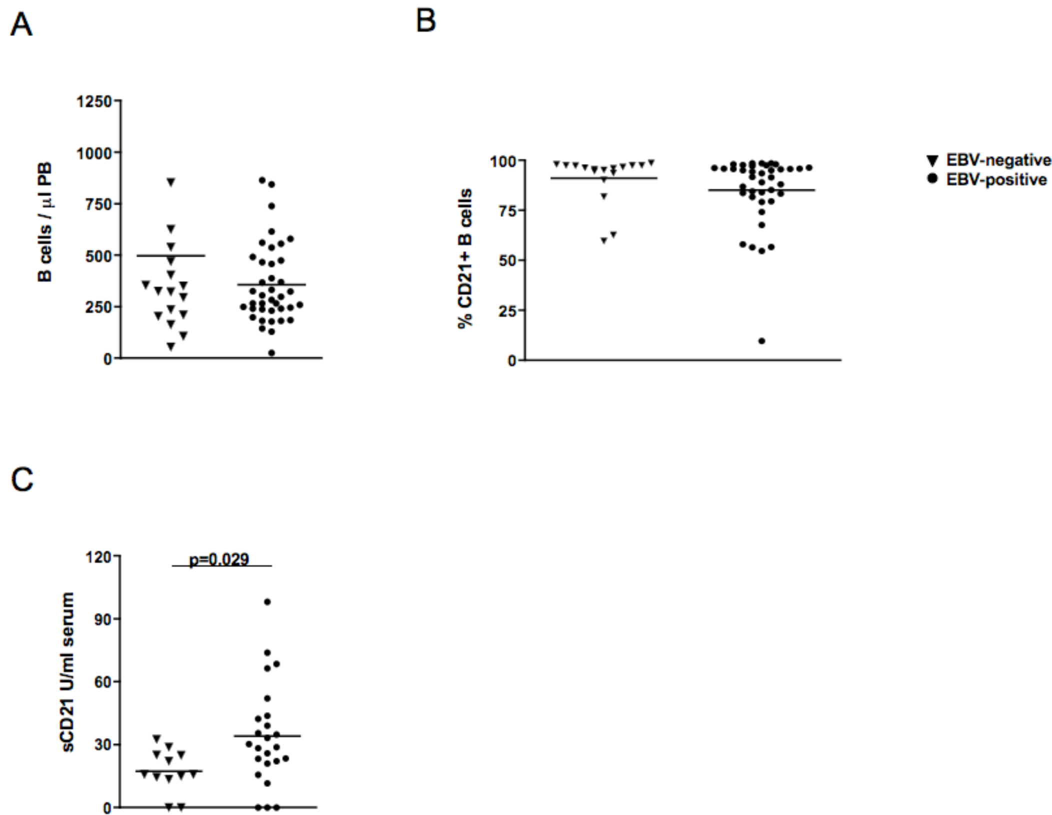


Figure 11 Absolute B cell numbers, expression of CD21 and levels of soluble CD21. **A)** No significant difference was found when comparing B cell numbers between EBV-negative and EBV-positive donors. **B)** For both groups the majority of donors expressed CD21 on >90% of B cells. **C)** Levels of soluble CD21 receptor in serum were significantly higher in EBV-positive donors (mean ± SD EBV-negative 17 ± 10 U/ml vs EBV-positive 34 ± 24 U/ml, p=0.029).

Soluble CD21 was shown to bind to EBV gp350/220 and to block EBV-induced B cell-transformation in vitro. We compared levels of sCD21 in sera of 12 EBV-negative and 20 EBV-positive donors to assess a possible role of sCD21 in modulating EBV infection. Soluble CD21 levels were lower in EBV-seronegative individuals with a mean of 17 U/ml as compared to EBV-seropositive donors 34 U/ml (p=0.029) (**Figure 11c**). Levels of sCD21 also

correlated with the number of peripheral B cells (r^2=0.123, p=0.039) irrespective of EBV status.

HLA class I and II allele- and KIR distribution

No significant difference was found when comparing single HLA class I and II and KIR alleles between EBV-positive and negative individuals. The distribution of activating and inhibitory KIR genes was balanced between the two groups with frequencies consistent with those of a Caucasoid population (69). KIR group A haplotypes showed no association with EBV-serostatus (haplotype A/A 6/17 EBV-negative vs 12/38 EBV-positive, p=1.0).

To test for a role of KIR and HLA class I combinations we first counted the number of inhibitory HLA-C interactions with their putative ligands KIR2DL1 and KIR2DL2 and compared them with activating interactions of HLA-C with KIR2DS1 and KIR2DS2, respectively. Inhibitory as well as activating HLA-C and KIR interactions were balanced between EBV-positive and EBV-negative donors. However, the frequency of HLA-B alleles with a Bw4 epitope was significantly higher among the EBV-negative group with 16/17 donors carrying at least one Bw4 epitope, versus 26/39 EBV-positive donors (p=0.043), thus permitting more inhibitory HLA-Bw4 and KIR interactions. Analyzing the presence of KIR3DL1 –the inhibitory receptor for HLA-Bw4– 94% of EBV-negative donors carried the receptor as compared to 90% of EBV-positive controls. The low frequency of KIR3DL1-negative donors precluded a meaningful combined analysis of KIR3DL1 and HLA-Bw4.

Discussion

In the immuno-competent host, persistent infection with EBV is thought to be accompanied by i) stable numbers of latently infected B cells, ii) serum antibodies specific for lytic and latent viral proteins and iii) EBV-specific memory T cells (70). Here, we screened 515 healthy individuals aged >60 years for EBV-specific humoral immunity by detection of IgG antibodies specific for the viral antigens VCA, EBNA-1 and EA. 17/515 (3.3%) donors repeatedly presenting negative on EBV-serology were further evaluated for EBV genome content and EBV-specific cellular immunity. In contrast to EBV-seropositive donors, in which EBV genome was detectable in >90% of cases, EBV-negative individuals were invariably negative. EBV-specific $CD8^+$ and $CD4^+$ T cell responses were only detected in EBV-positive individuals. Of note, EBV-seropositive donors who tested negative for EBV genome (3/25) had also no detectable $CD8^+$ and only borderline $CD4^+$ T cell reactivity to EBV. It is well established that T cell memory to some viruses is long-lived (71), however, it remains as yet unclear whether contact with antigen is required to maintain long-term memory. Our data would suggest that long-term cellular memory to EBV requires at least some contact with antigen. The question whether EBV-negative individuals are resistant to infection *or* have successfully eliminated virus and lost cellular and humoral immunity due to absence of antigen challenge remains unresolved.

Having identified a group of EBV-negative elderly people, we next asked the question whether these individuals dispose of *one* unique feature rendering them resistant to latent EBV infection, or if rather the interplay of multiple factors ranging from genetic to anatomic factors confers resistance to EBV infection. From an epidemiologic point of view, it is plausible that virtually all individuals get in contact with virus several times in life. After oral transmission from one infected individual to another, the virus enters the local lymphoid tissue of the tonsils to gain access to B cells. Once persistent infection is established, infectious virus is produced continuously from cells undergoing lytic replication (72). Human studies have confirmed that the majority of EBV–infected individuals shed virus into saliva,

suggesting chronic virus replication at some site in the oropharynx (70). As tonsillectomy was significantly more often found within the group of EBV-negative donors compared to matched EBV-positive controls, the tonsillar lymphoid tissue may indeed be essential for the long-term maintenance of EBV-carrier state, as it serves as reservoir for the virus. It is also conceivable, that tonsillectomy efficiently prevents EBV infection. Since the donor's age at tonsillectomy was equal for both, the EBV-negative as well as EBV-positive cohort, the latter possibility seems more unlikely.

In vitro, B cells of EBV-negative individuals could be readily infected with EBV, leading to outgrowth of lymphoblastoid cell lines. Transformation of B cells was actually more efficient in EBV-negative donors with a transformation rate of 100% as compared to 55% for EBV-positives, most probably due to missing EBV-specific T cell memory in virus-negative individuals. Although we did not look into distinct steps of B cell transformation, such as virus binding to the B cell surface, internalization of viral genome and expression of virus-encoded proteins, our data present a strong argument against a B cell intrinsic factor leading to absence of EBV latency in 5-10% of individuals aged >60 years.

Iatrogenic immunosuppression is a well-established risk factor for EBV reactivation and development of EBV-associated malignancies, pointing out the important role of adaptive immunity in controlling EBV infection. Initial acute infection of the immunocompetent host, however, is usually only mildly symptomatic, if at all, and has therefore raised only little attention. It is conceivable that upon primoinfection only subtle functional differences of the adaptive and/or innate immunity decide upon outcome of infection, either leading to latent infection, as for most, or in rare cases clearance of the virus. Combinations of HLA class I molecules and KIR have been described as important immunogenetic factors influencing the course of viral infection. In Hepatitis C infection, clearance of virus is associated with homozygosity for KIR2DL3 and group C1 allotypes (73), a combination predicted to mount a weak KIR2DL-mediated NK cell inhibition. We found no association of KIR2DL3 and HLA group C1 with EBV or CMV seropositivity. In HIV infected patients progression to AIDS is delayed in individuals who have both, an HLA-Bw4 epitope with 80Ile and KIR3DS1 (35)

implicating a better lymphocyte activation in HLA-Bw4 80Ile-positive individuals over such with an HLA-Bw4 80Thr epitope, which represents a weaker ligand. Analogously, we found a significantly higher number of HLA-Bw4 alleles within the EBV-negative group compared to EBV-positive controls. In contrast to studies in HIV infection, however, the increased prevalence of Bw4 alleles was not paralleled by KIR3DS1, and KIR3DS1 was even less frequent in EBV-negative donors. As KIR3DS1 and KIR3DL1 represent alleles, absence of KIR3DS1 in EBV-negative donors would implicate homozygosity for KIR3DL1. HLA-Bw4 and KIR3DL1 interactions were more often found in EBV-negative than in EBV-positive individuals (p=0.004). No overrepresentation of either HLA-Bw4 motif with regard to position 80 was found.

Of note in this context is, that EBV was reported to transcriptionally activate the env gene of the endogenous retrovirus HERV-K18 (74) upon infection of B cells. Env encodes a protein with superantigen activity that can elicit potent, antigen-independent T cell responses. Experiments in severe combined immunodeficient (SCID) mice engrafted with human PBMC show that the presence of T cells is required for the development of EBV-driven lymphoproliferative disease (75, 76). It is conceivable that enhanced T cell activation is detrimental in EBV control either by reducing efficiency of virus recognition and clearance or by activation of latently infected B cells. Our data implicates a role for inhibitory HLA class I and KIR interactions in resistance to latent EBV infection. This observation might thus be favorable in the context of excessive lymphocyte activation.

Future Perspectives

Our data from the cohort of EBV-negative individuals aged >60 years implicate a role for inhibitory HLA class I and KIR interactions in resistance to latent EBV infection. This observation is opposite to recent epidemiologic data that associate KIR/HLA compound genotypes with activating profiles with resistance to viral infection such as Hepatitis C (73) and HIV (77).

X-linked lymphoproliferative disorder (XLP) is a rare condition in which affected patients are able to control most pathogens, but upon infection with EBV show either uncontrolled B cell proliferation *or* fulminant infectious mononucleosis, pancytopenia or hypogamma-globulinemia, suggesting excessive immune activation. Most XLP patients have a mutation in within the signaling lymphocyte activation molecule (SLAM) gene family that encodes SLAM-associated proteins (SAP). The SAP receptor family is expressed in T cells, NK cells, and NK/T cells and it is involved in regulating cellular activation. Data derived from animal models also suggest a contributing role for T cells in the development of EBV-driven lymphoproliferative diseases (75, 76), demonstrating that tumor development can be prevented after depleting T cells or blocking T cell activation with agents such as Cyclosporin A. Excess immune activation has been also firmly established in other viral infections. In HIV, a general consensus was established that elevated $CD8^+$ T cell activation is a hallmark of pathogenic infections (78, 79).

Superantigens (SAg) are microbial proteins that sequentially bind to MHC class II proteins and the Vβ chain of the T cell receptor and thereby strongly stimulate and expand T cells (80) causing immune deregulation. Based on this property, superantigens have been implicated in the development of autoimmune diseases such as type 1 diabetes, rheumatoid arthritis, Kawasaki disease, and psoriasis (81). Interestingly, EBV has been linked to diseases with similar etiology such as systemic lupus erythematosus (82-84), multiple sclerosis (85) and Sjogren's syndrome (86, 87). Indeed, an EBV-associated SAg has been described, which is not encoded within the EBV genome, but by the envelope gene (env) of the human

endogenous retrovirus K18 (HERV-K18) (88). Under steady-state circumstances env is transcriptionally silent and the significance of its transactivation by EBV remains unclear.

We are currently testing the hypothesis, that EBV-induced T cell activation is enhanced by HERV-K18 env transactivation to the benefits of EBV rather then helping to resolve infection. This hypothesis is supported by the observation that marmosets, that do not contain the HERV-K18 provirus in their genome, can be infected by EBV in vitro, but are resistant to latent EBV infection. To test this hypothesis, we have established a quantitative RT-PCR enabling us to monitor HERV-K18 env transcription. Transcription levels will be assessed in resting human B cells and after in vitro infection with EBV. In a next step, we will compare env transactivation in EBV-positive with EBV-negative individuals.

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Curriculum Vitae

Name: Durovic Bojana

Address: Hirzbodenweg 78
CH-4052 Basel

Phone: +41 76 375 09 09
Email: Bojana.Durovic@stud.unibas.ch

Date of birth: 25.01.1980
Nationality: Swiss
Position: MD-PhD Student

Education

Since 2007 MD-PhD programme of the Swiss National Science Foundation

Department of Biomedicine, University Hospital Basel

Laboratory of Immunobiology, Prof. Christoph Hess

2001-2007 Degree in Medicine at the University of Basel

Thesis: *Soluble Ligands for the Immunoreceptor NKG2D in Leukemia.*

Department of Biomedicine, University Hospital Basel

Experimental Hematology, Prof. A. Filipowicz

1997-2001 High School at Neue Kantonsschule Aarau, Switzerland

1998-1999 High School exchange year at Schenley High School
Pittsburgh, USA

Continuing Education/Training

2010	Introductory course in laboratory animal science, LTK1 module Basel, Switzerland
2010	Participation at the World Immune Regulation Meeting, Davos
2007/08/09	Participation with poster at the Congress of Swiss Society of Allergology and Immunology in Basel, Freiburg, and Geneva
2009	Participation at the World Immune Regulation Meeting, Davos
2007	Course in Scientific English, Language Center of the University Basel
2007	Participation with shared oral presentation at the Annual Congress of the Swiss Society of Internal Medicine

Continuing Activities

Member of Youth for Understanding International Exchange Organisation (YFU)

Member of Young Internist of the European Federation of Internal Medicine (EFIM)

Member of the Lawn Tennis Club Basel

Publication List

1) Diermayr S, Himmelreich H, **Durovic B**, Mathys-Schneeberger A, Siegler U, Langenkamp U, Hofstenge J, Gratwohl A, Tichelli A, Paluszewska M, Wiktor-Jedrzejczak W, Kalberer CP, Wodnar-Filipowicz A. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. Blood. 2008;111:1428-36.

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7) Stefanie Fritz, Erik Mossdorf, Gabriela Zenhaeusern, Matthias Mehling, **Bojana Durovic**, Patricia Hilbert, Anna Conen, Ludwig Kapppos, Manuel Battegay, Martin Stern, Reto Nüesch, and Christoph Hess. In absence of HIV infection KIR2DS1 and 3DS1 genes are associated with enhanced CD4+ T cell-responses to influenza-vaccine. *(submitted)*

8) **Bojana Durovic**, Olivier Gasser, Jörg Sigle, Alexis Demoulin, Hans Hirsch, Andreas Buser, Christoph Hess. The EBV non-infected elderly – defining factors of resistance. *(in preparation)*