View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Immunity
Article
Cell PRESS

Cytomegalovirus Evasion of Innate Immunity by Subversion of the NKR-P1B:Clr-b Missing-Self Axis

Sebastian Voigt,[1,3,*] Aruz Mesci,[2,3] Jakob Ettinger,[1] Jason H. Fine,[2] Peter Chen,[2] Wayne Chou,[2] and James R. Carlyle[2,*]
[1] Division of Viral Infections, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany
[2] Department of Immunology, University of Toronto, Sunnybrook Research Institute, 2075 Bayview Ave, Toronto, ON M4N 3M5, Canada
[3] These authors contributed equally to this work.
*Correspondence: voigts@rki.de (S.V.), james.carlyle@utoronto.ca (J.R.C.)
DOI 10.1016/j.immuni.2007.03.013

SUMMARY

Cytomegaloviruses are known to encode several gene products that function to subvert MHC-dependent immune recognition. Here we characterize a rat cytomegalovirus (RCMV) C-type lectin-like (RCTL) gene product with homology to the Clr ligands for the NKR-P1 receptors. RCMV infection rapidly extinguished host Clr-b expression, thereby sensitizing infected cells to killing by natural killer (NK) cells. However, the RCTL protein functioned as a decoy ligand to protect infected cells from NK killing via direct interaction with the NKR-P1B inhibitory receptor. In vivo, an RCTL mutant virus displayed diminished virulence in an NK-dependent and strain-specific manner, suggesting that host NKR-P1 polymorphisms have evolved to avert the viral decoy mechanism while maintaining Clr-b recognition to preserve self tolerance. These findings reveal a unique strategy adopted by cytomegaloviruses to evade MHC-independent self-nonself discrimination. The existence of lectin-like genes in several poxviruses suggests that this may represent a common theme for viral evasion of innate immunity.

INTRODUCTION

Natural killer (NK) cells are innate immune lymphocytes capable of recognizing and destroying a wide variety of target cells, including transformed, infected, transplanted, antibody-coated, and stressed cells (Lanier, 2005). NK-mediated self-nonself discrimination is controlled at the molecular level by an integrated sum of stimulatory and inhibitory signals, transmitted via receptor-ligand interactions between NK cells and their targets, respectively. On normal cells, inhibitory ligands predominate, leading to self-tolerance through attenuation of NK cell activation (Kumar and McNerney, 2005; Raulet and Vance, 2006). However, in pathological states, target cells often lose inhibitory "self" ligands, leading to enhanced cytotoxicity via disinhibition of NK cells. This mode of detection has been designated "missing-self" recognition (Karre et al., 1986).

Several NK receptor families have been described, including the NKR-P1, Ly49, and CD94-NKG2 families (Yokoyama and Plougastel, 2003). The best-characterized missing-self ligands are the MHC I alleles recognized by the Ly49 receptors in rodents and KIR receptors in humans (Lanier, 2005). Nevertheless, non-MHC missing-self ligands for the NKR-P1 and 2B4 (CD244) receptors have recently been identified (Kumar and McNerney, 2005; Mesci et al., 2006).

The NKR-P1 family consists of homodimeric type II transmembrane C-type lectin-like proteins found primarily on NK cells, NKT cells, and activated $CD8^+$ T cells. Both stimulatory and inhibitory NKR-P1 isoforms have been characterized, encoded by distinct but closely related genes found within the NK gene complex (NKC) (Mesci et al., 2006; Yokoyama and Plougastel, 2003). Genes encoding the NKR-P1 family (designated *Klrb1*) appear to be conserved among birds, rodents, humans, and other mammals, suggesting that the gene products play an important role in innate immunity across species boundaries (Hao et al., 2006; Mesci et al., 2006).

Whereas the NKR-P1 receptors were originally thought to recognize carbohydrates (Bezouska et al., 1994), physiological ligands were recently discovered and shown to comprise a group of C-type lectin-like proteins themselves (Carlyle et al., 2004; Iizuka et al., 2003). Specifically, the cognate ligands are encoded by a gene family designated *Clec2* (also known as *Ocil* [Zhou et al., 2001] or *Clr* [Plougastel et al., 2001a]), which is genetically intermingled among the *Klrb1* receptor genes within the NKC. Genetic linkage of the *Clec2* and *Klrb1* genes underscores the importance of this system as a particularly unique self-nonself discrimination tool, because the "self" ligand is always coinherited with its cognate receptor (Carlyle et al., 2004; Iizuka et al., 2003; Mesci et al., 2006).

Only two ligands for the NKR-P1 receptors are currently known: Clr-b is a ligand for the inhibitory NKR-P1B receptor, whereas Clr-g is a ligand for the stimulatory NKR-P1F

receptor (Carlyle et al., 2004; Iizuka et al., 2003). In general, Clr transcripts exhibit restricted expression (Plougastel et al., 2001a); however, Clr-b displays a broad expression pattern similar to that of the MHC I ligands for the Ly49 receptors (Carlyle et al., 2004). Moreover, like MHC I, Clr-b is frequently downregulated on many tumor cell lines. This MHC-independent ''missing-self'' state results in increased sensitivity of tumor cells to NK cytotoxicity (Mesci et al., 2006). Nonetheless, the importance of this receptor-ligand system in recognition of infected cells remains unknown.

To date, several strategies for viral evasion of innate and adaptive immunity have been documented, mainly via cytomegalovirus (CMV) models (Lodoen and Lanier, 2005; Reddehase, 2002; Vidal and Lanier, 2006). The majority of CMV immunomodulatory gene products prevent T and NK stimulation via downregulation of host proteins, including MHC I molecules (e.g., MCMV m06, m152; HCMV US2, US3, US6, US11) and NKG2D ligands (e.g., MCMV m138, m145, m152, m155; HCMV UL16, UL142) (Lenac et al., 2006; Lodoen and Lanier, 2005; Vidal and Lanier, 2006; Wills et al., 2005). However, cytomegaloviruses also encode ''decoy'' ligands, including MHC I homologs that directly or indirectly inhibit T and NK function (e.g., MCMV m04, m144, m157; HCMV UL18, UL40) (Lodoen and Lanier, 2005; Vidal and Lanier, 2006). For example, the MCMV m157 gene product interacts directly with the Ly49I inhibitory receptor, whose cognate ligands include H-2K alleles (Arase et al., 2002; Smith et al., 2002; Vidal and Lanier, 2006). Thus, m157 plays a direct decoy role in evasion of MHC-dependent NK recognition. However, MHC-independent missing-self viral decoy strategies have yet to be documented.

In this report, we characterize an orphan RCMV gene product (RCTL) that closely resembles rat Clr-b (Mesci et al., 2006; Voigt et al., 2001). By using a recently developed monoclonal antibody (R3A8 mAb), we show that RCMV infection stimulates loss of rClr-b that is rapidly countered by upregulation of RCTL surface expression. RCTL inhibits NK killing of infected cells via direct interaction with NKR-P1B. Thus, RCTL functions as a decoy ligand to subvert NKR-P1B-mediated missing-self recognition by NK cells. Notably, an RCTL mutant (ΔRCTL) virus displays diminished virulence in vivo in a strain-dependent manner controlled by host NKR-P1 polymorphisms. Allelic divergence of rodent NKR-P1 receptors suggests that the host genomes are evolving under selection pressure to avert this viral evasion strategy (Carlyle et al., 2006; Mesci et al., 2006). These findings reveal a nonredundant role for NKR-P1B:Clr-b interactions in NK-mediated immunity to infection.

RESULTS

Rat Cytomegalovirus Encodes a Viral Ortholog of the Host Clr-b Ligand

The role of the NKR-P1B:Clr-b self-nonself discrimination system in the detection of infected cells by NK cells remains unknown (Kumar and McNerney, 2005; Mesci et al., 2006). Because previous work identified a spliced RCMV C-type lectin-like (*rctl*) gene with substantial homology to both *Klrb1* and *Clec2* genes (Voigt et al., 2001), we analyzed this sequence further. The RCTL gene product displays 50% and 48% amino acid identity to mouse Clr-b and Clr-g, respectively, and 60% identity to rat Clr-b (also known as rCLEC2D11; Figure 1A and Table S1 in the Supplemental Data available online; Hao et al., 2006; Plougastel et al., 2001a; Zhou et al., 2001). Interestingly, the *rctl* gene also retains an intron-exon structure similar to that of the rodent *Clec2d* genes (Figure 1B). This suggests a recent evolutionary acquisition of the viral gene from the host during a replication cycle. However, the *rctl* gene has markedly diverged from the rat *Clec2d11* gene. In particular, most intronic sequences have contracted, and the region encoding the cytoplasmic domain has been truncated and altered (Figures 1A and 1B). This is expected to have consequences for the independent regulation of RCTL expression and function relative to the host Clr-b gene product. Therefore, we analyzed RCTL and rClr-b expression in RCMV-infected rat embryonic fibroblast (REF) cells in vitro.

Reciprocal Regulation of RCTL and rClr-b Expression during RCMV Infection In Vitro

As previously shown via RNA blotting (Voigt et al., 2001), RT-PCR analysis demonstrated that RCTL was rapidly expressed upon RCMV infection (Figure 2A). Similar results were seen via quantitative real-time RT-PCR analysis (Figure S1A). Notably, RCTL transcripts were first detectable by 3 hr after infection, and expression was inhibited by cycloheximide but not phosphonoacetic acid, classifying *rctl* as an early gene (Figure S1B and data not shown; Voigt et al., 2001). In contrast, expression of host rClr-b was rapidly extinguished after RCMV infection, while expression of another Clr family member, rCLEC2D5 (Hao et al., 2006), and β-actin remained relatively unaltered (Figure 2A). The selective loss of rClr-b transcripts indicated that this effect was not due to global viral interference with host gene expression (Figure 2A; Figure S1A), nor was it a direct consequence of RCTL expression, because a ΔRCTL mutant virus (Voigt et al., 2001) exhibited the same phenotype (Figure S1A). Rather, host ligand downregulation appeared to be an innate response triggered within the cell upon virus infection.

To directly investigate expression of RCTL protein during infection, we generated RCTL mAb (Mesci and Carlyle, 2007). One clone (R3A8 mAb, mIgMκ) recognized both RCTL and rClr-b but did not crossreact with any other Clr (CLEC2 family) proteins (Figure 2B and data not shown; Hao et al., 2006). The dual specificity of R3A8 mAb further underscores the similarity of RCTL and rClr-b. Moreover, R3A8 reactivity with fresh splenocytes and bone-marrow cells confirmed a broad expression profile of rClr-b on hematopoietic cells ex vivo (Figure 2C), similar to that seen for mClr-b (Carlyle et al., 2004). In order to distinguish between the viral and host proteins, we infected REF cells in parallel with wild-type (WT) RCMV versus ΔRCTL virus. In keeping with rClr-b transcript results

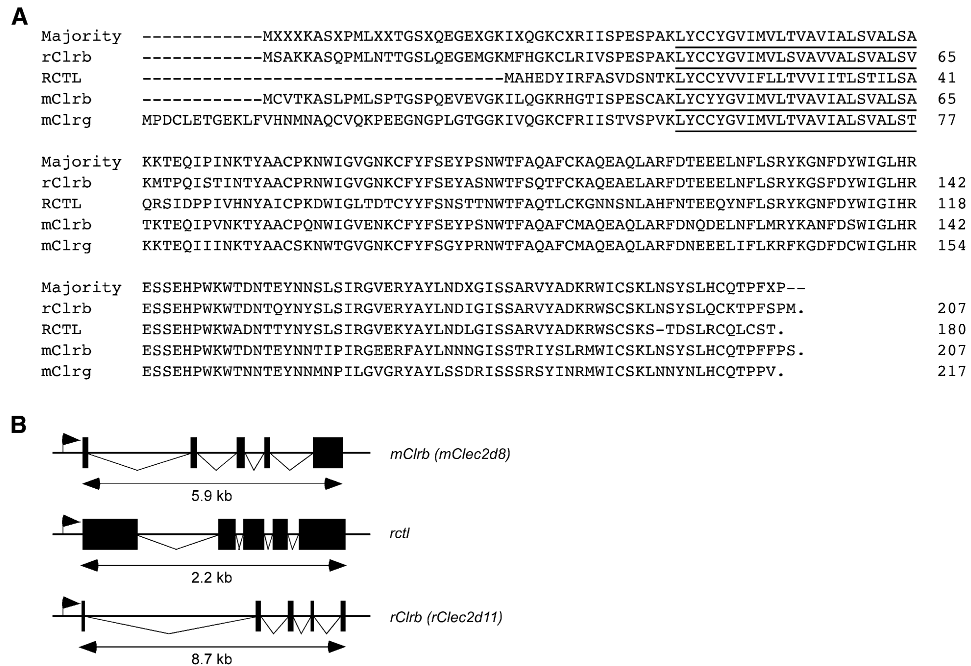

Figure 1. Identification of a Clr-b Ortholog in the RCMV Genome

(A) Alignment of the rat Clr-b (rClrb), RCMV C-type lectin (RCTL), mouse Clr-b (mClrb), and mouse Clr-g (mClrg) coding sequences. Consensus sequence (majority) is shown at the top, and amino acid positions are shown at the right; the transmembrane region is underlined. GenBank sequences are listed in Table S3.

(B) Diagram of the intron-exon structure of *Clec2d* orthologs from the mouse (*mClrb;* designated *mClec2d8*), rat (*rClrb;* designated *rClec2d11*), and RCMV (*rctl*). Arrows represent promoter orientation, boxes and lines indicate exons and introns, respectively, and lengths are given in kilobases below each gene. Analyses were performed with DNAStar Lasergene 7.0 software.

(Figure 2A), REF cells rapidly lost R3A8 staining in response to infection with ΔRCTL virus (Figure 2D), confirming that rClr-b surface protein was downregulated during infection. In contrast, REF cells retained then upregulated R3A8 staining upon infection with WT RCMV (Figure 2D), demonstrating that RCTL was maintained at the cell surface. At present, we cannot rule out partial heterodimerization of RCTL and rClr-b during WT RCMV infection; however, heterologous transfection experiments (Figure 2B and data not shown) suggest that RCTL can be expressed alone at the cell surface, likely as a homodimer. The kinetics of rClr-b downregulation suggest that turnover of cell-surface protein is rapid. Yet in contrast to MHC I downregulation, which occurs by intracellular retention and degradation of ligand protein (Lodoen and Lanier, 2005), the loss of rClr-b transcripts upon RCMV infection (Figure 2A) suggests that loss of surface protein can occur independently of other RCMV gene products. These findings demonstrate that RCMV infection results in modulation of rClr-b expression in a manner expected for a missing-self ligand, whereas RCTL may function as a decoy ligand.

Direct Interaction of RCTL with the Host NKR-P1B Inhibitory Receptor

To determine the host receptor recognized by RCTL, we employed our previous modification of the BWZ reporter cell assay, in which a CD3ζ fusion protein of the receptor of interest generates β-galactosidase activity in response to receptor ligation by cognate ligand or specific antibody (Carlyle et al., 2004; Mesci and Carlyle, 2007; Sanderson and Shastri, 1994). Here, BWZ reporter cells expressing a CD3ζ–RCTL fusion receptor (BWZ.RCTL) but not BWZ− control cells responded well when stimulated with plate-bound R3A8 mAb (Figure 3A). BWZ.RCTL cells were also stimulated by fresh ex vivo splenocytes and bone-marrow cells (Figure 3B) and by day 5 lymphokine-activated killer (LAK) cells (Figure 3C). Of note, the BWZ.RCTL response to fresh splenocytes was augmented relative to bone-marrow cells, whereas the response to splenic LAK was diminished relative to bone-marrow LAK (Figure 3B versus Figure 3C). These data are consistent with direct interaction of RCTL with a receptor expressed on NK cells and parallel the expression pattern of the rNKR-P1B inhibitory receptor, which proportionately decreases during splenic LAK culture (Kveberg et al., 2006). Collectively, these results suggest that RCTL may interact with host NKR-P1B, an interaction that is also predicted because of the strong homology between RCTL and Clr-b, the cognate ligand for NKR-P1B in mice (Carlyle et al., 2004).

To test this interaction, BWZ.RCTL cells were incubated with 293T transfectants bearing rat NKR-P1 receptors. Indeed, strong reporter activity was detected upon

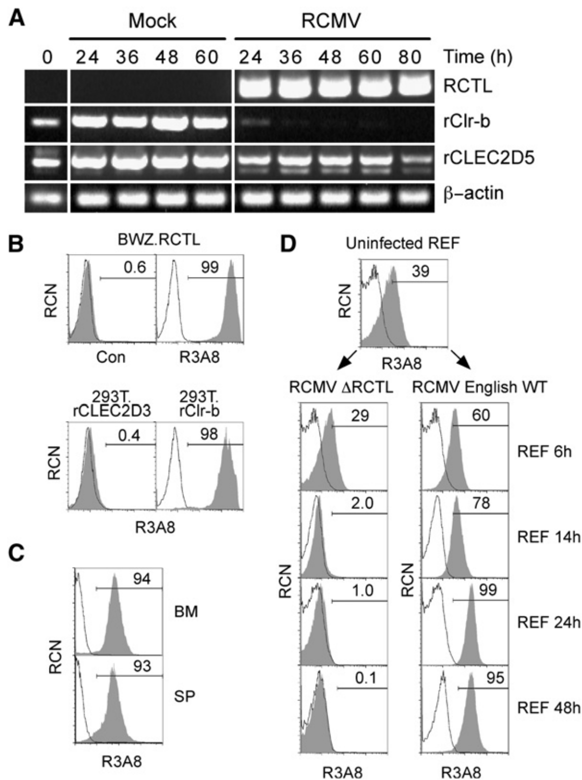

Figure 2. Reciprocal Regulation of RCTL and rClr-b during RCMV Infection

(A) Time-course RT-PCR analysis of transcript expression in REF cells during mock treatment or infection with WT-English RCMV. Lanes show equal cDNA input via limited cycle number. Representative of three experiments.

(B) Dual specificity of the R3A8 mAb for the viral RCTL (top) and host rClr-b (bottom) proteins, as revealed by flow cytometric analysis. Also shown are control stains: top, BWZ.RCTL transductants (Con; 10/78 mAb, α-NKR-P1A/B); and bottom, 293T.rCLEC2D3 transfectants (R3A8 mAb). Gray shading, transductants or transfectants (IRES-GFP^{+} gated); thin line, parental cell controls; RCN, relative cell number. Representative of three experiments.

(C) Surface staining of host rClr-b protein on fresh ex vivo rat bone-marrow cells (BM) and splenocytes (SP) via R3A8 mAb (gray shading) relative to secondary reagent alone (thin line). Representative of two experiments.

(D) R3A8 surface staining (gray shading) of host rClr-b versus viral RCTL protein on uninfected REF cells (top), or REF cells infected with ΔRCTL virus (left) or WT RCMV (right) over a 48 hr time course. Control staining, 10/78 mAb (thin line). Representative of three experiments. Percent positive populations are given in the upper right corner of each panel.

BWZ.RCTL stimulation with 293T transfectants expressing rNKR-P1B, but not with untransfected 293T cells (data not shown) or 293T transfectants expressing the related rNKR-P1F receptor (Figure 3D; Carlyle et al., 2006; Hao et al., 2006; Mesci et al., 2006). These results reveal that the RCTL protein interacts directly with the rNKR-P1B receptor. Like the related mNKR-P1B (Carlyle et al., 1999; Kung et al., 1999) and hNKR-P1A (Aldemir et al., 2005; Rosen et al., 2005) receptors, rNKR-P1B inhibits NK cell function (Kveberg et al., 2006; Li et al., 2003).

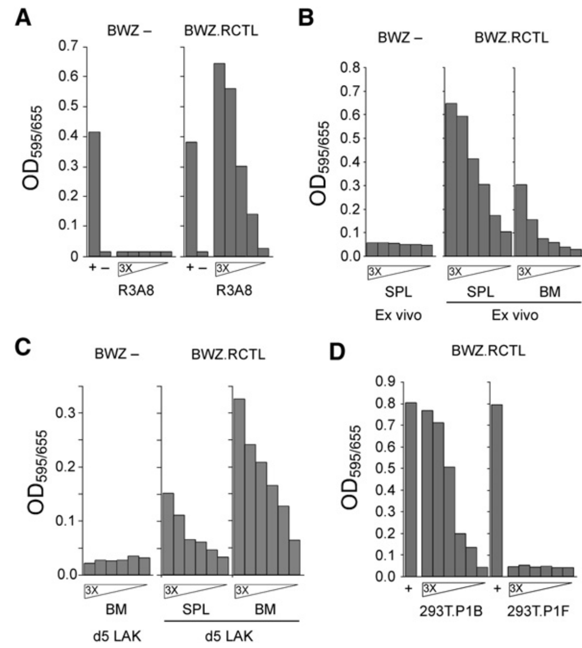

Figure 3. RCTL Interacts Directly with the NKR-P1B Inhibitory NK Cell Receptor

(A) Untransduced BWZ reporter cells (BWZ−) or BWZ cells expressing a CD3ζ–RCTL fusion-receptor (BWZ.RCTL) were stimulated overnight with immobilized R3A8 mAb titrated in 3-fold serial dilutions (initial concentration, 30 μg/mL in PBS) on 96-well plates, and β-galactosidase activity was quantitated. Controls: PMA/ionomycin (+); media alone (−). Representative of four experiments.

(B–D) Quantitation of β-galactosidase activity after BWZ.RCTL and BWZ− reporter cell responses to various stimulator cells: (B) fresh ex vivo hematopoietic cells (SPL, splenocytes; BM, bone-marrow cells), (C) day 5 LAK cells, or (D) 293T transfectants expressing the rat NKR-P1B or NKR-P1F receptors. Stimulator cells were titrated in 3-fold serial dilutions (starting with 10^5 cells) prior to addition of BWZ reporter cells (5×10^4 cells). Representative of two experiments.

Together, these findings are consistent with a decoy role for RCTL.

The RCTL Decoy Ligand Inhibits NK Cell Function and Augments RCMV Virulence

To assess the function of RCTL, we examined NK cytotoxicity of REF cells either uninfected or infected with WT versus ΔRCTL virus. Notably, WT-infected REF target cells were more resistant to NK killing than ΔRCTL-infected REF targets at 24 hr after infection (Figure 4A). Although the magnitude of this inhibition (WT versus ΔRCTL) appeared to be modest, it is important to note that NKR-P1B is expressed only by a subset of rodent NK cells (Carlyle et al., 1999; Kung et al., 1999; Liu et al., 2000); thus, in the rat (Kveberg et al., 2006), only half of LAK effectors are expected to be inhibited by RCTL, and other modes of recognition on distinct or overlapping NK subsets may mask RCTL inhibition. Because no specific mAb for rNKR-P1B is commercially available (Kveberg et al., 2006), this precluded prospective isolation of rNKR-P1B^{+} effectors from their rNKR-P1B^{-} counterparts.

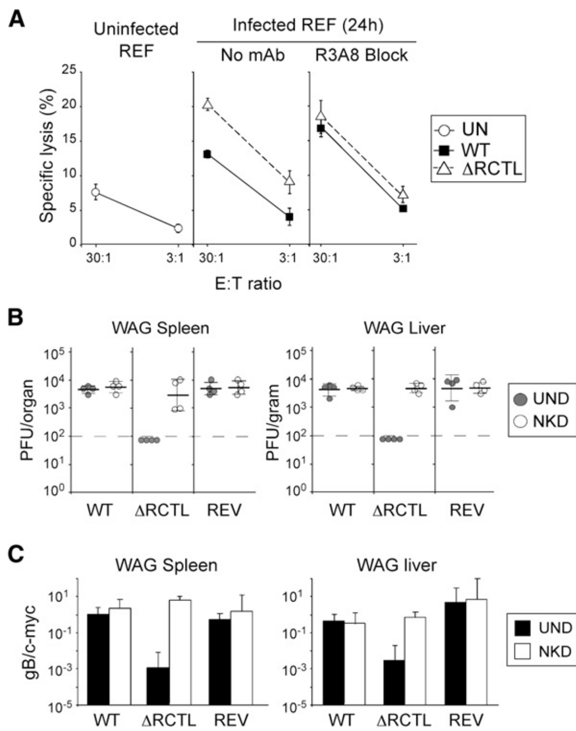

Figure 4. RCTL Inhibits NK Cell Function In Vitro and Augments RCMV Virulence In Vivo

(A) ^{51}Cr-release cytotoxicity analysis with d5 LAK effectors (from WAG-*nu/nu* rats) and REF targets. Uninfected REF cells or REF cells infected overnight (24 hr) with WT or ΔRCTL virus (moi = 5) were used as targets, either in the absence (no mAb) or presence (R3A8 block; 25 μg/mL) of blocking R3A8 mAb. Circles, uninfected REF cells; squares, WT-infected REF cells; triangles, ΔRCTL-infected REF cells. Percent specific lysis values (±SD) obtained at 30:1 and 3:1 effector to target (E:T) ratios are shown. Representative of three experiments.

(B) Viral titers (PFU) derived from spleens and livers of WAG rats infected with WT, ΔRCTL, or REV virus at day 3 after infection. Viral titers are plotted on a $\log_{10}$ scale, with bars indicating geometric means (±SD). The dashed line indicates the limit of detection; both NK-depleted (NKD) and undepleted (UND) animals are shown. ΔRCTL-UND PFU titers are significantly different from all other cohorts ($p < 0.05$).

(C) Viral loads of WAG tissues shown in (B) were assessed in parallel by quantitative real-time PCR (qPCR) analysis of normalized RCMV genome copy numbers (see Experimental Procedures). Viral loads are plotted on a $\log_{10}$ scale as geometric means (±SEM). ΔRCTL-UND qPCR values are significantly different from all other cohorts ($p < 0.05$), except WT-NKD-Liver ($p > 0.05$).

Nonetheless, the differential killing of WT versus ΔRCTL-infected targets was fully neutralized by R3A8 mAb (Figure 4A; R3A8 block), directly demonstrating that RCTL inhibits NK cell function. Because R3A8 is an IgM isotype mAb (Mesci and Carlyle, 2007), increased killing of WT-infected cells in the presence of R3A8 mAb is not due to antibody-dependent cellular cytotoxicity. These data support a direct decoy role for RCTL in functional inhibition of NK cytotoxicity in vitro.

To determine whether RCTL functions in evasion of innate immunity in vivo, we infected rats (WAG strain) with RCMV (WT, ΔRCTL, or an RCTL-revertant [REV] virus) and assayed infected rats for viral burden early after infection (day 3). To this end, splenic and liver infectious virus titers (PFU) were analyzed in parallel with RCMV genomic copy numbers, the latter determined by quantitative real-time PCR (qPCR) analysis, by normalizing RCMV glycoprotein-B (*gB*) relative to host c-*myc* genomic copy numbers. Importantly, both splenic and liver PFU titers were uniformly high in rats infected with WT or REV virus, whereas titers from ΔRCTL-infected animals were below the limit of detection (Figure 4B). The inability to detect PFU from rats infected with ΔRCTL virus was not due to lack of infection, because RCMV genomic DNA could be detected by qPCR analysis of the same tissues, albeit at reduced amounts compared to tissues from animals infected with WT or REV virus (Figure 4C). These data demonstrate that the *rctl* gene is sufficient to confer early virulence of RCMV in vivo and that the absence of the gene product results in more efficient clearance of RCMV, likely by innate immunity.

To demonstrate that this effect is NK dependent, a cohort of rats was pretreated with the NK-depleting antibodies, anti-asialo-GM-1 (Keller et al., 1983) and 3.2.3 mAb (Chambers et al., 1989). Notably, ΔRCTL virulence was significantly enhanced in NK-depleted animals, such that the differential viral burden between ΔRCTL-infected animals versus WT- or REV-infected animals was abrogated by in vivo NK depletion (Figures 4B and 4C). Collectively, these results demonstrate that the *rctl* gene represents a nonredundant virulence factor involved in RCMV evasion of NK recognition in vivo.

Allelic Divergence of Host NKR-P1B Receptor Sequences Alters RCTL Recognition

Coding sequence alignment of RCTL with known Clr (CLEC2 family) and NKR-P1 (KLRB1 family) sequences revealed a number of interesting observations (Figures 5A and 5B; Table S3; Hao et al., 2006). First, the RCTL sequence most closely resembles rClr-b (rCLEC2D11) among all known CLEC2 family members (Figure 5A; Hao et al., 2006). Second, NKR-P1B sequences display greater allelic polymorphism than other rodent NKR-P1 isoforms (Figure 5B; Hao et al., 2006). Notably, rNKR-P1B alleles differ substantially among the SD, BN, F344, BS, PVG, WAG, and TO strains (Figure 5B). Indeed, this divergence has led to the alternate designation of the NKR-P1B$^{BS/WAG}$ allele as a novel inhibitory NKR-P1C^{PVG} isoform (Figure 5B; these sequences are identical; Kveberg et al., 2006). Such allelic diversity also exists among the mNKR-P1B and mNKR-P1C gene products from the B6, SJL, BALB/c, and 129 strains (Carlyle et al., 2006; Mesci et al., 2006). Historically, this divergence is responsible for the alloreactivity that led to the identification of the mouse NK1.1 antigen (Glimcher et al., 1977) and for the alternate designation of the NKR-P1B^{B6} allele as a novel inhibitory NKR-P1D^{B6} isoform (Figure 5B; these sequences are identical; Carlyle et al., 1999, 2006; Kung et al., 1999; Plougastel et al., 2001b). However, only one functional inhibitory class *Klrb1* locus has been documented in the genomes of all species to date, supporting the notion that the

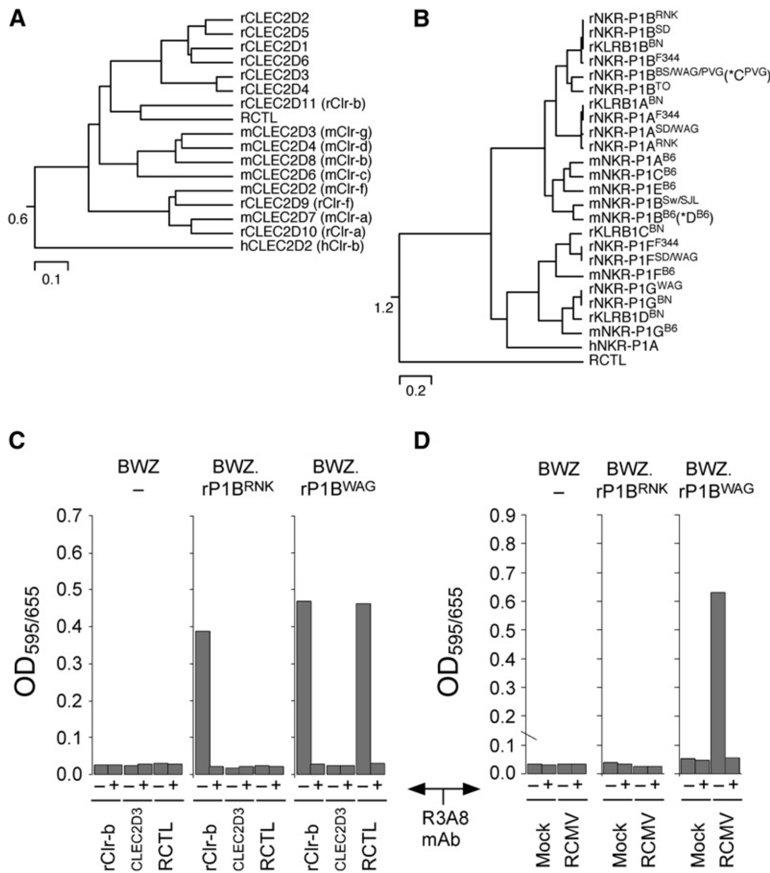

Figure 5. Allele-Specific Interaction of NKR-P1B with RCTL

(A) Phylogram of coding sequences of RCTL versus Clr family members from various species (see Table S3 for GenBank sequences). Scale bar indicates amino acid divergence, determined with DNAStar LaserGene 7.0 software.

(B) Phylogram of coding sequences of RCTL versus NKR-P1 family members from various species and strains (see Table S3 for GenBank sequences). An asterisk (*) indicates an alternate designation given to an allele derived from the *Klrb1b* locus.

(C) BWZ reporter analysis of allele-specific NKR-P1B interaction with 293T transfectants expressing rClr-b, rCLEC2D3, or RCTL. Quantitation of β-galactosidase activity after responses of BWZ− cells or BWZ cells expressing CD3ζ–NKR-P1B^{RNK} or CD3ζ–NKR-P1B^{WAG} fusion-receptors are shown, either with (+) or without (−) R3A8 blocking mAb (25 μg/mL). Representative of two experiments.

(D) As in (C), except shown are the responses of BWZ cells to primary REF cells, either uninfected (mock) or infected with WT RCMV (24 hr prior to analysis; moi = 5). Representative of two experiments.

Klrb1 family exhibits intraspecies allelic polymorphism rather than discrepant gene content (Carlyle et al., 2006; Hao et al., 2006). We have previously speculated that such directed divergence of host receptor genes may have occurred under selection pressure from viral evasion of NKR-P1B-mediated recognition (Mesci et al., 2006). Strain-dependent variation in host gene content also exists in the *Klra* system, where the MCMV m157 gene product directs host resistance or susceptibility via interactions with the stimulatory Ly49H^{B6} or inhibitory Ly49I^{129} receptors, respectively (Arase et al., 2002; Bubic et al., 2004; Smith et al., 2002). Therefore, we tested other rNKR-P1B alleles for differential interaction with the RCTL decoy ligand.

Previously, we analyzed the rNKR-P1B^{WAG} allele, which is identical to the published rNKR-P1B$^{BS/PVG}$ sequences (Figure 5B). Interestingly, the rNKR-P1B$^{BS/PVG/WAG}$ and rNKR-P1B^{TO} alleles have diverged substantially, whereas the rNKR-P1B^{RNK,SD,BN,F344} sequences are closely related to one another (Figure 5B). Therefore, with the BWZ assay, we analyzed the interaction specificities of two rNKR-P1B alleles, rNKR-P1B^{WAG} and rNKR-P1B^{RNK} (the latter obtained from RNK-16 cells, reportedly of F344 origin [Reynolds et al., 1984]). BWZ− cells or BWZ cells expressing CD3ζ–NKR-P1B fusion receptors were stimulated with 293T transfectants bearing various ligands. As expected, rNKR-P1B^{RNK} functionally recognized rClr-b but not a related family member, rCLEC2D3 (Figure 5C; Carlyle et al., 2004; Hao et al., 2006). This interaction is specific, because it could be blocked with R3A8 mAb (Figure 5C). However, rNKR-P1B^{RNK} failed to recognize RCTL (Figure 5C). In contrast, rNKR-P1B^{WAG} functionally recognized both rClr-b and RCTL (but not control rCLEC2D3; Figure 5C). Importantly, BWZ.P1B^{WAG} cells also recognized native RCTL on RCMV-infected REF cells, but BWZ.P1B^{RNK} cells failed to respond (Figure 5D). These data suggest that differential strain-dependent RCMV susceptibility may exist in rats, similar to the strain-dependent MCMV susceptibility observed in mice (Arase et al., 2002; Bubic et al., 2004; Smith et al., 2002).

Weak Recognition of RCTL by NKR-P1A and NKR-P1B Alleles Attenuates RCMV Virulence

We further analyzed the BWZ.RCTL reporter response to 293T transfectants bearing stimulatory and inhibitory NKR-P1 isoforms from other rat strains. The use of BWZ.RCTL cells also facilitated semiquantitative comparisons of RCTL interactions with different receptors (Mesci and Carlyle, 2007). Notably, the rNKR-P1B^{WAG} allele consistently yielded a strong BWZ.RCTL reporter signal, while only a weak response was detected for the rNKR-P1B^{SD} allele (Figure 6A). Moreover, weak interactions were detected for both the stimulatory rNKR-P1A^{WAG} and rNKR-P1A^{SD} alleles (Figure 6A). Collectively, these data

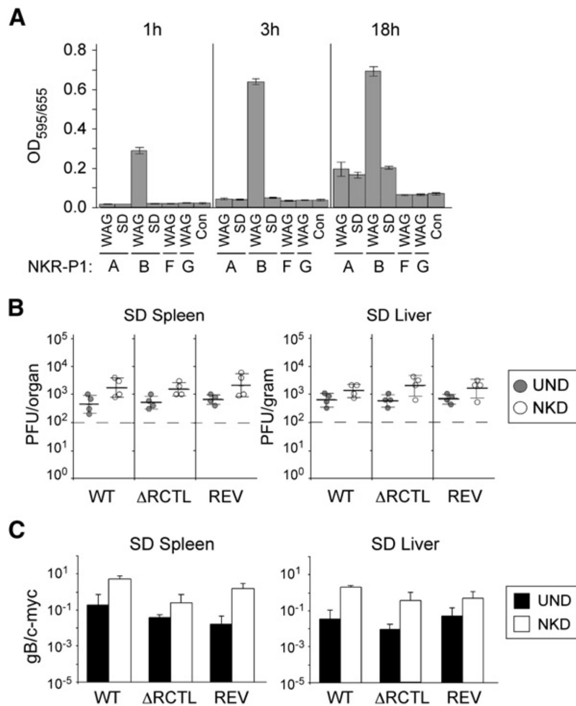

Figure 6. Weak Interaction of NKR-P1A and NKR-P1B Alleles with RCTL Modulates RCMV Virulence in a Strain-Dependent Manner

(A) Kinetic analysis of BWZ.RCTL responses to 293T transfectants expressing allelic NKR-P1 isoforms. Strong versus weak interactions were determined by extended time-course analysis (Mesci and Carlyle, 2007). Various rat NKR-P1 isoforms (A, B, F, G) are shown, with allelic designations according to rat strain (WAG, SD); also shown are untransfected 293T cells (Con). Time course of CPRG development: left, 1 hr; middle, 3 hr; right, 18 hr. Values indicate mean (±SD). Representative of three experiments.

(B) Viral titers (PFU) derived from tissues of SD rats infected with WT, ΔRCTL, and REV virus at day 3 after infection. The dashed line indicates the limit of detection; both NK-depleted (NKD) and undepleted (UND) animals are shown. ΔRCTL-UND PFU titers are not significantly different from all other cohorts ($p > 0.05$).

(C) Viral loads of SD tissues shown in (B) were assessed in parallel by qPCR analysis of normalized RCMV genome copy numbers (see Experimental Procedures and Figure 4C for details). ΔRCTL-UND qPCR values are significantly different from WT-NKD and REV-NKD cohorts ($p < 0.05$), but not ΔRCTL-NKD ($p = 0.057$).

suggest that RCMV-infected cells should be directly (albeit weakly) recognized by both stimulatory and inhibitory NKR-P1 receptors in SD rats. These opposing signals (on either distinct or overlapping NK subsets) would be expected to abrogate the RCTL decoy function, by concurrently weakening inhibition and inducing partial stimulation (Mesci et al., 2006).

Therefore, we infected SD rats with the WT, ΔRCTL, and REV viruses and assayed viral burdens in infected tissues. As shown in Figures 6B and 6C, no differences could be detected between the three cohorts in vivo, even though viral loads were augmented by NK depletion. Interestingly, the PFU titers and RCMV genomic copy numbers were also consistently lower in SD versus WAG rats in the absence of NK depletion (note: experiments in Figures 4B, 4C, 6B, and 6C were performed in parallel). These findings demonstrate that strain-dependent allelic divergence of NKR-P1 receptor sequences (Carlyle et al., 2004, 2006; Mesci et al., 2006) can modulate the host-pathogen interaction: both innate host resistance to RCMV and inherent RCMV virulence factors contribute to the outcome of RCMV infection in vivo.

DISCUSSION

Several strategies for evasion of NK-mediated immunity have been documented by the MCMV and HCMV models (Lodoen and Lanier, 2005; Reddehase, 2002; Vidal and Lanier, 2006). Here we provide evidence of a unique and MHC-independent strategy used by RCMV to subvert missing-self recognition mediated by the NKR-P1B:Clr-b receptor-ligand system (Kumar and McNerney, 2005; Mesci et al., 2006). The original discovery in the RCMV-English genome of a C-type lectin-like sequence with significant homology to several families of NK receptors, such as CD69, Ly49, and NKR-P1, implicated it in regulation of innate immunity (Voigt et al., 2001). Ironically, we show here that RCTL actually represents a viral ortholog of the host ligand for one of these receptors (Carlyle et al., 1999, 2004). We have previously shown that the mouse NKR-P1B:Clr-b system is involved in missing-self recognition of tumor cells by NK cells (Carlyle et al., 2004). Our new findings show that this mechanism also functions in NK immunity to infectious disease.

In keeping with a proposed role in "missing-self" recognition, rClr-b is rapidly lost on normal cells after RCMV infection, and this sensitizes infected cells to NK killing. Conversely, RCTL is rapidly expressed on the surface of RCMV-infected cells in a manner expected of a decoy ligand. Indeed, RCTL interacts directly with rNKR-P1B on NK cells and functions as a bona fide decoy ligand to inhibit NK function in vitro. Moreover, ΔRCTL virus is rendered less virulent in vivo at an early time point after infection, demonstrating that RCTL is vitally involved in RCMV evasion of innate immunity. Notably, ΔRCTL attenuation in vivo can be reversed to wild-type levels by depletion of NK cells, demonstrating that the immune evasion is NK dependent. Remarkably, certain rat strains have evolved rNKR-P1B allelic polymorphisms (Kveberg et al., 2006; Li et al., 2003) that disrupt the RCTL decoy interaction yet maintain host rClr-b recognition. Others have evolved stimulatory rNKR-P1A isoforms or paralogs (Chambers et al., 1989; Giorda et al., 1990) that directly recognize RCTL, albeit weakly. Thus, the host genome appears to be rapidly evolving mechanisms to counterbalance the role of RCTL in evasion of NK recognition. Together, these findings give us novel insights into NKR-P1 biology and underscore the importance of NKR-P1B:Clr-b interactions in NK-mediated immunity to infection (Carlyle et al., 2006; Mesci et al., 2006).

The striking homology of RCTL to the rodent Clr-b sequences, including structural amino acid residues, nucleotide similarity, and conservation of intron-exon structure,

highlights the likelihood that RCMV-English acquired the gene from the host during an infection cycle. CMV genomes are comparatively large among viruses, making it possible for them to acquire large contiguous sequences. However, *rctl* is one of few spliced genes found in CMV genomes (Voigt et al., 2001), and the contraction of its intronic elements relative to the host gene emphasizes that selective pressure does exist to reduce genome size. Additionally, divergence between the host and viral cytoplasmic domain sequences is substantial. This suggests that important features of the host Clr-b protein (including putative CK2, TRAF2, ubiquitinylation, and endocytosis motifs) may have been selected against for reasons that reflect the functional dichotomy between the viral decoy and physiological host proteins (Mesci et al., 2006). The dispensable nature of the *rctl* gene in vitro (Voigt et al., 2001), combined with its large size, suggests that the absence of similar lectin-like genes in MCMV and HCMV isolates may reflect a loss of these elements during extended adaptation to tissue culture (Wills et al., 2005), or alternatively during the evolution of host-pathogen interactions. The discovery of a second lectin-like sequence (r153) conserved between RCMV-English and RCMV-Maastricht (Brocchieri et al., 2005), as well as other lectin-like genes encoded by several poxviruses (cited in Voigt et al., 2001), suggests that the impact of these genes on innate immunity is just beginning to be understood. It is possible that functional redundancy may exist or that these new gene products may facilitate intracellular retention of ''induced-self'' Clr ligands (Lodoen and Lanier, 2005, 2006; Raulet and Vance, 2006). On the other hand, these gene products may disrupt NK:DC interactions during innate priming of adaptive immunity as a distinct viral evasion strategy (Iizuka et al., 2003). This latter mechanism may be especially important in NK cell interactions with RCMV-infected dendritic cells.

The missing-self loss of MHC I on virus-infected and tumor cells is thought to occur under selection pressure to evade T cell immunity. This inadvertently makes the target cells sensitive to NK killing, so other mechanisms must be invoked to counter NK recognition. Viral mechanisms for targeting MHC I downregulation are well known (Lodoen and Lanier, 2005; Reddehase, 2002; Vidal and Lanier, 2006), but strategies for exploiting MHC-independent missing-self recognition are undocumented (Kumar and McNerney, 2005). Moreover, no mechanistic basis or cause-and-effect relationship has yet been established regarding missing-self Clr-b downregulation on tumor cells (Carlyle et al., 2004; Mesci et al., 2006). Neither T nor NK cells possess any known stimulatory receptors for Clr-b (Carlyle et al., 2004; Iizuka et al., 2003), arguing against extrinsic selective pressure that forces loss of the ligand. While this collectively suggests a novel mechanism behind the missing-self regulation of Clr-b, the rationale for cells undergoing pathological transformation to selectively lose ligand, by proxy rendering them sensitive to NK killing, remains elusive.

Our new findings shed light on this matter, in that infection alone, perhaps as a cell-autonomous stimulus, appears to be sufficient to trigger a loss of Clr-b from the surface of RCMV-infected cells. Moreover, the rapid rate of downregulation indicates that rClr-b may normally undergo considerable turnover, or that post-translational mechanisms may actively internalize ligand (Mesci et al., 2006). Importantly, RCTL itself is not responsible for targeted rClr-b downregulation; ΔRCTL virus induces the same response. In fact, the loss of rClr-b at the RNA level indicates that RCMV likely evolved the RCTL decoy to prevent a functional loss of host ligand on infected cells. The absence of rClr-b transcripts also renders unnecessary any hypothetical viral gene products that might function to internalize the host ligand. Rather, our findings suggest that a programmed biological ''missing-self response'' may exist, such that innate cellular ''danger'' signals (Matzinger, 2002) may trigger an automatic loss of Clr-b from the cell surface. The pattern-recognition systems of innate immunity represent candidate effector mechanisms (Hargreaves and Medzhitov, 2005; Kufer et al., 2005), because infection may initiate signals that intersect with the control pathways regulating Clr-b expression. Of note, attempts to induce downregulation of Clr-b on normal cells via type I interferons or select TLR agonists have been unsuccessful to date (data not shown). Thus, elucidation of the causal mechanisms behind the missing-self response remains an important focus of future work.

Intriguingly, the rodent *Klrb1* sequences exhibit remarkable polymorphism. We have previously suggested that such allelic diversity could have evolved under selection pressure from viral evasion of NKR-P1-mediated recognition (Mesci et al., 2006). Notably, receptor sequence divergence appears to be limited to the *Klrb1c*–*Klrb1b* genes in mice and the putatively orthologous *Klrb1a*–*Klrb1b* genes in rats (Carlyle et al., 2006; Hao et al., 2006). This directed *Klrb1* polymorphism is in stark contrast to the genetic conservation of *Clec2* among strains (Carlyle et al., 2006). Our new findings suggest that the inhibitory NKR-P1B gene products are under pressure to circumvent binding to viral decoys while maintaining recognition of the conserved host ligands. Conversely, the stimulatory NKR-P1A and NKR-P1C gene products are diverging in parallel yet exhibit the reciprocal specificity. These findings uncouple the genetic protection of the *Klrb1* receptor and *Clec2* ligand genes, arguing against sequence conservation resulting from a mutation-suppression effect from genetic linkage (Iizuka et al., 2003).

The findings reported here have several implications. The rapid loss of rClr-b in response to RCMV infection suggests that the NKR-P1B:Clr-b missing-self recognition system may function as a general self-nonself discrimination rheostat to detect viral and bacterial infection, in addition to its proposed role in tumor cell immunosurveillance (Carlyle et al., 2004; Mesci et al., 2006). In addition, the fact that RCMV-English has evolved a decoy ligand to subvert NKR-P1B-mediated recognition suggests that the NKR-P1:Clr system may be a general target of evasion strategies in the evolution of infectious disease. The finding that NKR-P1-mediated recognition is conserved in humans (Aldemir et al., 2005; Rosen et al., 2005), together

with the existence of lectin-like genes in poxviruses (cited in Voigt et al., 2001), suggests that this interaction may be clinically relevant, because such decoy proteins may represent attractive targets for therapeutic antiviral drugs.

These results profoundly influence the scope of the original missing-self hypothesis, proposed some 20 years ago (Karre et al., 1986). This is a direct demonstration of MHC-independent missing-self recognition of infected cells by NK cells, as well as a discovery of a non-MHC decoy ligand involved in viral evasion of NK recognition. These findings broaden the importance of the NKR-P1:Clr receptor-ligand system in self-nonself discrimination and innate immunity to infectious disease. Future elucidation of the proposed "missing-self response" pathway may have implications for the regulation of both MHC (Hassink et al., 2005) and non-MHC (Carlyle et al., 2004) ligands during infection and transformation.

EXPERIMENTAL PROCEDURES

Animals and Viruses

Tissues derived from WAG-*nu/nu* rats were generously provided by P. Poussier (University of Toronto, Canada). For in vivo experiments, 4- to 6-week-old female WAG or SD rats were infected intraperitoneally with 3×10^6 PFU of WT, ΔRCTL, or REV virus (RCMV-English isolate). Rats were sacrificed on day 3 after infection, and blood, spleens, and livers were harvested. NK-depleted animals received one intravenous injection of rabbit polyclonal anti-asialo-GM-1 (150 μg) and 3.2.3 mAb (150 μg) 1 day prior to infection.

The ΔRCTL virus was described previously (Voigt et al., 2001). The REV virus was generated via a pSK Bluescript transfer vector containing the 2.4 kb *Kpn*R genomic fragment of RCMV-E that contains the *rctl* gene. Transfer vector and ΔRCTL virion DNA were cotransfected into REF cells, and recombinant virus was isolated as described previously (Voigt et al., 2001). Rescue of the *rctl* gene was confirmed by Southern blot and sequencing (data not shown). Single and multistep growth curves were performed to confirm the replication capacity of the revertant, which was identical to the WT virus (data not shown).

Cell Culture and BWZ Reporter Assays

BWZ.36 (Sanderson and Shastri, 1994), 293T, and REF cells were cultured as described (Carlyle et al., 2006; Voigt et al., 2001). Rat hematopoietic cells were processed with Lympholyte-Mammal (CedarLane Laboratories, Mississauga, Canada), washed, and used directly, or LAK cultured for cytotoxicity assays (see below). Eukaryotic and retroviral vectors, including the CD3ζ–NKR-P1B–RCTL fusion construct, were generated and transfected as described elsewhere (Carlyle et al., 2006; Mesci and Carlyle, 2007). BWZ assays were performed as described (Mesci and Carlyle, 2007; Sanderson and Shastri, 1994) and quantitated via optical density readings at 595 nm versus 655 nm ($OD_{595/655}$) with CPRG substrate. REF cells were infected (WT, moi = 5) 18 hr prior to BWZ analysis.

RT-PCR and Real-Time RT-PCR

RNA extraction and cDNA preparation were performed as described previously (Carlyle et al., 2006). Primer sequences are shown in Table S2. All constructs were sequenced (Macrogen Inc., Seoul, South Korea) to confirm cDNA identities. For real-time RT-PCR, RNA was harvested from RCMV-infected REF cells (moi = 5) with the RNeasy Mini Kit (Qiagen, Hilden, Germany), including on-column DNase treatment. cDNA was made with RevertAid RT (Fermentas, St. Leon-Rot, Germany), and samples were run on an ABI Prism 7900 HT Fast Real-Time PCR System. RT-PCR experiments were controlled with equal cDNA input.

Flow Cytometry

The generation and characterization of R3A8 mAb has been described in detail elsewhere (Mesci and Carlyle, 2007). In brief, mice were immunized with BWZ.RCTL cells and immune splenocytes were used to generate anti-RCTL hybridomas. Hybridomas were screened via BWZ.RCTL stimulation on anti-(mouse IgG/M)-captured, immobilized 96-well arrays of hybridoma supernatants. One hybridoma clone (R3A8) was selected for large-scale production and purification of mAb, as described previously (Carlyle et al., 2004; Mesci and Carlyle, 2007). All other mAbs were purchased from BD Biosciences (Mississauga, ON, Canada), e-Bioscience (San Diego, CA), or CedarLane or were produced and purified in our own facilities. Streptavidin-PE was purchased from Invitrogen (Burlington, ON, Canada). Flow cytometry was performed as previously described (Carlyle et al., 2006). NK depletion in vivo was confirmed by staining of splenocytes and blood cells with 10/78 mAb, anti-NKR-P1A/B (CedarLane).

^{51}Cr-Release Assays

Cytotoxicity assays were performed as previously described (Carlyle et al., 2004). LAK cells were prepared by culturing splenocytes or bone-marrow cells for 5 days in medium containing 2000 U/ml rhIL-2 plus 50 ng/mL mIL-15. REF target cells were infected with RCMV WT or ΔRCTL virus overnight (moi = 5) or used directly, with or without R3A8 blocking (25 μg/mL).

Quantification of Virus by Quantitative Real-Time PCR

DNA was isolated from tissues with the DNeasy Tissue Kit (Qiagen). For quantitative real-time PCR (qPCR), parallel amplifications of the RCMV-English glycoprotein B (*gB*) gene and host c-*myc* gene were performed with standard conditions and specific primers and probes (Table S2). Reactions were denatured (95°C, 10 min), then monitored for 45 cycles (95°C, 15 s; 60°C, 30 s) in parallel with control amplifications: negative controls, water; positive controls, plasmid dilutions used to establish standard curves. Duplicated samples were averaged, and C_T-values were further analyzed with ABI Prism SDS-Software 2.2.2. Absolute quantification of *gB* and c-*myc* copy numbers was obtained by standard curve analysis. Values were then normalized by determination of the viral *gB* to host c-*myc* genomic copy ratio.

Quantification of Virus by Plaque-Forming Unit Analysis

Viral titers in spleens and livers were determined by a standard plaque-forming unit (PFU) assays. Whole spleens and weighed liver samples were homogenized, serially diluted, and plated on REF cells in 24-well plates. Titers were read 6 days later after methylene blue staining.

Statistical Analysis

Statistical significance of differences between experimental groups for RCMV infectious titers (PFU) and genomic copy numbers (qPCR) were assessed by the Mann-Whitney exact rank sum test. Normalized PFU titers and *gB*/c-*myc* ratios were considered significantly different for $p < 0.05$.

Supplemental Data

Two figures and three tables are available at http://www.immunity.com/cgi/content/full/26/5/617/DC1/.

ACKNOWLEDGMENTS

We thank M. Whang and A. Jamieson for database searches, J.C. Zúñiga-Pflücker and D.H. Raulet for support, P. Poussier for providing animal tissues and mAbs, A. Martin and T.H. Watts for critical reading of the manuscript, W. Brune for helpful discussion, A. Nitsche for Taqman probe design, and C. Hasselberg-Christoph for technical assistance. This work was supported by an Operating Grant from the Canadian Institutes of Health Research (CIHR MOP 74754 to J.R.C.) and by funds from the Robert Koch Institute (to S.V.). A.M. was supported by a University of Toronto Life Sciences Award. J.H.F. was supported by an Aventis-Pasteur OGSST Award and a CGS-M Award from the Natural

Sciences and Engineering Research Council of Canada (NSERC). J.R.C. is supported by a Career Development Award from the International Human Frontier Science Program Organization (CDA0037-2005).

Received: November 10, 2006
Revised: February 26, 2007
Accepted: March 23, 2007
Published online: April 26, 2007

REFERENCES

Aldemir, H., Prod'homme, V., Dumaurier, M.J., Retiere, C., Poupon, G., Cazareth, J., Bihl, F., and Braud, V.M. (2005). Cutting edge: lectin-like transcript 1 is a ligand for the CD161 receptor. J. Immunol. *175*, 7791–7795.

Arase, H., Mocarski, E.S., Campbell, A.E., Hill, A.B., and Lanier, L.L. (2002). Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science *296*, 1323–1326.

Bezouska, K., Yuen, C.T., O'Brien, J., Childs, R.A., Chai, W., Lawson, A.M., Drbal, K., Fiserova, A., Pospisil, M., and Feizi, T. (1994). Oligosaccharide ligands for NKR-P1 protein activate NK cells and cytotoxicity. Nature *372*, 150–157.

Brocchieri, L., Kledal, T.N., Karlin, S., and Mocarski, E.S. (2005). Predicting coding potential from genome sequence: application to betaherpesviruses infecting rats and mice. J. Virol. *79*, 7570–7596.

Bubic, I., Wagner, M., Krmpotic, A., Saulig, T., Kim, S., Yokoyama, W.M., Jonjic, S., and Koszinowski, U.H. (2004). Gain of virulence caused by loss of a gene in murine cytomegalovirus. J. Virol. *78*, 7536–7544.

Carlyle, J.R., Martin, A., Mehra, A., Attisano, L., Tsui, F.W., and Zuniga-Pflucker, J.C. (1999). Mouse NKR-P1B, a novel NK1.1 antigen with inhibitory function. J. Immunol. *162*, 5917–5923.

Carlyle, J.R., Jamieson, A.M., Gasser, S., Clingan, C.S., Arase, H., and Raulet, D.H. (2004). Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. Proc. Natl. Acad. Sci. USA *101*, 3527–3532.

Carlyle, J.R., Mesci, A., Ljutic, B., Belanger, S., Tai, L.H., Rousselle, E., Troke, A.D., Proteau, M.F., and Makrigiannis, A.P. (2006). Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. J. Immunol. *176*, 7511–7524.

Chambers, W.H., Vujanovic, N.L., DeLeo, A.B., Olszowy, M.W., Herberman, R.B., and Hiserodt, J.C. (1989). Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine-activated killer cells. J. Exp. Med. *169*, 1373–1389.

Giorda, R., Rudert, W.A., Vavassori, C., Chambers, W.H., Hiserodt, J.C., and Trucco, M. (1990). NKR-P1, a signal transduction molecule on natural killer cells. Science *249*, 1298–1300.

Glimcher, L., Shen, F.W., and Cantor, H. (1977). Identification of a cell-surface antigen selectively expressed on the natural killer cell. J. Exp. Med. *145*, 1–9.

Hao, L., Klein, J., and Nei, M. (2006). Heterogeneous but conserved natural killer receptor gene complexes in four major orders of mammals. Proc. Natl. Acad. Sci. USA *103*, 3192–3197.

Hargreaves, D.C., and Medzhitov, R. (2005). Innate sensors of microbial infection. J. Clin. Immunol. *25*, 503–510.

Hassink, G.C., Duijvestijn-van Dam, J.G., Koppers-Lalic, D., van Gaans-van den Brink, J., van Leeuwen, D., Vink, C., Bruggeman, C.A., and Wiertz, E.J. (2005). Rat cytomegalovirus induces a temporal downregulation of major histocompatibility complex class I cell surface expression. Viral Immunol. *18*, 607–615.

Iizuka, K., Naidenko, O.V., Plougastel, B.F., Fremont, D.H., and Yokoyama, W.M. (2003). Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. Nat. Immunol. *4*, 801–807.

Karre, K., Ljunggren, H.G., Piontek, G., and Kiessling, R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature *319*, 675–678.

Keller, R., Bachi, T., and Okumura, K. (1983). Discrimination between macrophage-and NK-type tumoricidal activities via anti-asialo GM1 antibody. Exp. Cell Biol. *51*, 158–164.

Kufer, T.A., Fritz, J.H., and Philpott, D.J. (2005). NACHT-LRR proteins (NLRs) in bacterial infection and immunity. Trends Microbiol. *13*, 381–388.

Kumar, V., and McNerney, M.E. (2005). A new self: MHC-class-I-independent natural-killer-cell self-tolerance. Nat. Rev. Immunol. *5*, 363–374.

Kung, S.K., Su, R.C., Shannon, J., and Miller, R.G. (1999). The NKR-P1B gene product is an inhibitory receptor on SJL/J NK cells. J. Immunol. *162*, 5876–5887.

Kveberg, L., Back, C.J., Dai, K.Z., Inngjerdingen, M., Rolstad, B., Ryan, J.C., Vaage, J.T., and Naper, C. (2006). The novel inhibitory NKR-P1C receptor and Ly49s3 identify two complementary, functionally distinct NK cell subsets in rats. J. Immunol. *176*, 4133–4140.

Lanier, L.L. (2005). NK cell recognition. Annu. Rev. Immunol. *23*, 225–274.

Lenac, T., Budt, M., Arapovic, J., Hasan, M., Zimmermann, A., Simic, H., Krmpotic, A., Messerle, M., Ruzsics, Z., Koszinowski, U.H., et al. (2006). The herpesviral Fc receptor fcr-1 down-regulates the NKG2D ligands MULT-1 and H60. J. Exp. Med. *203*, 1843–1850.

Li, J., Rabinovich, B.A., Hurren, R., Shannon, J., and Miller, R.G. (2003). Expression cloning and function of the rat NK activating and inhibitory receptors NKR-P1A and -P1B. Int. Immunol. *15*, 411–416.

Liu, J., Morris, M.A., Nguyen, P., George, T.C., Koulich, E., Lai, W.C., Schatzle, J.D., Kumar, V., and Bennett, M. (2000). Ly49I NK cell receptor transgene inhibition of rejection of H2b mouse bone marrow transplants. J. Immunol. *164*, 1793–1799.

Lodoen, M.B., and Lanier, L.L. (2005). Viral modulation of NK cell immunity. Nat. Rev. Microbiol. *3*, 59–69.

Lodoen, M.B., and Lanier, L.L. (2006). Natural killer cells as an initial defense against pathogens. Curr. Opin. Immunol. *18*, 391–398.

Matzinger, P. (2002). The danger model: a renewed sense of self. Science *296*, 301–305.

Mesci, A., and Carlyle, J.R. (2007). A rapid and efficient method for the generation and screening of monoclonal antibodies specific for cell surface antigens. J. Immunol. Methods, in press. Published online March 15, 2007. 10.1016/j.jim.2007.02.007.

Mesci, A., Ljutic, B., Makrigiannis, A.P., and Carlyle, J.R. (2006). NKR-P1 biology: from prototype to missing self. Immunol. Res. *35*, 13–26.

Plougastel, B., Dubbelde, C., and Yokoyama, W.M. (2001a). Cloning of Clr, a new family of lectin-like genes localized between mouse Nkrp1a and Cd69. Immunogenetics *53*, 209–214.

Plougastel, B., Matsumoto, K., Dubbelde, C., and Yokoyama, W.M. (2001b). Analysis of a 1-Mb BAC contig overlapping the mouse Nkrp1 cluster of genes: cloning of three new Nkrp1 members, Nkrp1d, Nkrp1e, and Nkrp1f. Immunogenetics *53*, 592–598.

Raulet, D.H., and Vance, R.E. (2006). Self-tolerance of natural killer cells. Nat. Rev. Immunol. *6*, 520–531.

Reddehase, M.J. (2002). Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. Nat. Rev. Immunol. *2*, 831–844.

Reynolds, C.W., Bere, E.W., Jr., and Ward, J.M. (1984). Natural killer activity in the rat. III. Characterization of transplantable large granular lymphocyte (LGL) leukemias in the F344 rat. J. Immunol. *132*, 534–540.

Rosen, D.B., Bettadapura, J., Alsharifi, M., Mathew, P.A., Warren, H.S., and Lanier, L.L. (2005). Cutting edge: lectin-like transcript-1 is a ligand for the inhibitory human NKR-P1A receptor. J. Immunol. *175*, 7796–7799.

Sanderson, S., and Shastri, N. (1994). LacZ inducible, antigen/MHC-specific T cell hybrids. Int. Immunol. *6*, 369–376.

Smith, H.R., Heusel, J.W., Mehta, I.K., Kim, S., Dorner, B.G., Naidenko, O.V., Iizuka, K., Furukawa, H., Beckman, D.L., Pingel, J.T., et al. (2002). Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc. Natl. Acad. Sci. USA *99*, 8826–8831.

Vidal, S.M., and Lanier, L.L. (2006). NK cell recognition of mouse cytomegalovirus-infected cells. Curr. Top. Microbiol. Immunol. *298*, 183–206.

Voigt, S., Sandford, G.R., Ding, L., and Burns, W.H. (2001). Identification and characterization of a spliced C-type lectin-like gene encoded by rat cytomegalovirus. J. Virol. *75*, 603–611.

Wills, M.R., Ashiru, O., Reeves, M.B., Okecha, G., Trowsdale, J., Tomasec, P., Wilkinson, G.W., Sinclair, J., and Sissons, J.G. (2005). Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis. J. Immunol. *175*, 7457–7465.

Yokoyama, W.M., and Plougastel, B.F. (2003). Immune functions encoded by the natural killer gene complex. Nat. Rev. Immunol. *3*, 304–316.

Zhou, H., Kartsogiannis, V., Hu, Y.S., Elliott, J., Quinn, J.M., McKinstry, W.J., Gillespie, M.T., and Ng, K.W. (2001). A novel osteoblast-derived C-type lectin that inhibits osteoclast formation. J. Biol. Chem. *276*, 14916–14923.