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Article

Aging Promotes Neutrophil-Induced Mortality by Augmenting IL-17 Production during Viral Infection

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SUMMARY

Morbidity and mortality associated with viral infections increase with age, although the underlying mechanisms are unclear. Here, we investigated whether aging alters inflammatory responses during systemic viral infection and thereby contributes to virus-induced death. We found that infection of aged mice with systemic herpes viruses led to rapid increases in serum IL-17, neutrophil activation, and mortality due to hepatocyte necrosis. In contrast, all young mice survived infection, displaying weaker IL-17 induction and neutrophil activation. Natural killer T (NKT) cells isolated from the livers of aged mice produced more IL-17 than did young cells, and adoptively transferred aged NKT cells induced liver injury in young mice impaired in viral control. Importantly, IL-17 neutralization or neutrophil depletion during viral infection reduced liver damage and prevented death of aged mice. These results demonstrate that, during systemic viral infection, aging alters the host-pathogen interaction to overproduce IL-17, contributing to liver injury and death.

INTRODUCTION

The elderly are at increased risk for microbial infection and malignancy (Linton and Dorshkind, 2004), indicating that aging impairs immunity. These individuals are less able to overcome viral infections and subsequently exhibit high morbidity and mortality after infection (Gorina et al., 2008), imposing a heavy burden on health care resources (Han et al., 1999; Kaplan and Angus, 2003; Kaplan et al., 2002). Several studies have demonstrated that aging adversely affects various components of immunity (Linton and Dorshkind, 2004; Miller, 1996). However, most studies have focused on adaptive T cell responses (Garcia and Miller, 2001; Haynes et al., 1999; Linton et al., 1996; Linton and Dorshkind, 2004; Thoman and Weigle, 1982), leaving it unclear as to how aging modifies other host defenses against infection.

Studies in humans and mice have shown that aging is associated with aberrant cytokine production and inflammation (Brüünsgard and Pedersen, 2003; Rosenstiel et al., 2008). Aging is associated with elevated levels of several proinflammatory cytokines, including IL-1, IL-6, and TNF-α. The elevation in IL-6 has been of particular interest, as this cytokine has recently been shown to be important for the differentiation of Th17 $CD4^+$ T cells (Korn et al., 2007; Weaver et al., 2007). However, whether age-related aberrancies in inflammatory responses result from impaired host-pathogen interactions and whether these aberrant responses affect the outcome of systemic viral infections in older individuals is unclear. In this study, we compared inflammatory responses to systemic infection with either herpes simplex type 2 virus (HSV-2) or murine cytomegalovirus (MCMV) between young and aged mice. Our findings reveal that aberrant IL-17A responses to systemic viral infection contribute to the death of aged hosts via a neutrophil-dependent process. These findings suggest that therapies aimed at inactivating IL-17A may avert immune pathology in older individuals with systemic viral infections.

RESULTS

Systemic Viral Infection Induces Proinflammatory Cytokine Overproduction and Death in Aged Mice

To begin to understand how aging modifies the inflammatory response to systemic viral infection, we infected young (2–4 months), middle-aged (8–10 months), and aged (18–20 months) C57BL/6 mice with HSV-2, a human viral pathogen that activates plasmacytoid dendritic cells (pDCs) (Lund et al., 2003). We found that all young and middle-aged mice survived infection, whereas all aged mice succumbed to it (Figure 1A). Mortality was associated with a significant elevation in systemic levels of IL-17A, IL-6 (Figures 1B and 1C), and TNF-α (Figure S1). The elevation in IL-17A levels and mortality in response to HSV-2 infection was not limited to one genetic background of mice, as similar responses were found in the BALB/c strain (Figure S2). We also found that infection of aged BALB/c mice with the alternate pathogen, MCMV, induced death as well as an elevation in IL-17A, IL-6, and TNF-α levels (Figure S3). On the other hand, young BALB/c mice survived infection and exhibited lower levels of these cytokines (Figure S3). Thus, in aged mice, systemic viral infection induces an aberrantly high IL-17A response, which is associated with mortality.

Systemic HSV-2 Infection Elevates Serum IL-17A Levels in Aged Mice in a Dose-Dependent Fashion

Next, the age-dependent augmentation of IL-17A responses to systemic viral infection was further characterized using dose-response experiments. We first detected differences in serum

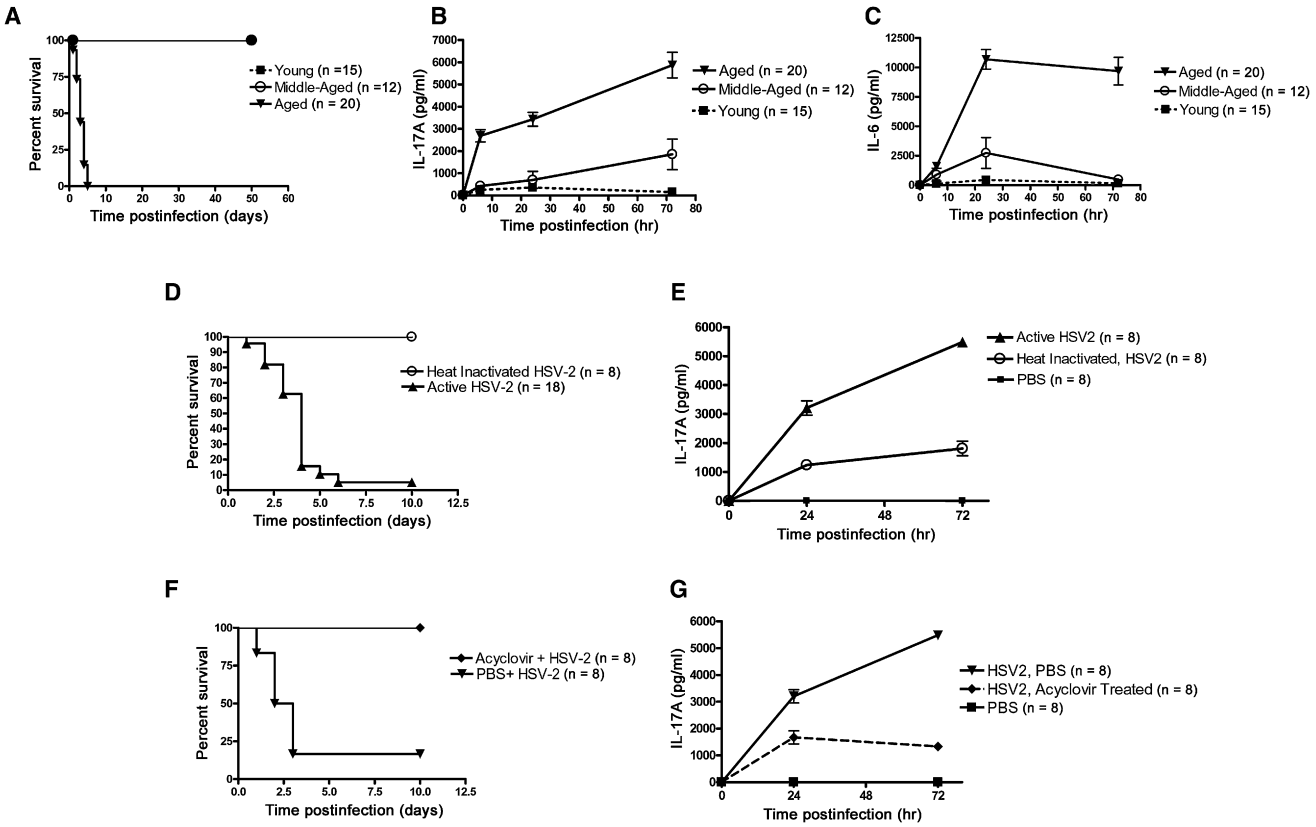

Figure 1. Aged Mice Succumb to Systemic Active HSV-2 Infection and Exhibit Elevated Serum Inflammatory Cytokine Levels

(A) Survival of aged (18–20 months), middle-aged (8–10 months), and young (2–4 months) C57BL/6 mice following HSV-2 infection. All aged mice died within 5 days of infection, whereas the other groups all survived up to 50 days after infection, at which time the experiment was terminated. $p < 0.001$, aged group versus the other groups (Log rank test).

(B and C) Serum levels of IL-17A (B) and IL-6 (C) following HSV-2 infection. Values represent the mean ±SD of three independent experiments, each performed in triplicate. $p < 0.01$, aged group versus the other groups (ANOVA).

(D and E) Survival (D) and IL-17A responses (E) in aged mice infected with active or heat-inactivated HSV-2. The difference in survival between the active and heat-inactivated groups was significant ($p < 0.001$, Log rank).

(F and G) Effect of acyclovir or PBS on survival ($p = 0.005$, Log rank) (F) and IL-17A responses (G) in aged mice infected with HSV-2. Values in (E) and (G) represent the mean ±SD of two independent experiments, each performed in triplicate.

IL-17A levels between aged mice and their younger counterparts at a 1×10^5 pfu dose of HSV-2 (Figure S4A). The difference in systemic IL-17A levels between young and aged mice grew larger with increasing doses of HSV-2 (Figure S4A). In contrast to this age-dependent elevation in the systemic IL-17A response, systemic IFNα responses to viral infection were lower in aged mice than in young mice (Figure S4B). Moreover, measurement of viral plaques revealed that these age-related changes in IL-17A and IFNα responses were accompanied by a reduced ability to clear HSV-2 (Figure S4C). Finally, mortality was noted in aged mice infected with 1×10^7 pfu of HSV-2, but not in those infected with a 1×10^5 pfu (Figure S5). A comparison of aged mice infected with these two doses revealed that the higher infecting dose induced a greater increase in systemic IL-6, IL-17A, and TNF-α levels (Figure S5).

Infection with Active Virus Promotes Higher IL-17A Levels and Mortality Than Infection with Heat-Inactivated Virus

To determine whether systemic infection with active virus promotes death of aged mice, we infected aged mice with either active HSV-2 or heat-inactivated HSV-2. We found that, in aged mice, infection with 10^7 pfu of the active form of the virus was lethal and increased systemic IL-17A levels, whereas infection with the same dose of inactive form was not lethal and increased IL-17A to a lesser extent (Figures 1D and 1E). Moreover, administration of the antiviral agent, acyclovir, to aged mice just before HSV-2 infection and 24 hr after infection reduced IL-17A production and prevented mortality (Figures 1F and 1G). Taken together, these results imply that the active form of the HSV-2 is superior to the inactive form in elevating IL-17A and inducing mortality in aged mice.

Aging Augments Neutrophil-Attracting Chemokine Production, Neutrophil Activity, and Liver Neutrophil Accumulation Following HSV-2 Infection

The finding that aging increases the IL-17A response prompted us to measure the production of macrophage inflammatory protein-1α (MIP-1α), a neutrophil- and macrophage-homing chemokine that is induced by IL-17A (Aggarwal and Gurney, 2002). We found that, following HSV-2 infection, serum levels of MIP-1α and myeloperoxidase (MPO, a marker of neutrophil

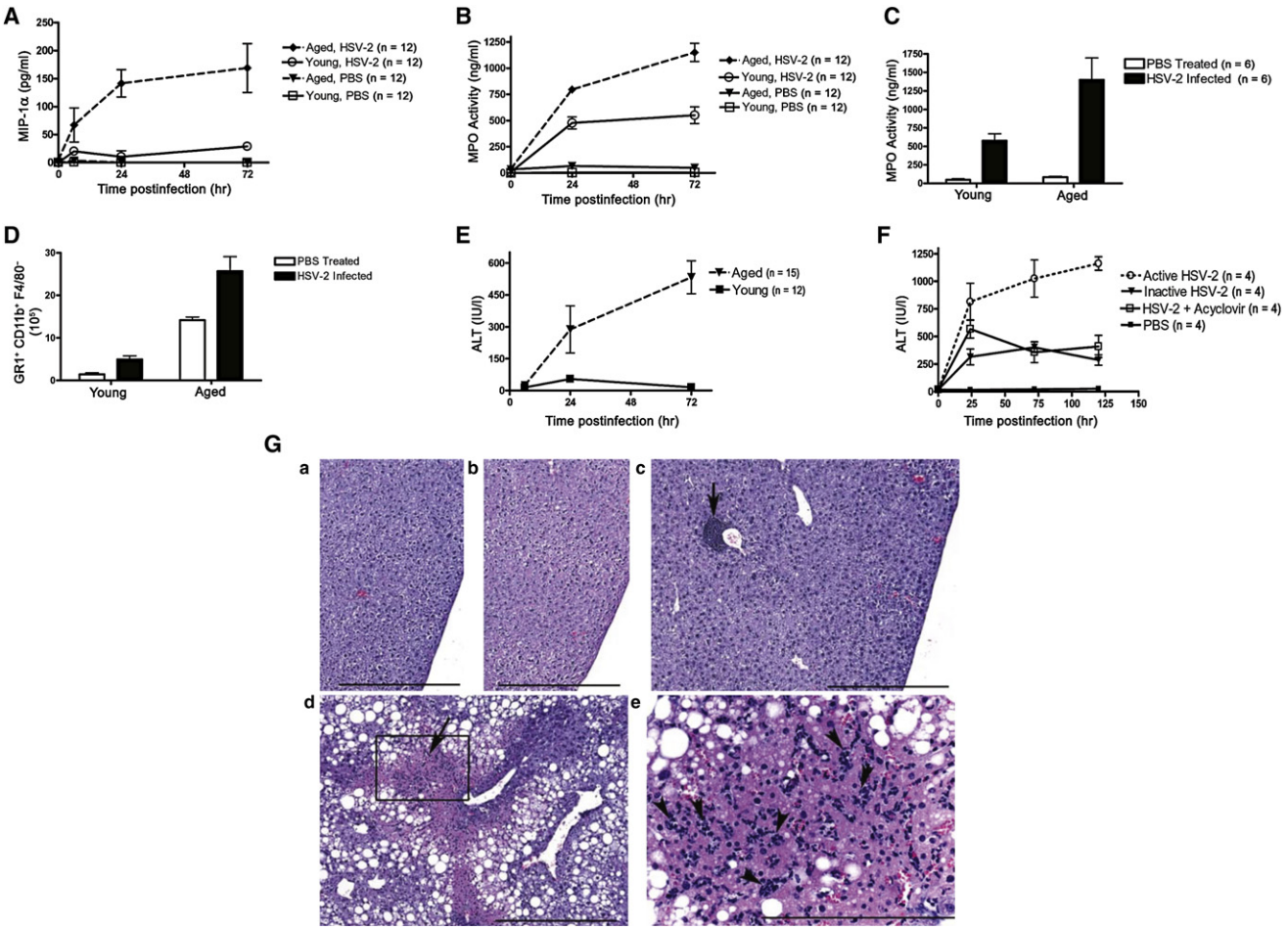

Figure 2. Aged Mice Exhibit Greater MIP-1α Production, Neutrophil Activity, Liver Neutrophil Accumulation, and Hepatocyte Necrosis Than Young Mice after HSV-2 Infection

(A) Effect of HSV-2 infection on serum MIP-1α levels in aged and young mice. $p < 0.01$, aged infected versus young infected (ANOVA).

(B) Serum neutrophil activity (MPO levels) in aged and young HSV-2-infected mice. $p = 0.007$, aged infected versus young infected (ANOVA).

(C) Liver MPO levels in aged and young mice 12 hr after HSV-2 infection. $p = 0.05$, aged infected versus young infected (t test).

(D) Accumulation of liver $GR1^+CD11b^+$ $F4/80^-$ cells in aged and young HSV-2-infected mice at 12 hr after infection. $p < 0.01$, aged infected versus young infected (t test), n = 4/group.

(E) Serum ALT levels (measure of liver necrosis) in aged and young HSV-2-infected mice. $p < 0.01$, aged infected versus young infected (ANOVA).

(F) Serum ALT levels in aged HSV-2-infected mice treated with acyclovir or in aged mice infected with heat-inactivated HSV-2. Values in (A), (B), and (E) represent the mean ±SD of three independent experiments, each performed in triplicate. Values in (C), (D), and (F) represent the mean ±SD of two independent experiments, each performed in triplicate.

(G) Histological images of livers from young and aged mice that were left uninfected (young [a] and aged [c]) or infected for 24 hr (young [b] and aged [d]). Noninfected aged livers were similar to young infected and noninfected livers, except for normal age-related changes such as perivascular collections of lymphocytes and plasma cells (c, arrow). Livers from infected aged mice exhibited marked multifocal hepatocellular necrosis (d, arrow), with multifocal-to-diffuse hepatocellular fatty changes. Numerous polymorphonuclear cells were present in necrotic areas (arrowhead, e [inset of d]). Scale bars: a–d = 500 μm; e = 200 μm. n = 3–6 mice per group.

activation) were higher in aged mice than in young mice (Figures 2A and 2B). MPO levels were also present at higher concentrations in the livers of aged infected mice than in those from young infected mice (Figure 2C). Accordingly, livers from aged infected mice contained a greater number of $GR1^+CD11b^+$ $F4/80^-$ cells than livers from young infected mice (Figure 2D).

HSV-2 Infection Induces Hepatocyte Necrosis in Aged Mice, but Not in Young Mice

Neutrophils can induce acute hepatocyte injury and necrosis (Ramaiah and Jaeschke, 2007), which are accompanied by release of alanine aminotransferase (ALT). We found that, during the first 72 hr after HSV-2 infection, aged mice had higher serum ALT levels than young mice (Figure 2E). Similar findings were noted in BALB/c mice infected with MCMV (Figure S6). Furthermore, a comparison of serum ALT levels in aged mice infected with either 10^5 or 10^7 pfu of HSV-2 demonstrated that the higher infecting dose was superior in inducing ALT release than the lower dose (Figure S7). Importantly, this age-related increase in serum ALT levels was partially prevented when mice were infected with heat-inactivated HSV-2 or given acyclovir just prior to viral infection (Figure 2F). Liver damage in aged mice was

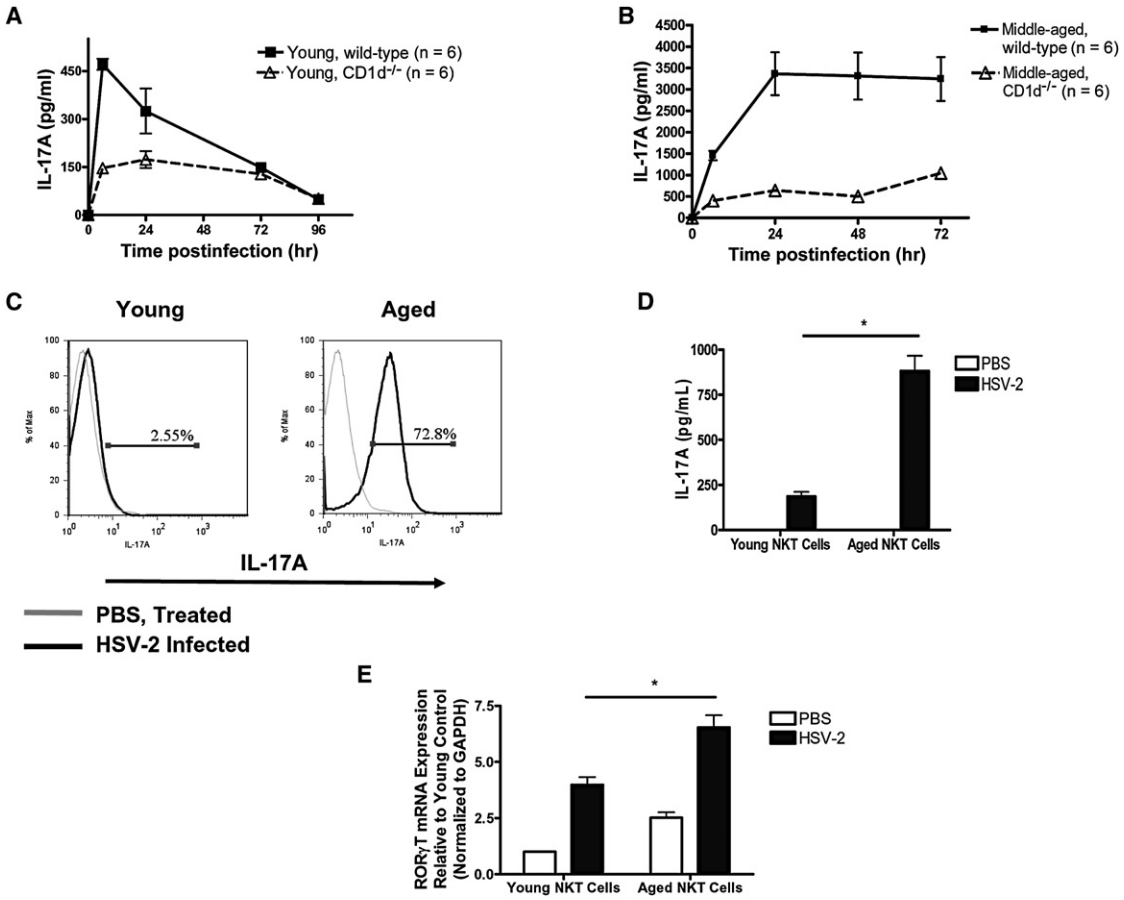

Figure 3. Aging Augments HSV-2-Induced Production of IL-17A by NKT Cells

(A and B) IL-17A serum levels in young (A) and middle-aged (9 months of age) (B) WT and NKT cell-deficient (*CD1d*$^{-/-}$) mice infected with HSV-2. p = 0.03, middle-aged WT versus middle-aged *CD1d*$^{-/-}$ (ANOVA).

(C) Intracellular IL-17A labeling in NKT cells (glycolipid-loaded CD1d tetramer^{+} TCRβ^{+} cells) gated within liver lymphocytes from aged and young mice receiving PBS or HSV-2. A representative experiment is shown from six independent experiments.

(D) HSV-2-induced IL-17A production in cultured liver NKT lymphocytes isolated from aged and young mice. *p < 0.01, young infected versus aged infected cells (t test). Values represent the mean ±SD of three independent experiments, each performed in triplicate.

(E) *RORγT* gene expression in liver NKT cells at rest and after HSV-2 infection. *p = 0.01, young infected versus aged infected cells (t test). Values in (A), (B), and (E) represent the mean ±SD of two independent experiments, each performed in triplicate. In all cases, n = 3 mice/group/experiment.

confirmed by histological analysis. As shown in Figure 2G, liver tissue from aged HSV-2-infected mice showed clear evidence of hepatocyte necrosis, while livers from young HSV-2-infected mice showed no histopathological changes. Histological examination of other organs and tissues from infected animals did not reveal any signs of age-specific necrosis (data not shown).

Aging Augments IL-17A Production by NKT Cells after HSV-2 Infection

The rapid kinetics of IL-17A induction in aged HSV-2-infected mice indicate that IL-17A release primarily arises from the innate immune response. Recent studies have demonstrated that NKT cells produce IL-17A in response to synthetic NKT cell ligands (Michel et al., 2007; Rachitskaya et al., 2008). Furthermore, aging induces an accumulation of NKT cells (Faunce et al., 2005), which can mediate age-dependent liver injury (Inui et al., 2002; Kawabata et al., 2008). Hence, we infected 2- to 4-month-old WT and NKT cell-deficient (*CD1d*$^{-/-}$) mice with HSV-2 and measured the serum IL-17A response. Six hours after infection, systemic IL-17A levels were lower in *CD1d*$^{-/-}$ mice than in WT mice (Figure 3A). We repeated this experiment with middle-aged mice (i.e., 9 months of age) and found that the difference in serum IL-17A levels between WT and *CD1d*$^{-/-}$ mice was even larger (Figure 3B). Similar results were noted in an alternate form of mutant mice that were deficient in NKT cells (*Jα18*$^{-/-}$) (Figure S8).

Next, we analyzed IL-17A responses in liver NKT cells (PBS-57-loaded CD1d tetramer^{+}, TCRβ^{+}) from young and aged mice at 12 hr after HSV-2 infection. The proportion of liver NKT cells that expressed IL-17A was greater in aged mice than in young mice (Figure 3C). Consistent with prior work (Inui et al., 2002), noninfected aged mice also manifested higher numbers of liver NKT cells than their younger counterparts, though these numbers did not increase after infection (Figure S9). To determine whether aging augments IL-17A responses on a per cell basis, we isolated liver NKT cells from noninfected aged and

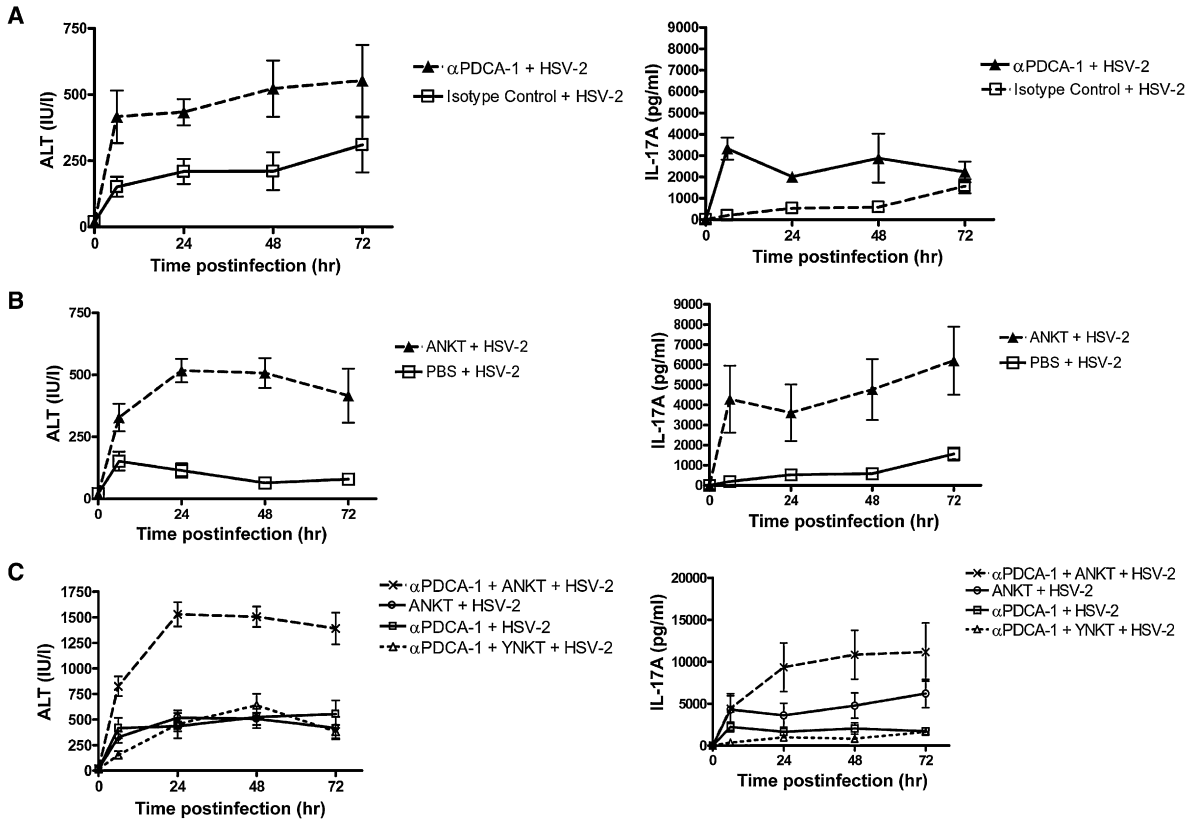

Figure 4. Impaired Viral Control and Aged NKT Cells Synergize to Induce Liver Injury during HSV-2 Infection

(A) Serum ALT and IL-17A levels were measured in young HSV-2-infected mice that were subjected to pDC depletion, adoptive transfer of NKT cells, or a combination of these procedures. pDC depletion with α-PDCA-1 antibody (α-PDCA-1 antibody group versus isotype control group, $p = 0.01$ for ALT and $p < 0.05$ for IL-17A [ANOVA]) is shown in (A).

(B) Adoptive transfer of aged NKT (ANKT) cells (ANKT versus PBS, $p < 0.001$ for ALT and $p < 0.05$ for IL-17A [ANOVA]).

(C) Combination of pDC depletion and adoptive transfer of NKT cells. Animals received ANKT cells or young NKT (YNKT) cells. ALT and IL-17A levels in mice undergoing a combination of pDC depletion and adoptive transfer of ANKT cells significantly differed from those in all other groups ($p < 0.001$, ANOVA). Values in (A)–(C) represent the mean ±SD of two independent experiments, each performed in triplicate. n = 4 mice per group/experiment. Young uninfected mice undergoing pDC depletion or adoptive transfer with NKT cells did not exhibit elevations in IL-17A and ALT (data not shown).

young mice. We then activated these cells ex vivo with HSV-2. We found that, during in vitro HSV-2 activation, aged NKT cells produced higher levels of IL-17A than young NKT cells (Figure 3D). Moreover, mRNA levels of *RORγT*, an IL-17-promoting transcription factor (Ivanov et al., 2006; Michel et al., 2007; Rachitskaya et al., 2008), were greater in aged NKT cells than in young NKT cells, both at rest and after HSV-2 infection (Figure 3E). Finally, NKT cells have been shown to produce IL-17A in an IL-6-independent manner, and experiments with middle-aged WT and *IL-6*-deficient mice revealed that HSV-2-induced production of IL-17A was IL-6 independent (Figure S10).

Impaired Viral Control and Aged NKT Cells Synergize to Induce Liver Injury during Viral Infection

Our results indicate that, in aged animals, liver injury and mortality during viral infection are associated with two factors: impaired containment of the virus (Figure S4C) and the presence of a cell with superior IL-17A-producing capabilities (i.e., NKT cell) (Figure 3C). Therefore, we investigated whether the presence of these two factors synergized to induce liver injury during viral infection in young mice. To impair viral control in young mice, we depleted these hosts of pDCs, which are critical to host defense to HSV-2 (Lund et al., 2006; Stout-Delgado et al., 2008). In young hosts, pDC depletion before viral infection using αPDCA-1 antibody led to a greater viral load in the liver than treatment with isotype control (Figure S11), confirming the critical role of pDCs in host defense to HSV-2.

Next, we measured serum ALT and IL-17A levels in young mice that underwent either pDC depletion or adoptive transfer of aged NKT cells. Our results revealed that ALT and IL-17A levels were greater in mice undergoing pDC depletion than in control animals (mice receiving isotype control antibody) (Figure 4A). ALT and IL-17A levels were also greater in young infected hosts undergoing adoptive transfer of aged NKT cells than in those not receiving these cells (Figure 4B). However, elevating serum IL-17A levels in the absence of infection through administration of recombinant IL-17A did not induce ALT release or mortality, regardless of host age (Figure S12). In young infected hosts, a combination of pDC depletion and adoptive transfer of aged NKT cells increased ALT levels to a greater

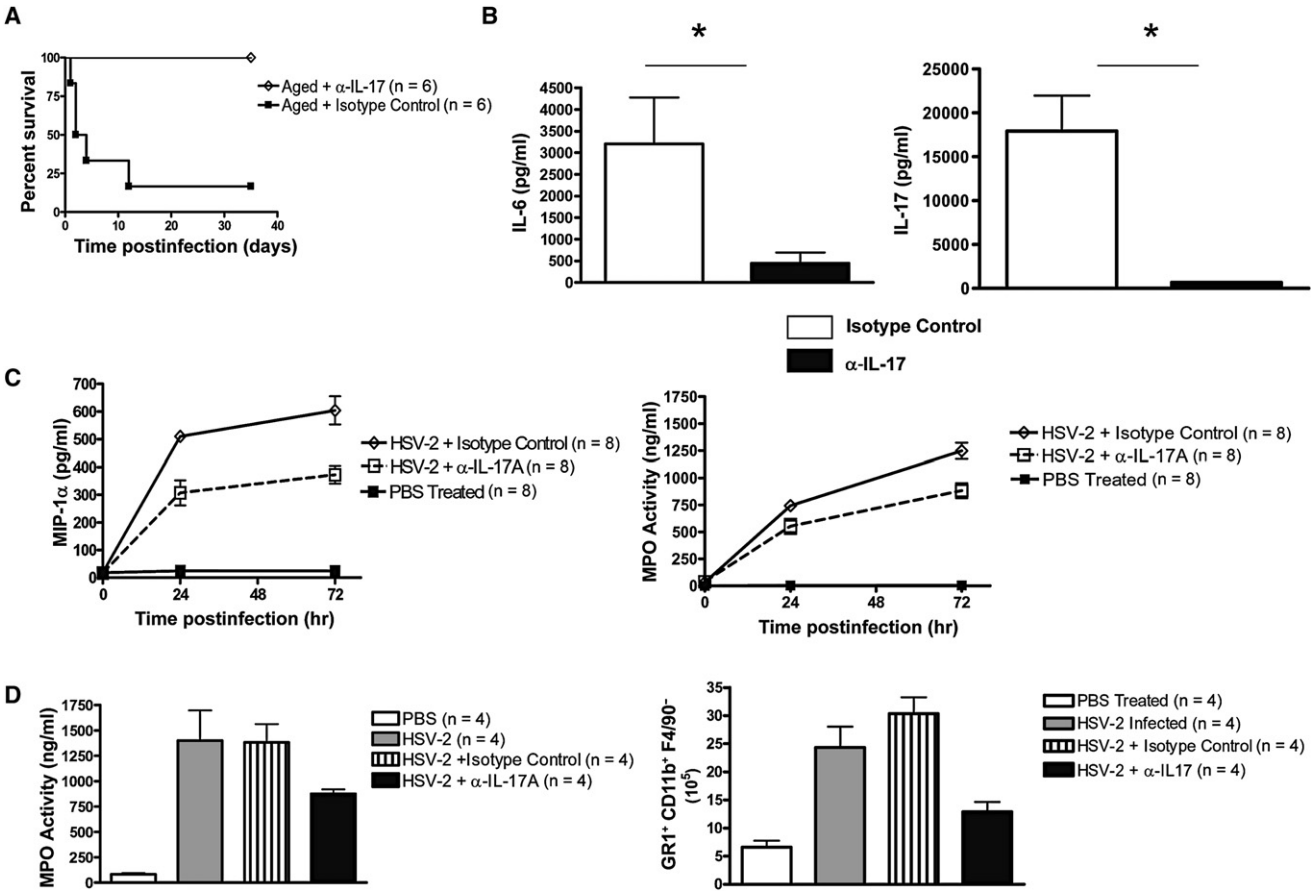

Figure 5. IL-17A Neutralization Blocks HSV-2-Induced Death, MIP-1α Production, and Neutrophil Activity in Aged Mice

(A–D) Effect of anti-IL-17A antibody on survival of aged mice (A) ($p = 0.007$, anti-IL-17A antibody versus isotype control, Log rank test), serum IL-17A and IL-6 levels (B) (n = 6 mice per group; *$p < 0.01$, anti-IL-17A antibody versus isotype control, t test), serum MIP-1α levels and MPO activity (C) ($p < 0.01$, anti-IL-17A antibody versus isotype control, ANOVA), and liver MPO production and $GR1^+CD11b^+$ $F4/80^-$ accumulation at 12 hr after infection (D) ($p < 0.01$, anti-IL-17A antibody versus isotype control, ANOVA). Values in (B)–(D) represent the mean ±SD of two independent experiments, each performed in triplicate.

extent than a comparable degree of pDC depletion combined with adoptive transfer of young NKT cells (Figure 4C). The pDC-depleted young mice that were adoptively transferred with aged, but not young, NKT cells exhibited signs of illness during viral infection (e.g., reduced movement, grooming, and feeding); however, they eventually survived infection. Finally, we found that young infected hosts that underwent pDC depletion and received aged NKT cells exhibited higher ALT levels and IL-17A levels than young HSV-2-infected mice undergoing only pDC depletion or adoptive transfer of aged NKT cells (Figure 4C). Importantly, the effect of these combined manipulations on ALT and IL-17A production was greater than the sum of the effects resulting from either manipulation alone (Figure 4C). These data indicate that impaired viral control and the presence of aged NKT cells synergize to induce liver injury during HSV-2 infection.

IL-17A Inhibition Prevents Lethality of HSV-2 in Aged Mice

To understand the role of IL-17A in HSV-2-induced death of aged mice, we tested the effect of an IL-17A-neutralizing antibody on survival. Treatment of aged mice with the IL-17A-neutralizing antibody at the time of HSV-2 infection did not affect viral clearance from either the spleen or liver during the time course of study (Figure S13A). Similar findings were noted when serum viral load was measured by PCR (Figure S13B). Nevertheless, this treatment led to 100% survival in this group (6/6) (Figure 5A). On the other hand, administration of an isotype control antibody to aged mice led to the survival of only a single mouse (1/6 or 17%) (Figure 5A). A comparison of cytokine production between HSV-2-infected mice receiving IL-17A-neutralizing antibody and receiving isotype control antibody revealed that IL-17A neutralization not only prevented death, but also significantly reduced serum IL-17A, IL-6, and TNF-α levels at 24 hr after infection (Figures 5B and S14). These changes were accompanied by a reduction in MIP-1α and MPO serum levels (Figure 5C). Liver MPO levels and neutrophil accumulation were also lower in the IL-17A neutralization group than in the isotype control group (Figure 5D). Importantly, these protective effects of IL-17A neutralization were associated with a reduction in ALT levels and hepatocyte necrosis (Figures 6A and 6B). In addition, the ability of anti-IL-17A treatment to prevent mortality was noted in aged BALB/c mice infected with MCMV (Figures S15A and S15B). IL-17A neutralization was also capable of preventing

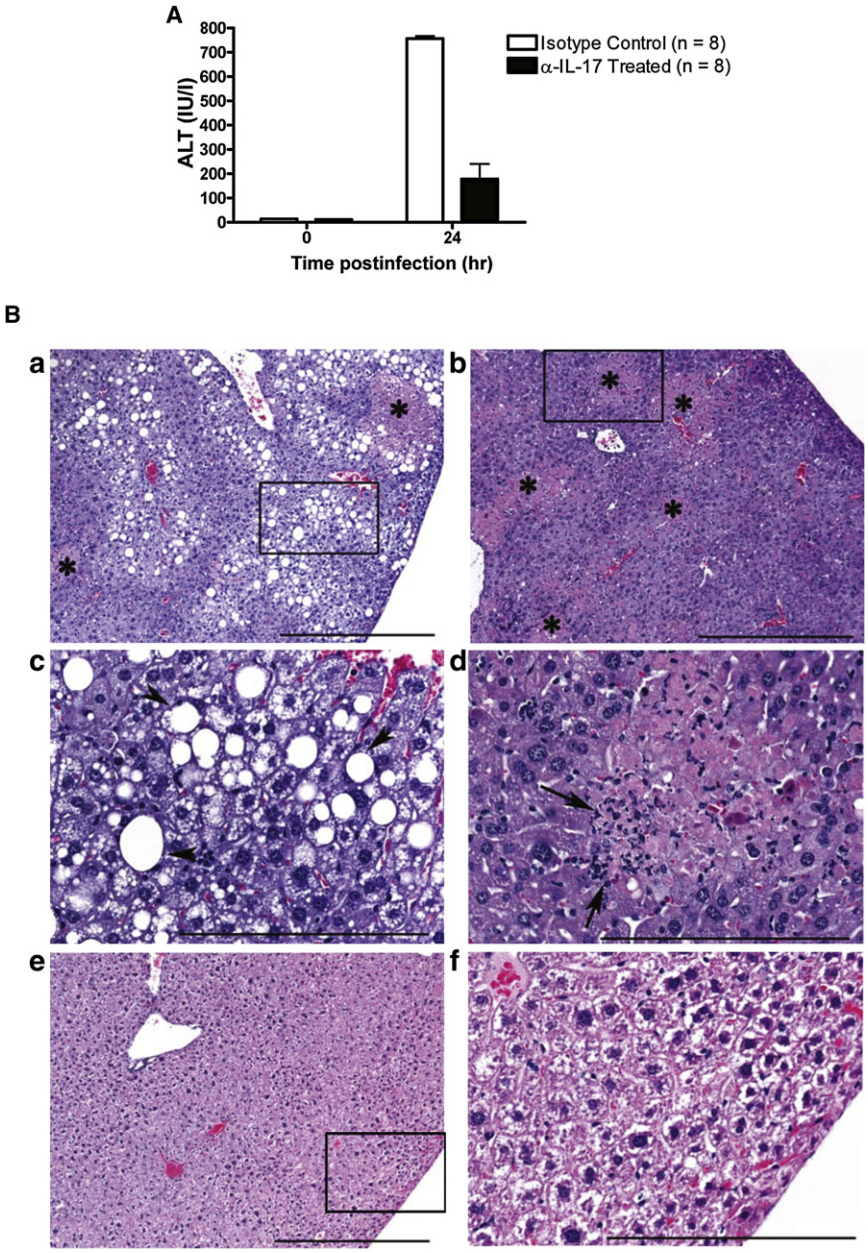

Figure 6. IL-17A Neutralization Inhibits HSV-2-Induced Hepatocyte Necrosis in Aged Mice

(A) Effect of anti-IL-17A antibody on HSV-2-induced increases in systemic ALT levels. Values represent the mean ±SD of two independent experiments, each performed in triplicate.

(B) Effect of anti-IL-17A antibody on HSV-2-induced histopathological changes in the aged liver at 48 hr after infection (n = 3 mice per group). Isotype antibody treatment (a–d) was associated with random multifocal necrosis (a, b *), scattered polymorphonuclear cells (arrows, d [inset of b]), and marked multifocal-to-diffuse fatty changes (steatosis) in some hepatic lobes (c; arrowheads [inset of a]). Treatment with anti-IL-17A prior to infection (e, f [inset of e]) prevented the induction of histopathological changes. Scale bars: a, b, and e = 500 μm; c, d, and f = 200 μm.

death when administered at 24 hr after HSV-2 infection (Figures S15C and S15D). These data indicate that HSV-2-induced death in aged mice is IL-17A dependent.

Neutrophil Depletion Prevents HSV-2-Induced Death in Aged Mice

The above results suggest that neutrophils are the cellular mediators of HSV-2-induced liver damage and death in aged mice. To further investigate this possibility, we depleted aged mice of neutrophils with a specific neutrophil-depleting mAb (Daley et al., 2008) and then infected these animals with HSV-2. All neutrophil-depleted mice (8/8) survived HSV-2 infection, while none of the isotype control mice survived (0/8) (Figure 7A). In addition, ALT levels were lower in neutrophil-depleted mice than in isotype control animals (Figure 7B). Similar results were noted in aged BALB/c mice infected with MCMV (Figures 7C and 7D). These data indicate that neutrophils are the cellular mediators of herpes virus-induced death in aged mice.

DISCUSSION

Aging is known to augment inflammatory responses, but whether these alterations contribute to death following viral infection is unknown. In the current study, we found that, following either HSV-2 or MCMV infection, aged mice produced higher serum levels of several inflammatory cytokines, in particular IL-17A. Importantly, neutralizing IL-17A in aged mice during HSV-2 or MCMV infection reduced liver injury and increased survival (Figures 5 and 6). These findings are consistent with prior work showing that IL-17 signaling induces liver injury in nonaging models (Nagata et al., 2008). IL-17A neutralization also reduced serum levels of other inflammatory cytokines, including IL-6 and TNF-α, indicating that elevated IL-17A levels control the production of other cytokines in this experimental system. This is consistent with prior work demonstrating that IL-17A can induce the production of IL-6 by a variety of cells (Fossiez et al., 1996). Our findings demonstrate that aging induces an aberrant overproduction of IL-17A during systemic viral infection and that this IL-17A overproduction can lead to death secondary to liver damage.

In the aged mice studied here, neutrophil-mediated liver necrosis was responsible for HSV-2-induced death. Prior work in nonaging models has demonstrated that neutrophils are key mediators of liver cell injury (Ramaiah and Jaeschke, 2007). We found that 24 hr after infection, aged virally infected mice, but

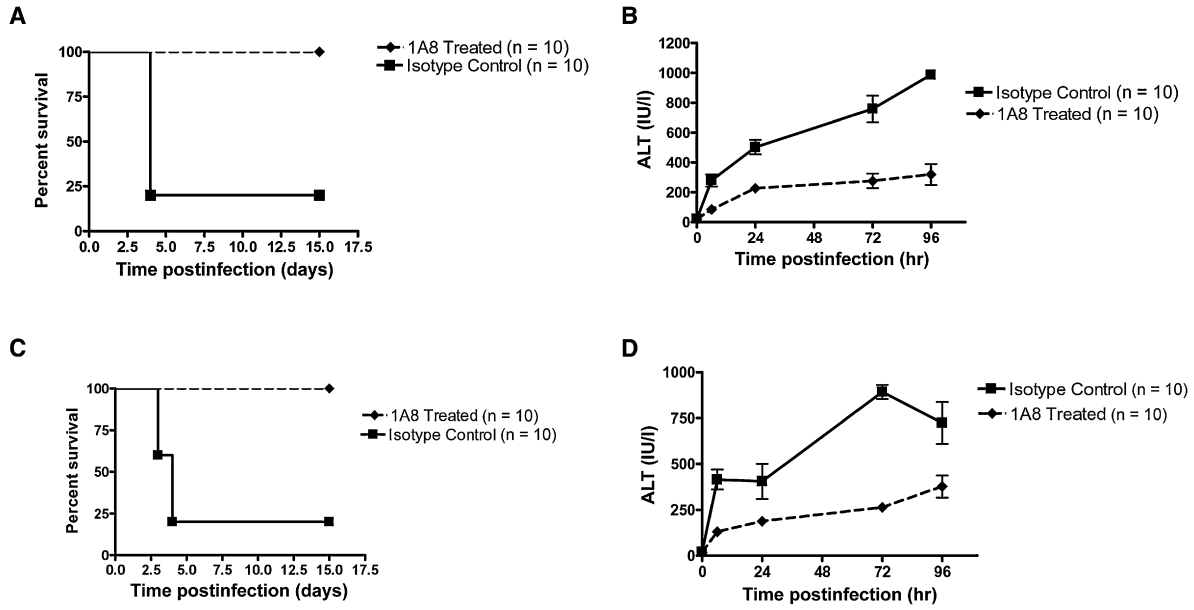

Figure 7. Depletion of 1A8$^+$ Cells Prevents HSV-2 or MCMV-Induced Death in Aged Mice

(A and B) Effect of depletion of 1A8$^+$ cells on survival (A) ($p < 0.001$, Log rank test) and serum ALT levels (B) (1A8$^+$ versus isotype, $p < 0.001$, ANOVA) in aged C57BL/6 mice infected with HSV-2. Noninfected mice did not exhibit an increase in serum ALT (data not shown).

(C and D) Effect of depletion of 1A8$^+$ cells on survival (C) ($p < 0.001$, Log rank test) and serum ALT levels (D) (1A8$^+$ versus isotype, $p < 0.001$, ANOVA) in aged BALB/c mice infected with MCMV. Noninfected mice did not exhibit an increase in serum ALT (data not shown). Values in (B) and (D) represent the mean ±SD of two independent experiments, each performed in triplicate.

not their young infected counterparts, exhibited severe hepatocyte necrosis that was associated with neutrophil activation (Figure 2). Moreover, both liver injury and mortality were blocked by neutrophil depletion (Figure 7). Both outcomes were driven by IL-17A, as seen by the ability of IL-17A neutralization to reduce levels of MIP-1α (a neutrophil-attracting chemokine) and decrease neutrophil enzyme activity (Figure 5). These data indicate that neutrophils are a key cellular mediator of IL-17A-dependent death in aged mice with systemic viral infections.

Our study provides evidence that NKT cells are important for the rapid production of IL-17A during HSV-2 infection, because NKT-deficient mice exhibited a much weaker IL-17A response than WT mice (Figure 3). These results indicate that NKT cells are a major source of IL-17A in our experimental systems but do not exclude the possibility that other cell types with known IL-17-producing capabilities, such as $\gamma\delta$ T cells and CD4$^+$ T cells (O'Brien et al., 2009; Stockinger and Veldhoen, 2007), contribute to the age-induced IL-17 response in our study. While recent studies have demonstrated that NKT cells produce IL-17A when activated by α-galactosylceramide or LPS instillation (Michel et al., 2007; Rachitskaya et al., 2008), here we demonstrate and characterize the ability of NKT cells to produce IL-17A during a viral infection. Our work suggests that IL-17-producing NKT cells may have pathological consequences during viral infection. Prior work indicates that NKT cells are important for host defenses against infection with HSV and other viruses (Grubor-Bauk et al., 2003, 2008). Furthermore, studies with MCMV have indicated that NKT cells are activated via the presence of an accessory cell to maximally induce IFNγ production during infection (Tyznik et al., 2008; Wesley et al., 2008). Our results indicate that NKT cells can respond directly to in vitro stimulation by HSV-2 to produce IL-17A, although our study does not exclude the possibility that an accessory cell is involved in vivo. In the future, it will be important to identify the viral components (e.g., viral glycolipids) that stimulate NKT cells to produce IL-17A.

We found that aging increased expression of *RORγT*, a transcription factor that may control IL-17A production by NKT cells (Michel et al., 2007; Rachitskaya et al., 2008). Why aging led to an upregulation of the *RORγT* gene in NKT cells is unclear. One possible avenue for future investigation is exploration of the role of oxidative stress, as many studies have shown that age-induced phenotypes correlate with increasing levels of oxidative stress (reviewed in Bokov et al., 2004).

Our work provides evidence that two factors (i.e., impaired viral control and aged NKT cells) cooperate in inducing liver injury during viral infection in our experimental models. A recent study demonstrated that aging impairs pDC function and, in this way, disrupts the IFNα response, inhibiting HSV-2 or MCMV clearance in aged mice (Stout-Delgado et al., 2008). In young infected mice, impeding viral clearance via pDC depletion and adoptively transferring aged, but not young, NKT cells induced greater ALT release than either manipulation alone and imparted a phenotype similar to that seen in aged mice (Figure 4). Although these young mice became ill, they eventually survived viral infection, suggesting that young hosts contain unidentified protective factors or that aged hosts contain unidentified disease-promoting factors that contribute to mortality during viral infection. A recent study found that IL-17A inhibits NK function, reducing clearance of vaccinia virus (Kawakami et al., 2009). This study may explain the synergy between impaired viral control and elevated IL-17A levels in our model, as it is possible that the augmented IL-17A

response alters other components of the aging immune system, further exacerbating liver injury. Nevertheless, our results indicate that, in aged hosts, viral infection induces liver injury through at least two factors: impaired containment of the virus and aged NKT cells, which exhibit an exaggerated IL-17A response.

In future studies, it will be important to determine whether local or organ-specific infection in aged hosts induces a phenotype similar to that described here in response to systemic viral infections. This may depend on whether NKT cells accumulate with age at local sites of infection. In addition, clinical studies should be conducted to determine whether older people exhibit increased levels of IL-17 during viral infection. A recent study showed that Th17 cells enhance viral persistence in a murine chronic viral infection model (Hou et al., 2009). If Th17 cells are found to be elevated in aged humans with viral infections, then perhaps age-dependent increases in IL-17 responses may not only contribute to immune pathology during systemic viral infections but also promote chronic viral infections. In any case, our data clearly show that aging alters the host-virus interaction by inducing liver damage and mortality through a pathway involving IL-17A. Thus, anti-IL-17A therapies may hold promise for preventing immune pathology and increasing survival in older individuals with systemic viral infections.

EXPERIMENTAL PROCEDURES

Mice

Aged (18–20 months), middle-aged (8–10 months), and young (2–4 months) C57BL/6 or BALB/c mice were purchased from the NIA rodent facility. C57BL/6 *IL-6*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 *CD1d*$^{-/-}$ mice and BALB/c *Jα18*$^{-/-}$ were generously provided by Dr. Erol Fikrig and Dr. McMahon-Pratt, respectively (both of Yale University; New Haven, CT), and were aged in our colony. The institutional animal care and use committee at Yale University approved the use of animals in this study. Animals with any evidence of skin lesions, weight loss, or lymphadenopathy were excluded from the study.

In Vivo Infection

HSV-2 virus was generously provided by Dr. Akiko Iwasaki (Yale University) and grown in Vero cells as previously described (Lund et al., 2003). Gradient-purified virus was administered via tail vein injection at a dose of 1×10^7 pfu or at other doses as indicated. HSV-2 viremia was measured via a plaque assay as previously described (Gadjeva et al., 2004). Briefly, diluted serum or supernatants obtained from homogenized spleen and liver samples were plated on a confluent layer of Vero cells, seeded at 1×10^6 cells/well in 10% FCS DMEM. The cells were incubated overnight at 37°C, and then plaques were counted by visual inspection following a Giemsa stain. Results are expressed as units/ml of serum or units/liver. Viral load was also assessed by real-time PCR (see below). HSV-2 was heat inactivated at 56°C for 1 hr. The MCMV Smith strain was generously provided by Anthony Van Den Pol (Yale University). Mice received 1×10^3 pfu of MCMV via intravenous injection, as described elsewhere (Delale et al., 2005).

In Vivo Reagents

IL-17A-neutralizing antibody (rat anti-mouse mAb, clone M210, IgG2a) was generously provided by Amgen (Seattle, WA) (Chu et al., 2007). It was administered i.p. at a dose of 150 μg/mouse at 24 hr prior to viral infection or at other times indicated. Anti-1A8 mAb and isotype control were obtained from BioXcell (Lebanon, NH). Both were used at a concentration of 0.5 mg/mouse and administered i.p. 72 hr prior to infection. Acyclovir (Sigma-Aldrich; St. Louis) or PBS (control) was administered i.p. at a dose of 0.6 mg/mouse. These agents were administered at the time of HSV-2 infection and 24 hr after infection. For pDC depletion, mice received 0.5 mg of anti-PDCA-1 monoclonal antibody (Miltenyi Biotec; Bergisch Gladbach, Germany) or isotype control (BioXcell) i.p. 24 hr before infection, as previously reported (Stout-Delgado et al., 2008). Recombinant IL-17A (eBioscience; San Diego, CA) was administered i.p. at a dose of 250 pg/gm body weight.

Histology

A routine selection of tissues from all organ systems was subjected to histopathologic examination. The tissues were processed, embedded in paraffin, sliced into 5 μm thick sections, and stained with hematoxylin and eosin using routine methods. Samples were evaluated by investigators blinded to the experimental group. Digital light microscopic images were acquired using a Zeiss Axioskop microscope, an AxioCam MRC Camera, and AxioVision 4.4 imaging software (Carl Zeiss MicroImaging, Inc.; Thornwood, NY). The resulting images were optimized using Adobe Photoshop 8.0 (San Jose, CA).

Cell Sorting, Flow Cytometry, and NKT Cell Culture

Liver lymphocytes were isolated as previously described (Crispe, 2003). These cells were stained with fluorescently labeled PBS-57 glycolipid-loaded or unloaded CD1d tetramers (NIAID Tetramer Facility; Atlanta) and FITC-tagged rat anti-mouse TCRβ$^+$ mAb (eBioscience). The cells were subjected to intracellular cytokine staining with PE-tagged rat anti-mouse IL-17 mAb (eBioscience), and NKT cells were identified by gating on the PBS-57-loaded CD1d tetramer$^+$ TCRβ$^+$ population, as previously described (Liu et al., 2006). Liver NKT cells (i.e., PBS-57-loaded CD1d tetramer$^+$ TCRβ$^+$) were also sorted by FACs for further in vitro studies. For cytokine staining, NKT cells were harvested 12 hr after HSV-2 infection and then cultured in the presence of cell permeability agents and Golgi stop (eBioscience). For in vitro studies, 1×10^5 FACs-purified NKT cells were plated in RPMI supplemented with 10% FBS (200 μl) and treated with 50 pfu heat-inactivated HSV-2 for 18 hr at 37°C. Cells were then processed for real-time PCR and cell supernatants for ELISA.

Adoptive Transfer of NKT Cells

Liver NKT cells were purified as described above, and 1×10^5 cells were adoptively transferred via intravenous tail vein injection at 72 hr prior to infection.

RNA and DNA Purification

RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN; Valencia, CA) and quantified by $A_{260/280}$ absorbance readings. SuperScript III Reverse Transcriptase (Invitrogen) was used, according to the manufacturer's instructions, to create complementary DNA from mRNA. DNA was extracted from 10 μl of serum samples using the QIAGEN DNeasy Blood and Tissue Kit; eluted into 50 μl of sterile, double-distilled water; and quantified by $A_{260/280}$ absorbance readings.

Real-Time PCR

Real-time PCR for *RORγT* was performed using TaqMan gene expression probe/primer sets (Applied Biosystems; Foster City, CA). Reaction mixtures had a final volume of 25 μl and contained complementary DNA from 20 ng of reverse-transcribed total RNA, TaqMan Gene expression primer/probe mix, and TaqMan Gene Expression Master Mix. Real-time PCR for *HSV-2* DNA was performed using SYBR Green Universal PCR Master Mix (Applied Biosystems). The subsequent real-time PCR was performed in a final volume of 25 μl containing 20 ng of DNA, 150 nM forward and reverse primers (Wada et al., 2009), and SYBR Green Universal PCR Master Mix. Known concentrations of HSV-2 DNA were included in all PCR reactions to assess sensitivity. PCR was performed in 96-well plates using the MJ Research detection system (MJ Research, Inc.; Waltham, MA). All reactions were performed in triplicate. An amplification curve analysis was performed, and dilution curve standards were generated to identify primer sets and conditions yielding specific products with 100% amplification efficiency. Relative mRNA levels were calculated using the comparative cycle threshold method (User Bulletin #2, Applied Biosystems). *GAPDH* and *cyclophilin* mRNA levels served as invariant controls.

ELISA

Culture supernatants or sera were analyzed for IL-6, IL-17A, IFNα, TNF-α, and MIP-1α using ELISA kits from BD Biosciences (San Diego, CA), R&D Systems (Minneapolis), or PBL (Piscataway, NJ) according to the manufacturers' instructions. Neutrophil activity was determined by measuring MPO levels, which were also analyzed using a commercially available ELISA kit (Hycult Biotechnology; Uden, The Netherlands) according to the manufacturer's protocol.

ALT Assay

Serum ALT levels were measured using the Infinity ALT Assay Kit (Fisher Scientific; Suwance, GA) according to the supplied instructions.

Statistical Analysis

Survival analysis was performed using the Log rank method. Comparison of means was performed using a two-tailed t test or repeated-measures ANOVA. All data were analyzed using GraphPad prism software (San Diego, CA). Values of p less than 0.05 were considered significant.

SUPPLEMENTAL DATA

Supplemental Data include 15 figures and can be found online at http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00347-3.

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