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Viral Induced CD28 Loss Evokes Costimulation Independent Alloimmunity

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Abstract

Background—Belatacept, a B7-specific fusion protein, blocks CD28-B7 costimulation and prevents kidney allograft rejection. However, it is ineffective in a sizable minority of patients. Although T cell receptor and CD28 engagement is known to initiate T cell activation, many human antigen-experienced T cells lose CD28, and can be activated independent of CD28 signals. We posit that these cells are central drivers of costimulation blockade resistant rejection (CoBRR) and propose that CoBRR might relate to an accumulation of CD28⁻ T cells resulting from viral antigen exposure.

Materials and Methods—We infected C57BL/6 mice with Polyomavirus (a BK virus analog), murine cytomegalovirus (mCMV; a human CMV analog), and gammaherpes virus (HV68; an Epstein-Barr virus analog) and assessed for CD28 expression relative to mock infection controls. We then employed mixed lymphocyte reactions (MLR) assays to assess the alloreactive response of these mice against MHC-mismatched cells.

Results—We demonstrated that infection with Polyomavirus, murine CMV, and HV68 can induce CD28 down-regulation in mice. We showed that these analogs of clinically relevant human viruses enable lymphocytes from infected mice to launch an anamnestic, costimulation blockade

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Author contributions: Danny Mou developed the idea of the project under the guidance of his mentor, Dr. Allan Kirk. He was the point person who conducted the bulk of all aspects of the experiments and data analysis. He wrote the totality of the manuscript under the guidance of Dr. Kirk. Jaclyn Espinosa helped with the project methodology development, mice bleeds, flow cytometric analyses, and MLR assays. She served as an indispensable resource throughout the project. Linda Stempora provided critical guidance in terms of flow cytometry and data analysis. She helped develop the multi-color flow panels and the tailored flow protocols necessary for the project. Neal Iwakoshi provided indispensable senior-level guidance throughout the project design and execution. He played a particularly large role as a thought leader in the flow cytometric analysis portion, and helped with all of the mouse bleeds and splenocyte isolations for the MLR. Dr. Allan Kirk, along with Danny Mou, devised the impetus and structure of the project, and kept an active role in guiding the overall direction of the project. He acted as the senior-PI throughout the project, and provided the funding. He played a critical role in guiding Danny Mou during the writing phase of the manuscript.

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resistant, alloreactive response against MHC-mismatched cells without prior alloantigen exposure. Further analysis revealed that gammaherpesvirus-induced oligoclonal T cell expansion is required for the increased alloreactivity.

Conclusion—Virus exposure results in reduced T cell expression of CD28, the target of costimulation blockade therapy. These viruses also contribute to increased alloreactivity. Thus, CD28 down-regulation following viral infection may play a seminal role in driving CoBRR.

Keywords

CD28; belatacept; alloimmunity; CMV; BK; HV68

1. Introduction

Solid organ transplantation is the therapy of choice for most end-stage organ diseases; it has the potential to drastically improve both quality of life and survival duration for patients with organ failure. Unfortunately, the complication of T-cell mediated rejection is unavoidable without significant immune modification of the recipient. Thus, transplant patients are inextricably tied to immunosuppressants, particularly agents that impair T cell function. T cell activation is a central driver of acquired immune responses, and is predicated on the receipt of antigen-specific stimulation through the T cell receptor (TCR), in combination with non-antigen specific costimulation signals, the most important of which is derived from CD28 engagement. However, once a cell has become activated it can in some circumstances lose CD28 and become capable to being activated upon subsequent antigen encounters without the need for CD28 binding. This is a known characteristic of some human antigen-experienced T cell subsets, including subsets of T cells characterized as Memory T cells (TMs).

CD28 down-regulation is well characterized in humans. The CD28⁻ T cell population is likely comprised of prematurely senescent lymphocytes due to persistent immune activation. High proportions of CD28⁻ T cells have been observed in human diseases, including inflammatory syndromes, chronic infections, and cancer.¹ Human CD28⁻ T cells are functionally active, resistant to apoptosis, and limited in proliferative capacity. Though well known in humans, CD28 loss has not been well characterized in mice. Indeed, as mice are typically studied in pathogen-free conditions, the phenomenon of CD28 down-regulation has not been rigorously evaluated, and as such, studies of transplantation immunosuppression have occurred without due appreciation of the potential consequences of this maturation. We have therefore sought to establish a mouse model of viral infection to investigate CD28 down-regulation, particularly as it relates to viral pathogen exposure.

To characterize virally-induced CD28 down-regulation and CoBRR in mice, we developed a poly-viral infection murine system that involved sequentially infecting mice with Polyomavirus, mCMV, and HV68. These viruses are murine analogs of highly prevalent human viruses, which makes them particularly relevant for the immunosuppressed transplant patient.

2. Materials and Methods

2.1 Mice and Viral Infections

C57BL/6 and BALB/c mice (6-12 weeks old, males) were obtained from Jackson Laboratory (Bar Harbor, ME). Virus stocks were grown and quantitated as previously described.² Mice were given sequential infections, 3 weeks apart, with 10^5 plaque-forming units of Polyomavirus (footpad injection), M187 CMV (i.p.), and HV68 (i.p.). 5 mice cohorts, each containing 10 mice, were defined as mock infections, single PyV infection, single mCMV infection, single HV68 infection, or 'all 3' infections. Of note, HV68 infection in C57BL/6 mice is known to generate a significant oligoclonal T cell V β 4 expansion, which could contribute to CD28 down-regulation and/or augmented alloreactivity.³ To control for this, an M1STOP mutant strain of HV68 engineered to not produce this V β 4 expansion was also used in the CD28 MFI and MLR assays. Viral latency was defined at 3 weeks post-infection.² All animal studies were approved by the Institutional Animal Care and Use Committee of Emory University (IACUC #: DAR-2002644-020317GN). All surgery was performed under anesthesia with isoflurane.

2.2 Costimulation Blockade Administration

CoB-treated mice received 500 mg each of hamster anti-mouse CD154 mAb (MR-1, Bio X Cell, West Lebanon, NH) and human CTLA-4-Ig (Abatacept, Bristol-Meyers Squibb, New York, NY) i.p. at transplantation and on post-transplant days 2, 4, and 6.

2.3 Flow Cytometry

Peripheral blood was prepared with fixative-free lysing solution (Invitrogen, Carlsbad, California) and stained with the relevant mAbs. Antibodies were used against KLRG1 (BV421), CD4 (BV510), and CD44 (PE-CF594) from BD Biosciences (Franklin Lakes, NJ), CD197 (PE), Ki67 (PE), CD28 (PE-Cy7), CD279 (BV605), CD62L (FITC), CD3 (PerCP), and CD8 (APC) from Biolegend (San Diego, CA). Intracellular staining for interferon-gamma (IFN γ) was performed against ICOS (FITC), and CD152 (APC) using the BD Biosciences Fixation/Permeabilization Solution Kit with brefeldin A. Samples were acquired on a BD Biosciences LSRII flow cytometer and analyzed using FlowJo (Tree Star, Ashland, OR). Flow analysis of T cells was performed on the day of infection, at peak infection, at the memory time point, and at multiple long-term time points. Statistical analyses were performed at the 5% significance level with GraphPad Prism 6 (La Jolla, CA).

2.4 Alloreactivity Assays

Balb/c to C57BL/6 splenocyte MLRs were conducted 40 days after the infections. Alloreactivity was assessed by measuring IFN γ -producing lymphocytes both as a percentage of total CD4s or CD8s and as an absolute lymphocyte cell count. Intracellular expression was induced in response to 5 hours of *ex vivo* restimulation with allogeneic stimulators, syngeneic stimulators, or PMA/ionomycin (PMA, 50 ng/ml; ionomycin, 500 ng/ml). In brief, responders were resuspended in cell culture media containing brefeldin (GolgiPlug, BD Pharmingen). All stimulations were performed for 5 h at 37°C. Intracellular staining for IFN γ was performed as per the manufacturer's instructions (Cytotfix/Cytoperm kit, BD

Pharmingen). Flow cytometry was performed on BD LSR II, and data were analyzed using FlowJo (TreeStar) software. Statistical analyses were performed at the 5% significance level with GraphPad Prism 6 (La Jolla, CA).

3. Results

3.1 Viral infection in mice leads to a decrease in T cell effector memory (TEM) CD28 expression

C57BL/6 mice infected with BK, mCMV, and HV68 exhibited statistically significant ($P<0.05$) decrease in CD28 mean fluorescence intensity (MFI) 21 days after initial infection (Figure 1). This CD28 MFI reduction was observed only in CD8 TEMs, which is the lymphocyte population most strongly implicated in mediating both viral immunity and cellular allograft rejection.⁴ Interestingly, the triply infected cohort exhibited a significantly greater degree of CD28 MFI reduction compared to singly infected BK and mCMV cohorts, but not more than the singly infected HV68 cohort, which suggested that HV68 might be responsible for the bulk of the CD28 MFI reduction (Figure 1). Additionally, HV68-infected cohorts exhibited decreased CD28 MFI in both CD4 and CD8 TEMs. Importantly, murine HV68 infections are known to generate a significant oligoclonal T cell V β 4 expansion,³ which could contribute to the CD28 down-regulation. To control for this, an M1STOP mutant strain of HV68 engineered to not produce this V β 4 expansion was used to investigate its impact on CD28 expression. Interestingly, the M1STOP HV68-infected strain did not exhibit significant CD28 MFI reduction in CD8 TEMs (Figure 1C). Overall, murine analogs of clinically relevant and highly prevalent human viruses have the ability to reduce CD28 MFI with the exception of M1STOP HV68 strain. It should be noted that though statistically significant CD28 down-regulation was observed, murine lymphocytes were not observed to lose surface expression of CD28 completely, as seen in humans. This may imply an inherent phenotypic immune system difference between mice and humans.

3.2 Splenocytes exhibited increased CD8 lymphocyte IFN γ production in the HV68 and triply infected cohorts

Balb/c to C57BL/6 splenocyte MLRs were conducted 40 days after receipt and resolution of all of the infections. Alloreactivity was assessed by measuring IFN γ -producing lymphocytes both as a percentage of total CD4s or CD8s and as an absolute lymphocyte cell count. The HV68 and triply infected cohorts exhibited significantly greater alloreactivity in MLR as measured by IFN γ production when compared with the naïve and the singly infected polyoma and mCMV cohorts (Figure 2A, 2B).

To investigate whether this virally-induced alloreactivity was resistant to costimulation blockade, MLRs of splenocytes at day 14 were conducted with combinations of costimulation blockade treatments, including CTLA4-Ig, MR1, or a combination of CTLA4-Ig and MR1 (Figure 2C, 2D). The triply infected cohort exhibited increased IFN γ production relative to the naïve cohort despite costimulation blockade treatment, suggesting that this virally-induced alloreactivity is costimulation-blockade resistant. The overall MLR reactivity was not appreciably reduced by the CoB.

3.3 Infection with the M1Stop mutant of HV68 does not result in increased IFN γ production

Infection of HV68 in mice is known to generate a significant oligoclonal T cell V β 4 expansion, which could contribute to the augmented alloreactivity.³ To control for this potential confounder, an M1STOP mutant strain of HV68 engineered to not produce this V β 4 expansion was used to investigate its impact on alloreactivity (Figure 3). Interestingly, the M1STOP cohort did not confer additional IFN γ production relative to the naïve cohort whereas the wild-type HV68 did, suggesting that the oligoclonal V β 4 expansion plays an important role in increasing the ability to produce IFN γ . Consequently, oligoclonal T cell expansion may be responsible for mediating CoBRR as opposed to a direct virally-mediated CoBRR mechanism.

4. Discussion

The emergence of T cell costimulation blockade-based immunosuppression therapies in solid organ transplantation has significantly increased the importance of understanding CD28 expression on T cells. Belatacept, a B7-specific fusion protein, blocks CD28-B7 costimulation and prevents kidney allograft rejection. However, it has been ineffective in preventing early cell mediated acute rejection in a sizable minority of patients.⁵ It has become increasingly evident that viral infections and alloimmune rejection are related via a process known as heterologous immunity.^{3,6-8} Over time, as individuals are exposed to viral antigens, they accumulate T cells with lower activation thresholds. These T cells also exhibit a phenomenon known as “experience by proxy”, whereby they enable an allo-naïve recipient to launch a significant anamnestic-like alloreactive cellular response against the transplanted organ. Specifically, TMs have been implicated in expanding during heterologous immune response^{6,9} and contributing to costimulation blockade resistance.^{4,10} Within this TM population, it is thought that the antigen-experienced TMs that exhibit CD28 down-regulation are the primary drivers of CoBRR. It is reasonable to suspect that belatacept, which blocks CD28-B7 costimulation, may not be as effective in TMs that have down-regulated CD28 expression. We posit that CoBRR might relate to the accumulation of CD28⁻ T cells resulting from viral antigen exposure. Though patients treated with belatacept enjoyed improved long-term renal function, they experienced a higher rate of acute rejection compared to patients on standard calcineurin inhibitor-based therapy.^{5,11} These findings suggest that the subset of patients who are at high risk for CoBRR may possess a CD28dim TM-skewed immune repertoire from viral exposure. It is thus critically important to elucidate the relationship between viral infections and heterologous alloimmunity.

The immune repertoire is continuously changing as a result of expansions and contractions of cells responding to environmental antigenic exposures. Given the emergence of costimulation blockade-based therapies for solid organ transplants, and the changes in costimulation molecules occurring as a result of these dynamic alterations, it is imperative to understand the impact of changes in one's immune repertoire on CoB-based regimens. Transplant patients are typically latently or persistently infected with numerous viruses, the most common of which are BK virus, CMV, and Epstein-Barr virus (EBV). While prior studies have established that acute viral infections can induce repertoire changes that lead to CoBRR, the impact of persistent infections, such as BK virus, or latent viruses such as CMV

and EBV have been poorly characterized.^{6,12-14} In addition, specific attention toward fluctuations in CD28 expression has been lacking, largely because these changes are less overt in mice than in humans. To model these aspects of the human condition, we established a murine model that employs a sequential polyviral infection of the most common relevant human virus homologs: Polyomavirus (BK virus homolog), mCMV (CMV homolog), and HV68 (EBV homolog). We used this model to investigate virally-induced CD28 down-regulation, and its impact on alloreactivity *in vitro* and in a murine heart transplant system. We find that these viruses indeed lead to increased alloimmunity and that this is associated with a subtle but statistically evident alteration in CD28 expression, with the degree of change in CD28 most associated with HV68 infection.

Employing our poly-viral infection model, we showed that Polyomavirus, mCMV, and HV68 are capable of inducing CD28 MFI reduction in CD8 TEMs (Figure 1). This CD28 MFI reduction is not correlated with a cell size reduction (data not shown), suggesting that it represents a true biologic CD28 down-regulation. During HV68 and mCMV infections, CD8⁺ T cells have been shown to be critical for both the resolution of acute infection^{15,16} and for the long-term control of persistent infection.¹⁷ Thus, these viruses may have an evolutionary impetus to compromise the host CD8 T cell function. Herpesviridae, including HV68, have been shown to release cytokines (IL-10), chemokines, and interferons to disrupt immune signaling and attenuate immune response.¹⁸ HV68 may therefore down-regulate host CD28 expression as a mechanism to stall immune response and maintain latent infection. Unlike HV68 and mCMV, Polyomavirus titers are strongly related to IFN γ , and not associated with MPyV-specific CD8 T cells.¹⁹ Given its relative independent function from CD8 T cell activation, a virally-mediated attenuation of CD8 T cells seems evolutionarily unnecessary. Instead of a pathologic virus-mediated phenomenon, CD28 down-regulation may be a physiologic response to Polyomavirus.

We have also demonstrated that HV68 have the unique ability to augment alloreactive response. Our MLR assays reveal that splenocytes of mice infected with HV68 exhibited significantly increased IFN γ production compared to splenocytes of mice infected with Polyomavirus and mCMV, suggesting that somehow HV68 uniquely increased alloreactivity (Figure 2). Indeed, latent HV68 has been previously shown to resist allograft tolerance in a mixed-chimerism model.³ Additionally, HV68 is known to generate a V β 4 CD8 T cell expansion.^{20,21} The M1STOP mutant of HV68 has been shown to establish a latent infection that is similar to wild-type HV68, but does not induce V β 4 CD8 T cell expansion.²² Interestingly, our MLR assays reveal that the M1STOP HV68 does not confer increased IFN γ production, suggesting that the V β 4 CD8 T cell expansion may be necessary for augmented alloreactivity (Figure 3A). This HV68 nuance has also been shown in a mixed chimerism model.³ Furthermore, this increased alloreactivity is resistant to both CTLA4-Ig and MR1, costimulation blockade therapies that inhibit CD28 and CD154, respectively (Figure 3B). Importantly, we also show that the M1STOP HV68-infected mice do not exhibit any CD28 MFI reduction, illustrating a correlative relationship between CD28 down-regulation and *in vitro* alloreactivity (Figure 1C).

The M1 protein has been shown to mediate the VB4 CD8 T cell expansion in a way similar to a viral superantigen.²³ As such, although it is an HV68-specific protein, its actions on T

cell expansion could be surmised to have effects similar to expansions in response to other viral mechanisms. Specific to HV68, the M1 protein is necessary and sufficient to mediate a VB4 expansion that appears to maintain viral latency and suppress viral reactivation. The teleological impetus of the M1 protein is poorly described, but it has been suggested that the M1 protein induces this VB4 CD8 T cell expansion, which in turn secretes IFN γ to suppress virus reactivation. Thus, the M1 protein may serve as a mechanism to modulate the threshold of HV68 virus reactivation. We have shown that the T cells from this VB4 CD8 T cell expansion downregulates CD28 expression, and this suggests that the T cell expansion may have resulted in an exhausted T cell phenotype. Indeed, CD28 downregulation has been previously associated with exhaustion in the context of chronic viral infections.²⁴

The average transplant patient is infected with multiple latent viruses, which can alter the immune repertoire by down-regulating CD28 surface expression, and thus predispose the patient to belatacept-resistant rejection. We have established a poly-viral murine model showing that clinically relevant viruses not only induce CD28 down-regulation, but also augment alloreactivity *in vitro* and *in vivo*. These findings provide a platform for studying costimulation blockade in mice, and suggest that viruses may lead to medically relevant and potentially anticipatable changes in the immune repertoire. Understanding the dynamic interface between latent viral infections and responsiveness to costimulation blockade therapy may enable prediction whether transplant patients will be responsive to costimulation blockade therapy based on their viral serologies.

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Figure 1.
Heterologous infections induce CD28 down-regulation. HV68 is the primary driver for CD28 down-regulation (A, B). However, the M1STOP HV68 infection does not induce CD28 down-regulation in CD8 TEMs (C).

Figure 2.

MLR of splenocytes demonstrate increased CD8 lymphocyte IFN γ production in the HV68 and triply infected cohorts (A, B). This increased IFN γ production is resistant to costimulation blockade (C, D).

Figure 3.

M1STOP HV68 does not result in increased IFN γ production of CD8 T cells (A). This effect holds persists with CTLA4-Ig treatment (B).