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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 are 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 (a human cytomegalovirus analog), and gammaherpesvirus (HV68; an Epstein–Barr virus analog) and assessed for CD28 expression relative to mock infection controls. We then used mixed lymphocyte reaction (MLR) assays to assess the alloreactive response of these mice against major histocompatibility complex-mismatched cells.

*Results:* We demonstrated that infection with polyomavirus, murine CMV, and HV68 can induce CD28 downregulation in mice. We showed that these analogs of clinically relevant human viruses enable lymphocytes from infected mice to launch an anamnestic, costimulation blockade resistant, alloreactive response against major histocompatibility complex-mismatched cells without prior alloantigen exposure. Further analysis revealed that gammherpesvirus–induced oligoclonal T-cell expansion is required for the increased alloreactivity.

*Conclusions:* 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 downregulation after viral infection may play a seminal role in driving CoBRR.



## 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

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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, 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 of being activated on 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 downregulation is well characterized in humans. The $CD28^-$ T-cell population is likely composed 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. Although 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 downregulation 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 downregulation, particularly as it relates to viral pathogen exposure.

To characterize virally induced CD28 downregulation and costimulation blockade resistant rejection (CoBRR) in mice, we developed a poly-viral infection murine system that involved sequentially infecting mice with polyomavirus, murine cytomegalovirus (mCMV), and HV68. These viruses are murine analogs of highly prevalent human viruses, which make 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-wk-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), ΔM187 cytomegalovirus (ΔM187 CMV; intraperitoneally [i.p.]), and HV68 (i.p.). Five mice cohorts, each containing 10 mice, were defined as mock infections, single PyV infection, single mCMV infection, single HV68 infection, or "all three" infections. Of note, HV68 infection in C57BL/6 mice is known to generate a significant oligoclonal T-cell Vβ4 expansion, which could contribute to CD28 downregulation and/or augmented alloreactivity [3]. To control for this, an M1STOP mutant strain of HV68 engineered to not produce this Vβ4 expansion was also used in the CD28 mean fluorescence intensity (MFI) and MLR assays. Viral latency was defined at 3 weeks after 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 monoclonal antibodies (MR-1; Bio X Cell, West Lebanon, NH) and human CTLA-4-Ig (Abatacept; Bristol-Myers 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, CA) and stained with the relevant monoclonal antibodies. Antibodies were used against KLRG1 (BV421), CD4 (BV510), and CD44 (PE-CF594) from BD Biosciences (Franklin Lakes, NJ) and 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 (IFNγ) 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 d after the infections. Alloreactivity was assessed by measuring IFNγ-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 h 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, San Jose, CA). All stimulations were performed for 5 h at 37°C. Intracellular staining for IFNγ was performed as per the manufacturer's instructions (Cytofix/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.

## 3. Results

### 3.1. Viral infection in mice leads to a decrease in T-cell effector memory CD28 expression

C57BL/6 mice infected with BK, mCMV, and HV68 exhibited a statistically significant ($P < 0.05$) decrease in CD28 MFI 21 d after initial infection (Fig. 1). This CD28 MFI reduction was observed only in CD8 T-cell effector memory (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