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1 **V α 14 β 1/NKT cells Promote Liver Pathology during Adenovirus Infection by Inducing CCL5 Production:**
2 **Implications for Gene Therapy**

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23
24 Abbreviations: V α 14 β 1/NKT cells, V α 14 invariant natural killer T cells; AdLacZ, replication-defective Adenovirus;
25 CCL5, CC ligand 5; ROS, reactive oxygen species; NAC, N acetylcysteine; ALT, alanine transaminase; $\gamma\delta$ T
26 cells, gamma delta T cells; Met-RANTES, Methionylated-RANTES; J α 18KO mice, V α 14 β 1/NKT cell deficient
27 mice

28
29

Abstract

31 Replication-defective recombinant Adenoviruses are the most widely studied replication-defective vectors for
32 the potential treatment of inherited human diseases. However, broad clinical application of replication-
33 defective Adenoviruses in gene therapy is being hindered by the induction of vigorous innate and adaptive
34 immune responses against the vector which causes deleterious effects in the liver. $V\alpha 14$ invariant natural
35 killer T ($V\alpha 14$ NKT) cells are thymic-derived innate T cells at the interface between the two arms of the immune
36 response and provide full engagement of host defense. The pathophysiological role of intrahepatic $V\alpha 14$ NKT
37 cells during replication-defective Adenovirus infection is not known, and is the main focus of our study. Our
38 data showed that intrahepatic $V\alpha 14$ NKT cells were activated in response to Adenovirus infection to induce
39 significant levels of hepatic CCL5 and subsequent liver toxicity. Moreover, intrahepatic CCL5 production was
40 selectively reduced by $V\alpha 14$ NKT cell deficiency. *In vivo* studies utilizing CCL5 deficient mice or $V\alpha 14$ NKT
41 cell deficient mice demonstrated that CCL5 or $V\alpha 14$ NKT cell deficiency were each associated with reduced
42 liver pathology. Similar results were seen after blocking the biological effects of the CCL5 receptors. In
43 conclusion, we have identified **an important pro-inflammatory role** for activated intrahepatic $V\alpha 14$ NKT cells in
44 positively influencing hepatic CCL5 production to promote acute liver inflammation and injury. Therefore, our
45 findings highlight the blockade of CCL5 interaction with cognate receptor(s) as an important potential strategy
46 to alleviate liver pathology associated with replication-defective Adenovirus infection.

47 Introduction

48 Replication-defective Adenoviruses, non enveloped dsDNA viruses with genomes of ~36kbp, are the
49 most widely studied and used replication-defective vectors [www.wiley.co.uk/genmed/clinical](21, 39, 42). This
50 is because they are easily produced in high titers, can be lyophilized, administered through various routes and
51 have broad host cell range (39). Clinically, universal application of replication-defective Adenoviruses in gene
52 therapy is hampered by robust immune responses against the vector which results in the loss of Adenovirus
53 transgene and deleterious effects in the liver of rodents (39), non-human primates (24) and humans (33). For
54 many years, several attempts to minimize/inhibit the ability of the host inflammatory and cellular immune
55 responses to eliminate the vector initially focused on manipulating the adaptive immune system (39).
56 However, in the last decade, several studies have demonstrated that innate immune cells such as neutrophils
57 (29), NK cells (23) and $\gamma\delta$ T cells (2) also promotes liver inflammation and injury. For this reason, the innate
58 immune response against the Adenovirus vector (rather than that mediated by adaptive immune response) and
59 subsequent hepatotoxicity are the biggest hurdle to the broad clinical application of replication-defective
60 Adenovirus vectors in gene therapy.

61 $V\alpha 14/NKT$ cells are a unique subset of thymic-derived innate T lymphocytes that are distinct from
62 conventional T cells in that they express a highly restricted T cell receptor (TCR) characterized by a $V\alpha 14$ - $J\alpha 18$
63 rearrangement with an invariant junction preferentially associated with $V\beta 8.2$, $V\beta 7$, or $V\beta 2$ (14), and may also
64 express cell surface markers for NK cells (usually NK1.1). In contrast to conventional T cells [i.e. CD4(+) and
65 CD8(+) T cells] which recognizes peptide antigens presented by MHC class I and II molecules, $V\alpha 14/NKT$ cells
66 respond to glycolipid antigens presented by CD1d bearing antigen presenting cells (15). A number of cellular
67 lipids have been shown to activate $V\alpha 14/NKT$ cells [see review by Randy Brutkiewicz (7)], but the specific
68 endogenous ligand(s) is presently unknown (31, 34). Compelling evidence from many studies using CD1d
69 tetramers loaded with the prototypical synthetic glycolipid antigen, α Galactosylceramide, shows that mouse
70 liver has the highest frequency of resident $V\alpha 14/NKT$ cells (1, 26). $V\alpha 14/NKT$ cells may be activated in a TCR-
71 dependent manner by lipids presented by CD1d (25) or by TCR independent mechanisms involving toll like
72 receptors [TLR](12, 25, 30, 36). Following activation, $V\alpha 14/NKT$ cells typically exert multiple effects including
73 the production of several cytokines, chemokines and cytotoxic proteins (25). Through these mediators,
74 activated $V\alpha 14/NKT$ cells can “bridge” the innate and adaptive immune systems by interacting with and
75 transactivating immune cells (4, 8, 35). In the present study, we evaluated the functional importance of
76 intrahepatic $V\alpha 14/NKT$ cells in the development of liver toxicity following replication-defective Adenovirus
77 infection.

78 **Materials and Methods**

79 **Mice.** Male C57BL6 mice, CCL5^{-/-} mice (on C57BL6 background), TCR- δ ^{-/-} mice (on C57BL6 background)
80 and IFN- γ ^{-/-} mice (on C57BL6 background) aged 5–7 weeks were all purchased from the Jackson Laboratory
81 (Bar Harbor, ME). Breeding pairs of J α 18^{-/-} mice (on C57BL6 background) were kindly provided by Dr. M.
82 Taniguchi [RIKEN Research Center, Japan](10, 12) and bred in a pathogen-free breeding facility at LSUHSC-
83 Shreveport (12). All experiments were conducted in accordance with institutional guidelines for animal care.

84
85 **Adenovirus virus infection and *in vivo* treatment protocol.** Endotoxin free, replication-defective E1/E3-
86 deleted type 5 Adenovirus vectors expressing *E. coli* LacZ gene (herein called AdLacZ) as reporter gene,
87 driven by the cytomegalovirus promoter, was purchased from Vector Development Laboratory [Baylor College
88 of Medicine, Houston] (2). Mice were injected with AdLacZ [10¹¹ virus particles, i.p.](2). **Mock-infected mice**
89 **received an equivalent volume of vehicle** (2). At indicated time-points, mice were anesthetized with a mixture
90 of xylazine and ketamine hydrochloride, blood serum was collected. Livers were then perfused with ice-cold
91 sterile PBS (to remove blood elements) and **harvested for the experimental assays described below.**

92 For CCL5 blocking experiment, 0.5ml/mouse of **goat anti-murine CCL5 serum** [supplied by Dr. Robert
93 Strieter, University of Virginia, Charlottesville](5) or control goat serum (Sigma Chem. Com; St. Louis) were
94 administered i.p. to naïve C57BL6 mice 16h before AdLacZ infection and additional dose given every 48h until
95 termination of the experiment. **Met-RANTES, a dual CCR1/CCR5 antagonist (32), is widely used to inhibit the**
96 **biological activities of CCR1/CCR5, cognate receptors for CCL5 (13).** Therefore, Met-RANTES was used in
97 **the present study to inhibit the effector functions of CCR1/CCR5** in the liver during AdLacZ infection.
98 Specifically, Met-RANTES [30 μ g/mouse](4) or vehicle PBS was administered to mice intraperitoneally 24h
99 before AdLacZ infection, and every 24h thereafter until termination of the experiment.

100
101 **Evaluation of acute liver injury.** Liver injury was determined by biochemical and histological means.
102 Biochemical liver damage was determined by measuring serum levels of the liver enzyme, alanine
103 aminotransferase (ALT), using a commercial kit [Thermo Electron, Waltham](2). For histological evaluation,
104 paraffin embedded liver sections (5 μ m thick) were deparaffinized, stained with H & E according to standard
105 protocols and then analyzed by light microscopy in a blinded fashion by a pathologist (PAA). The degree of
106 inflammation of the liver and hepatocyte damage was graded as mild, moderate or severe using a combination
107 of the severity of the inflammation, and the degree of hepatocyte degenerative changes including ballooning
108 degeneration, hepatocyte necrosis and frequency of acidophilic bodies (2).

109
110 **Agonistic Fas mAb Treatment.** Agonistic Fas mAb [clone Jo2; 0.5 μ g/g of body weight; BD Pharmingen] (2)
111 was administered intraperitoneally to naïve WT mice and naïve J α 18 KO mice for 5h **and liver injury was then**
112 **evaluated by measuring ALT levels.**

113

114 **Flow cytometric analysis of isolated hepatic lymphoid cells.** Hepatic lymphocytes were isolated using our
115 published protocols (1, 2, 12). For the specific identification of hepatic V α 14/NKT cells, isolated hepatic
116 lymphocytes were preincubated with anti-mouse CD16/32 mAb (clone 2.4G2; BD Pharmingen) to block Fc γ Rs
117 and then incubated simultaneously with fluorochrome-labeled TCR β mAb (clone H57-597; BD Pharmingen)
118 and fluorochrome-labeled V α 14/NKT cell tetramer [CD1d-PBS57; NIH Tetramer Core Facility, Atlanta] (2, 12).
119 Next, three-color staining was used to assess intracellular IFN- γ or intracellular active caspase 3 expression by
120 tetramer positive hepatic V α 14/NKT cells using fluorochrome-labeled murine IFN- γ mAb [clone XMG1.2; BD
121 Pharmingen] (2) and fluorochrome-labeled active caspase 3 mAb [clone C92-605; BD Pharmingen] (1, 12),
122 respectively. Fas expression on the surface of tetramer positive V α 14/NKT cells was determined using
123 fluorochrome-labeled murine Fas mAb [clone Jo2; BD Pharmingen] (9).

124 Hepatic $\gamma\delta$ T cells were identified using fluorochrome-labeled TCR $\gamma\delta$ mAb (clone GL3; BD Pharmingen)
125 and fluorochrome-labeled CD3 ϵ mAb (clone 145-2C11; BD Pharmingen) as we recently described (1, 2, 12).
126 Next, TCR $\gamma\delta$ -CD3(+) double positive T cells were permeabilized with Cytofix/Cytoperm plus and stained
127 intracellularly with fluorochrome-labeled murine IFN- γ mAb.

128 NK and CD8 (+) T cell infiltrates in the liver were evaluated using fluorochrome-labeled NK1.1 mAb
129 (clone PK136; BD Pharmingen) and fluorochrome-labeled CD8a mAb (clone 53-6.7; BD Pharmingen),
130 respectively. In all experiments, corresponding isotype antibodies/tetramer were used as controls. Viable
131 lymphoid populations were gated using forward and side scatter characteristics and analysis performed using
132 the FACS Calibur and FACS Scan Diva software (BD Biosciences). Notably, all intracellular cytokine stainings
133 were performed without further reactivation *in vitro*.

134

135 **Hepatic chemokine protein.** Murine CCL2, CCL3, CCL4 and CCL5 protein levels in the liver were measured
136 using the BioPlex array system (BioRad Laboratories) as described by us (2). Total protein levels in liver were
137 determined using the BCA protein assay reagent (Pierce Biotechnology).

138

139 **Statistical Analysis.** All data are shown as mean \pm SEM. For comparisons of means between 2 experimental
140 groups a Student unpaired *t* test was used. Comparison among three or more experimental groups was
141 performed using a one-way ANOVA, followed by either Dunnett's multiple comparison test or Newman-Kuels
142 post hoc test. A value of $p < 0.05$ was considered significant.

143 **Results**

144 **Hepatic V α 14/NKT cells undergo activation induced cell death in response to AdLacZ infection.** Hepatic
145 V α 14/NKT cells were activated in response to AdLacZ infection as demonstrated by significant increases in
146 V α 14/NKT cell intracellular IFN- γ production relative to uninfected mice (Fig. 1A). V α 14/NKT cell activation
147 was followed by a significant decrease/loss in the frequency of hepatic V α 14/NKT cells relative to uninfected
148 control (i.e. day 0; Fig. 1B and C). In contrast, the absolute number of hepatic V α 14/NKT cells was
149 significantly increased at day 6 (but not at day 1) post-AdLacZ infection (Fig. 1D). Notably, V α 14/NKT cell
150 activation occurred prior to significant accumulation of CD8(+) T cells in the liver (Fig. 1E). To determine if this
151 decline in intrahepatic V α 14/NKT cells frequency was due to death by apoptosis, Fas and active caspase 3
152 expressions were analysed by flow cytometry. Fas was highly upregulated on the surface of hepatic
153 V α 14/NKT cells post-AdLacZ infection (Fig. 2A and B), increasing >3-fold over that seen in uninfected control.
154 The frequency of intracellular active caspase 3 expressing hepatic V α 14/NKT cells was also significantly
155 increased (>10-fold) during AdLacZ infection (Fig. 2C and D). Furthermore, the absolute number of
156 intracellular active caspase 3 expressing hepatic V α 14/NKT cells was significantly increased at day 6 (but not
157 at day 1) post-AdLacZ infection in comparison to uninfected control (Fig. 2E). In summary, the decline in the
158 frequency of hepatic V α 14/NKT cells paralleled increases in Fas and active caspase 3 expression; an
159 indication that activated hepatic V α 14/NKT cells become susceptible to apoptotic death following AdLacZ
160 infection.

161

162 **Hepatic V α 14/NKT cells initiate liver toxicity in response to AdLacZ Infection.** To ascertain whether
163 hepatic V α 14/NKT cells initiate acute liver toxicity during AdLacZ infection, wildtype (WT) and J α 18^{-/-} (i.e.
164 V α 14/NKT cell deficient) mice were infected with AdLacZ. Mock-infected WT and J α 18^{-/-} mice received
165 vehicle. Acute liver injury was assessed biochemically (by ALT levels) and histologically 6 days later. This
166 endpoint was chosen due to considerable liver damage at this time-point as previously reported by us (2).
167 Mock-infected WT or J α 18^{-/-} mice had comparable baseline levels of serum ALT (Fig. 3A). However, in
168 response to AdLacZ infection, a significant increase in serum ALT level was observed in WT mice relative to
169 mock-infected WT or J α 18^{-/-} mice (Fig. 3A). In contrast, J α 18^{-/-} mice were highly resistant to acute liver injury
170 following AdLacZ infection as shown by significantly lower (>70% reduction) serum ALT levels (Fig. 3A). In
171 agreement with the biochemical data, liver sections obtained from infected WT mice exhibited extensive
172 hepatocyte damage and increased inflammatory cell infiltrates (Fig. 3B and C) whereas liver sections from
173 infected J α 18^{-/-} mice displayed reduced inflammatory cell infiltrates with little or no hepatocyte damage (Fig.
174 3B and C). Notably, V α 14/NKT cell deficiency was associated with reduced accumulation of immune cells,
175 CD8(+) T cells, $\gamma\delta$ T cells and NK cells, in the liver during AdLacZ infection (Fig. 3D). In summary, our data
176 demonstrates an important pro-inflammatory role for V α 14/NKT cells in promoting the development of acute
177 hepatic inflammation and injury during AdLacZ infection.

178

179 **Intrahepatic V α 14/NKT cells promote chemokine production during AdLacZ infection.** Activated
180 V α 14/NKT cells has been reported to secrete CCL5 during inflammation (11, 27). Therefore, we determined
181 whether CCR1/CCR5 ligands (i.e. CCL3, CCL4 and CCL5) are produced in the liver in response to AdLacZ
182 infection. These ligands were significantly increased in the liver of WT mice at day 6 post-AdLacZ infection
183 (Fig. 4A, B and C). We also observed that V α 14/NKT cell deficiency significantly suppressed hepatic
184 production of CCL4 and CCL5 (but not CCL3) during AdLacZ infection since hepatic levels of CCL4 and CCL5
185 in J α 18^{-/-} mice were significantly lower relative to levels in infected WT mice (Fig. 4D). Although activated
186 V α 14/NKT cells are also capable of secreting CCL2 during an inflammatory response (27), our study revealed
187 that CCL2 production in the liver of WT mice was comparable to that seen in the liver of J α 18^{-/-} mice following
188 AdLacZ infection (Fig. 4D).

189

190 **Effects of CCL5 blockade on AdLacZ-mediated acute liver toxicity.** The preceding results suggest that
191 activated hepatic V α 14/NKT cells could be a source of CCL5 during AdLacZ infection. For this reason, CCL5
192 gene deficient mice and a specific murine CCL5 blocking antibody were used to assess whether CCL5 is an
193 essential participant in the pro-inflammatory effects of hepatic V α 14/NKT cells during AdLacZ infection.
194 **Indeed, serum ALT levels in CCL5 deficient mice at day 6 post-AdLacZ infection was significantly lower (~52%**
195 **reduction) relative to WT mice** (Fig. 5A). Accordingly, liver sections from AdLacZ-infected CCL5 deficient mice
196 displayed reduced hepatocellular damage relative to liver sections derived from AdLacZ-infected WT mice (Fig.
197 5B). In parallel, murine CCL5 antiserum treatment of WT mice prior to AdLacZ infection also alleviated acute
198 hepatic injury (both biochemically and histologically) in comparison to AdLacZ infected WT mice given control
199 serum (Fig. 5A and B). The ability of the chemokine receptors CCR1/CCR5 to drive the effector functions of
200 chemokines CCL3, CCL4 and CCL5 are well defined (3). For this reason, we also evaluated the effect of
201 CCR1/CCR5 blockade on AdLacZ-mediated liver toxicity. Treatment of AdLacZ-infected WT mice with Met-
202 RANTES, a dual CCR1/CCR5 chemokine receptor antagonist, also attenuated acute liver injury (72%
203 reduction) relative to that seen in AdLacZ-infected WT mice administered vehicle (Fig. 5A), a finding that was
204 confirmed histologically (Fig. 5B). In Table I, we demonstrated that CCL5 may induce CCL3 and CCL4
205 production in the liver during AdLacZ infection since hepatic CCL3 and CCL4 production were significantly
206 **reduced** by CCL5 deficiency. Overall, our data highlights a key role for CCL5, acting possibly via CCR1/CCR5,
207 in promoting the pro-inflammatory effects of activated hepatic V α 14/NKT cells during AdLacZ infection.

208

209 **V α 14/NKT cells transactivate γ δ T and CD8(+) T cells, but not NK cells, during AdLacZ infection.**
210 Numerous studies have documented an important role for IFN- γ in inducing CCL5 production in many
211 inflammatory settings (19, 20). Moreover, **hepatic V α 14/NKT cells produce IFN- γ during AdLacZ infection** (Fig.
212 1A). Therefore, we investigated whether IFN- γ was capable of inducing CCL5 production in the liver in
213 response to AdLacZ infection. IFN- γ deficiency significantly suppressed hepatic production of CCL5 at day 6

214 post-AdLacZ infection (Fig. 6A). Next, we determined if $\gamma\delta$ T cells, an innate immune T cell, which we recently
215 reported to also be an important cellular source for IFN- γ during AdLacZ infection (2), may also mediate CCL5
216 production in the liver. Indeed, $\gamma\delta$ T cell deficiency also significantly inhibited CCL5 production in the liver post-
217 AdLacZ infection (Fig. 6A). Our data thus far, suggests that IFN- γ producing immune cells such as $V\alpha 14$ NKT
218 and $\gamma\delta$ T cells are capable of inducing CCL5 production in the liver during AdLacZ infection via IFN- γ release.

219 Activated hepatic $V\alpha 14$ NKT cells are able to initiate the inflammatory process by transactivating NK
220 and CD8(+) T cells (4, 8) and more recently, $\gamma\delta$ T cells (12). Therefore, we investigated if $V\alpha 14$ NKT cells
221 transactivate $\gamma\delta$ T cells, NK cells and CD8(+) T cells during AdLacZ infection. As shown in Fig. 6B, $V\alpha 14$ NKT
222 cell deficiency significantly impaired $\gamma\delta$ T cell intracellular IFN- γ production at day 6 (but not day 1) post-AdLacZ
223 infection. Similarly, the number of IFN- γ producing CD8(+) T cells in the liver of $J\alpha 18^{-/-}$ mice was significantly
224 reduced at day 6 (but not day 1) post-AdLacZ infection relative to infected WT mice (Fig. 6C). In contrast, the
225 number of IFN- γ producing NK cells in the liver of $J\alpha 18^{-/-}$ mice was not significantly reduced at days 1 or 6
226 following AdLacZ infection relative to WT mice (data not shown). Taken together, we propose that the $\gamma\delta$ T
227 cells and CD8(+) T cells promote CCL5 production in the liver via IFN- γ secretion and this response is
228 mediated/driven by activated hepatic $V\alpha 14$ NKT cells.

229

230 **Contribution of Fas expressing $V\alpha 14$ NKT cells to acute hepatic injury.** Activated $V\alpha 14$ NKT cells
231 upregulate Fas following AdLacZ infection (Fig. 2A). To assess whether activation of Fas expressing
232 $V\alpha 14$ NKT cells could promote hepatic inflammation and injury, we treated naïve WT mice and naïve $J\alpha 18^{-/-}$
233 mice with agonistic Fas mAb (Jo2) for 5h. Our results showed an almost complete inhibition (97% reduction)
234 of serum ALT level in mice deficient in $V\alpha 14$ NKT cells after Jo2 treatment when compared to WT mice [WT
235 mice: 3643 ± 548 IU/L relative to $96.75 \pm 37.62^*$ IU/L in $J\alpha 18^{-/-}$ mice; $*P \leq 0.05$ vs. WT mice; $n=3-4$
236 mice/group]. Furthermore, a marked improvement in hepatic histology was seen in $J\alpha 18^{-/-}$ relative to WT mice
237 after Jo2 treatment (data not shown). In summary, our data highlights an important role for Fas expressing
238 $V\alpha 14$ NKT cells in promoting acute liver injury in response to agonistic Fas mAb treatment.

239 **Discussion**

240 $V\alpha 14/NKT$ cells are an important link between the innate and adaptive immune systems and plays an
241 important immunoregulatory role in cardiovascular, infectious and autoimmune diseases as well as in tumor
242 immunity. Broad clinical application of replication-defective AdLacZ vectors in gene therapy remains elusive
243 due to vigorous innate and adaptive immune responses against the vector which causes harmful effects in the
244 liver and the subsequent loss of the AdLacZ transgene. Several studies have previously revealed the
245 importance of classical innate immune cells, NK cells (23) and $\gamma\delta T$ cells (2), in regulating the host immune
246 response during AdLacZ infection. Mouse liver contains the highest frequency of resident $V\alpha 14/NKT$ cells
247 relative to other peripheral organs, accounting for $\leq 30\%$ of murine hepatic T lymphocytes (1, 26). However,
248 nothing is known about how $V\alpha 14/NKT$ cells respond physiologically during AdLacZ infection. Consequently,
249 the functional importance of $V\alpha 14/NKT$ cells in the development of liver pathology is currently undefined, and
250 was the main focus of this study. Our study highlights an essential role for activated $V\alpha 14/NKT$ cells in
251 positively influencing CCL5 production to promote acute liver toxicity during AdLacZ infection.

252 Studies in murine models of infectious diseases have previously documented reduced hepatic
253 $V\alpha 14/NKT$ cell frequency during viral [LCMV (17), mCMV (38)] and bacterial [*M. bovis* (9)] infections. In
254 agreement, we found that hepatic $V\alpha 14/NKT$ cell activation in response to AdLacZ infection was followed by a
255 significant decline in the frequency of hepatic $V\alpha 14/NKT$ cells. On the contrary, the absolute number of hepatic
256 $V\alpha 14/NKT$ cells was significantly increased by AdLacZ infection at day 6 but not at day 1. Despite this, we
257 next determined the reason(s) underlying this decline in the frequency of hepatic $V\alpha 14/NKT$ cells by analyzing
258 changes in the expression of selected markers of apoptosis. Our data revealed that AdLacZ infection
259 significantly upregulated the frequency and absolute numbers of both extracellular Fas and intracellular active
260 caspase 3 positive hepatic $V\alpha 14/NKT$ cells, suggesting that a significant proportion of intrahepatic $V\alpha 14/NKT$
261 cells undergo apoptotic death after activation during AdLacZ infection. The discrepancy in the profile of
262 hepatic $V\alpha 14/NKT$ cell frequency versus absolute number during AdLacZ infection may indicate that resident
263 hepatic $V\alpha 14/NKT$ cells are susceptible to apoptotic death during the early phase (i.e. day 1) of AdLacZ
264 infection whereas infiltrating hepatic $V\alpha 14/NKT$ cells become susceptible to apoptotic death at day 6 (i.e. late
265 phase) of AdLacZ infection. Evidence in favor of this speculation derives from our observation that the
266 frequency (but not absolute number) of Fas-positive hepatic $V\alpha 14/NKT$ cells and active caspase 3-positive
267 $V\alpha 14/NKT$ cells was significantly elevated as early as day 1 of AdLacZ infection. Interestingly, the absolute
268 number of Fas-positive $V\alpha 14/NKT$ cells and active caspase 3-positive $V\alpha 14/NKT$ cells was only significantly
269 increased at day 6 post-AdLacZ infection. Collectively, our results show that the Fas/FasL pathway is an
270 important pathway through which apoptotic death of activated intrahepatic $V\alpha 14/NKT$ cells may occur during
271 AdLacZ infection and our finding is in agreement with a recent observation in the *M. bovis* infection model (9).
272 Moreover, it is clear from our data that Fas expressing activated $V\alpha 14/NKT$ cells could potentially contribute to
273 acute liver toxicity during AdLacZ infection since we found that activation of Fas expressing intrahepatic
274 $V\alpha 14/NKT$ cells using agonistic Fas mAb induces hepatocyte death.

275 To assess the functional consequences of intrahepatic V α 14/NKT cell activation on liver pathology,
276 J α 18^{-/-} mice which specifically lack V α 14/NKT cells were used (10, 12). Activated V α 14/NKT cells played a
277 central role in promoting the development of liver injury since J α 18^{-/-} mice exhibited significantly lower ALT
278 levels relative to WT mice following AdLacZ infection, a finding that was also confirmed histologically.
279 Additionally, we provided evidence that V α 14/NKT cell deficiency suppressed hepatic accumulation of innate
280 and adaptive lymphoid cells [i.e. CD8(+) T cells, NK cells and γ δ T cells]. In view of the fact that CD8(+) T cells
281 (41), γ δ T cells (2) and NK cells (23) independently contributes to liver damage in AdLacZ infected mice, our
282 findings raises the possibility that activated V α 14/NKT cells can initiate liver toxicity during AdLacZ infection by
283 mechanisms that influence accumulation of CD8(+) T cells, NK cells and γ δ T cells. Furthermore, our results
284 indicate that activated V α 14/NKT cells could “bridge/regulate” the innate and adaptive immune responses
285 during AdLacZ infection since hepatic V α 14/NKT cell activation and subsequent contraction during AdLacZ
286 infection in WT mice occurred prior to significant accumulation of adaptive [CD8(+) T cells, see Fig. 1E] and
287 innate [γ δ T cells (2) and NK cells (23)] immune cells in the liver. In addition to hepatotoxicity, Adenovirus
288 transgene loss remains a big hurdle to the broad clinical application of replication-defective Adenovirus vectors
289 in gene therapy. β -galactosidase activity is a biochemical marker widely used to evaluate AdLacZ transgene
290 expression in the liver of mice (2, 23, 28, 41). Our preliminary data have shown that V α 14/NKT cell deficiency
291 results in reduced hepatic β -galactosidase activity (M.N. Ajuebor, unpublished results). Several questions
292 remain regarding why V α 14/NKT cell deficiency is associated with decreased liver pathology and increased
293 Adenovirus transgene loss (i.e. reduced β -galactosidase activity) and ongoing studies will elucidate the specific
294 molecular pathways involved in these mechanisms.

295 Activated V α 14/NKT cells exert multiple effects during immune responses by inducing inflammatory
296 mediators including cytokines [such as IFN- γ and IL-4] (1), chemokines [RANTES/CCL5, MCP-1/CCL2 and
297 MIP-1 α /CCL3] (1, 2, 35) and cytotoxic proteins [Fas/FasL] (25). Indeed, our results clearly showed that
298 activated V α 14/NKT cells are capable of inducing CCL5 during AdLacZ infection since CCL5 production in the
299 liver was significantly suppressed by V α 14/NKT cell deficiency. However, we cannot completely rule out the
300 possibility that other hepatic cell types may also mediate CCL5 production during AdLacZ infection since
301 hepatic CCL5 production was not completely inhibited by V α 14/NKT cell deficiency. In contrast to the
302 documented role of activated V α 14/NKT cells at inducing CCL2 during immune responses (27), hepatic CCL2
303 production in response to AdLacZ infection was not suppressed by V α 14/NKT cell deficiency. Interestingly,
304 hepatic CCL4 production (but not CCL3) was also inhibited by V α 14/NKT cell deficiency during AdLacZ
305 infection. However, we believe that CCL3 and CCL4 production in the liver during AdLacZ infection are mostly
306 likely CCL5 dependent since these chemokines were significantly attenuated by CCL5 deficiency. Therefore,
307 we speculate that CCL3 production in the liver in response to AdLacZ infection is mediated by CCL5 derived
308 from hepatic cells other than V α 14/NKT cells since V α 14/NKT cell deficiency did not suppress hepatic CCL3
309 production. Overall, we have provided new and important information that chemokine production by activated

310 V α 14/NKT cells during AdLacZ infection was not a generalized response since only hepatic CCL5 production
311 was directly suppressed by V α 14/NKT cell deficiency.

312 For this reason, we next evaluated the potential contribution of CCL5 to the pro-inflammatory effects of
313 activated hepatic V α 14/NKT cells during AdLacZ infection. We showed that CCL5 promotes the development
314 of liver pathology during AdLacZ infection since CCL5 deficiency induced by CCL5 deficient mice or CCL5
315 antibody treatment caused a marked reduction in serum ALT levels and reduced hepatic histological injury.
316 Moreover, blockade of the CCL5 receptors using Met-RANTES, a CCR1/CCR5 antagonist, also alleviated liver
317 pathology associated with AdLacZ infection as shown by lower ALT levels and diminished histological injury.
318 Interestingly, immune cells [CD8(+) T cells, $\gamma\delta$ T cells and NK cells] in the liver were not inhibited by CCL5
319 deficiency or CCR1/CCR5 blockade (data not shown), suggesting that these immune cells may not contribute
320 to the pro-inflammatory effects of CCL5. In view of the fact that CCR1 (40) and CCR5 (37) are upregulated on
321 hepatocytes during inflammation, we propose that CCL5 may mediate direct cytotoxic effects on hepatocytes
322 during AdLacZ infection via interaction with cognate receptors. In summary, these novel studies provide
323 definite evidence of a crucial role for CCL5 derived from activated hepatic V α 14/NKT cells, acting in concert
324 with cognate receptors (i.e. CCR1/CCR5), to cause liver pathology during AdLacZ infection.

325 IFN- γ , a major cytokine produced by activated immune cells [including V α 14/NKT cells (Fig. 1A), $\gamma\delta$ T
326 cells (2) and NK cells (4)], is capable of promoting acute liver damage in experimental models of autoimmune,
327 viral (adenovirus) and toxin liver injuries (2, 18, 22), possibly by directly exerting a cytotoxic effect on
328 hepatocytes which express the IFN- γ receptor on their surface (6). We validated published reports that IFN- γ
329 is capable of mediating CCL5 production during inflammatory responses (19, 20), by demonstrating that CCL5
330 production in the liver during AdLacZ infection was markedly inhibited by IFN- γ deficiency and also by IFN- γ -
331 producing $\gamma\delta$ T cells. Next, we evaluated the effect of V α 14/NKT cell deficiency on IFN- γ production by CD8(+)
332 T cells and $\gamma\delta$ T cells. As expected, V α 14/NKT cell deficiency caused reduced IFN- γ production by $\gamma\delta$ T cells
333 and CD8(+) T cells post AdLacZ infection. Taken together, we propose that hepatic CCL5 production by IFN- γ -
334 producing $\gamma\delta$ T cells and CD8(+) T cells during AdLacZ infection is predominantly driven/initiated by activated
335 hepatic V α 14/NKT cells. A potential mediator of hepatic V α 14/NKT cell activation is reactive oxygen species
336 (ROS) since T cell activation has been associated with increased levels of endogenous ROS (16).
337 Furthermore, we recently reported that treatment of mice with the ROS scavenger, N-acetylcysteine (NAC),
338 dampened hepatic V α 14/NKT cell activation in response to polyriboinosinic:polycytidylic acid treatment (12).
339 Interestingly, we have preliminary evidence that treatment of mice with NAC alleviates liver toxicity associated
340 with AdLacZ infection (M.N. Ajuebor, unpublished observations), consistent with a role for endogenous ROS.
341 In future studies, we plan to specifically evaluate the functional importance of endogenous ROS in regulating
342 hepatic V α 14/NKT cell activation and function during AdLacZ infection.

343 As highlighted in introduction, V α 14/NKT cells may be activated in a TCR-dependent manner by lipids
344 presented by CD1d (25) or by TCR independent mechanisms involving TLRs (12, 25, 30, 36). Specifically,
345 V α 14/NKT cell activation (in the absence of foreign lipid antigen for their TCR) could be mediated by IL-12

346 and/or IL-18 secreted by TLR (4,7,8,9) activated APCs in response to bacterial and viral infections (12, 25, 30,
347 36). In addition, we recently proposed an important role for TLR3, a viral sensor, in promoting V α 14/NKT cell
348 activation in response to the specific TLR3 ligand, poly I:C (12). Although beyond the scope of the current
349 study, it is conceivable that IL-12 and/or IL-18 secreted by TLR expressing APCs are capable of inducing
350 hepatic V α 14/NKT cell activation during viral infection in response to the non enveloped DNA virus, AdLacZ.
351 This is a fertile area that will be investigated in a future study. In summary, our study highlighted the functional
352 relevance of activated hepatic V α 14/NKT cells in promoting the development of liver toxicity during replication-
353 Adenovirus infection by regulating innate and adaptive immune responses. Furthermore, we have provided
354 definite evidence that the effector functions of activated hepatic V α 14/NKT cells at initiating acute liver toxicity
355 during AdLacZ infection is largely driven by IFN- γ -dependent CCL5.

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363 University, Atlanta).

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477 **Figure Legends**478 **Figure 1: Kinetics of hepatic V α 14/NKT cell activation and decline/loss during AdLacZ infection.**

479 Hepatic V α 14/NKT cells were isolated from C57BL6 mice infected with AdLacZ and then identified by flow
 480 cytometry as described in materials and method. Uninfected mice (i.e. day 0) served as control. (a) tetramer-
 481 positive hepatic V α 14/NKT cells were stained with fluorochrome-labeled IFN- γ mAb for the determination of
 482 intracellular V α 14/NKT cell IFN- γ production. (b) depicts the frequency of hepatic V α 14/NKT cells before and
 483 after AdLacZ infection. (c) shows representative FACS dot plot of hepatic V α 14/NKT cell decline/loss during
 484 AdLacZ infection. (d) depicts the absolute of number of V α 14/NKT cell accumulation in the liver before and
 485 after AdLacZ infection. (e) CD8(+)T cell accumulation in the liver of C57BL6 mice before and after AdLacZ
 486 infection. Data from figures a, b, d and e are shown as mean \pm SEM with $n=4-6$ mice per group, from two
 487 separate experiments. * $P\leq 0.05$ vs. day 0.

488 **Figure 2: Time course of hepatic V α 14/NKT cell apoptosis during AdLacZ infection.** C57BL6 mice were
 489 infected with AdLacZ at indicated time-points whereas uninfected mice served as control. (a-b) isolated
 490 tetramer-positive hepatic V α 14/NKT cells were stained with fluorochrome-labeled Fas mAb for evaluation of
 491 the frequency and absolute number of extracellular Fas V α 14/NKT cell expression in the liver. Results from
 492 figures a and b are presented as mean \pm SEM with $n=4-7$ mice/group from two independent experiments.
 493 * $P\leq 0.05$ vs. day 0. (c-e) isolated tetramer-positive hepatic V α 14/NKT cells were stained with fluorochrome-
 494 labeled active caspase mAb for evaluation of the frequency (c and d) and absolute number (e) of intracellular
 495 active caspase 3 positive hepatic V α 14/NKT cells. A representative FACS histogram depicting active caspase
 496 3 positive expression by hepatic V α 14/NKT cells is shown in (d). Results from figures c and e are presented
 497 as mean \pm SEM with $n=4-5$ mice per group from two independent experiments. * $P\leq 0.05$ vs. day 0. .

498 **Figure 3: Effects of V α 14/NKT cell deficiency on acute liver inflammation and injury in AdLacZ-infected**

499 **mice.** WT and J α 18^{-/-} mice were infected with AdLacZ whereas mock-infected WT and J α 18^{-/-} mice received
 500 vehicle. Serum ALT levels and liver histology were analyzed 6 days later. (a) Serum samples were obtained
 501 for the determination of ALT levels (IU/L). All results are presented as mean \pm SEM; $n= 6$ mice per group; * $P\leq$
 502 0.05 vs. all mock-infected controls; # $P\leq 0.05$ vs. AdLacZ-infected WT mice. (b) Representative
 503 photomicrograph of H & E-stained representative liver sections derived from WT mice and J α 18^{-/-} mice at day 6
 504 post AdLacZ infection (Original magnification– X200). The degree of liver inflammation and hepatocyte
 505 damage was graded as mild, moderate or severe as described in materials and methods. Specifically, severe
 506 liver inflammation and hepatocyte damage was observed in liver sections obtained from AdLacZ-infected WT
 507 mice whereas only mild liver inflammation and hepatocyte damage was observed in liver sections obtained
 508 from AdLacZ-infected J α 18^{-/-} mice. (c-d) inflammatory cell infiltrates in the liver of WT and J α 18^{-/-} mice were
 509 evaluated post-AdLacZ infection. Specifically, isolated hepatic inflammatory cell infiltrates were stained in
 510 trypan blue to determine cell viability and then viable cells were counted using a hemocytometer. Hepatic NK
 511 cells, $\gamma\delta$ T cells and CD8(+)T cells were identified by flow cytometry (as described in materials and methods).
 512 The number of NK cells, $\gamma\delta$ T cells and CD8(+)T cells observed in the liver of naïve WT mice ($n=4$ mice; figure

513 3d) were 0.59 ± 0.14 ($\times 10^5$), 0.05 ± 0.01 ($\times 10^5$) and 0.26 ± 0.03 ($\times 10^5$), respectively. All data are presented
514 as mean \pm SEM of $n=4-8$ mice/group; # $P \leq 0.05$ vs. infected WT mice.

515 **Figure 4: Effects of $V\alpha 14/NKT$ cell deficiency on hepatic chemokine production during AdLacZ**
516 **infection.** (a-c) C57BL6 WT mice were infected with AdLacZ over several time points for the determination of
517 hepatic chemokine production by Bioplex assay. Results are shown as mean \pm SEM of $n=4-8$ mice per group;
518 * $P \leq 0.05$ vs. day 0 (i.e. uninfected group). (d) WT and $J\alpha 18^{-/-}$ mice were infected with AdLacZ for 6 days for
519 measurement of depicted chemokines (CCL2, CCL3, CCL4 and CCL5) in the liver. All results are presented
520 as mean \pm SEM of 4-8 mice per group; # $P \leq 0.05$ vs. infected WT mice.

521 **Figure 5: Effect of CCL5-CCR5 blockade on hepatic inflammation and injury during AdLacZ infection.**
522 C57BL6 mice were pre-treated with CCL5 Ab or Met-RANTES (as described in materials and methods) and
523 then infected with AdLacZ for 6 days. In addition, CCL5 KO mice (on C57BL6 background) and corresponding
524 WT mice were infected with AdLacZ for 6 days. The following parameters were then determined; (a) serum
525 ALT levels with data presented as mean \pm SEM of $n=5-11$ mice/group; * $P \leq 0.05$ vs. control. (b) **histological**
526 **liver injury (Original magnification– X200).** As expected, liver inflammation and hepatocyte damage in liver
527 sections obtained from AdLacZ-infected WT mice was severe. In contrast, moderate liver inflammation and
528 hepatocyte damage was observed in liver sections obtained from CCL5 Ab-treated mice and CCL5 deficient
529 mice during AdLacZ infection. In addition, we also observed that liver inflammation and hepatocyte damage in
530 the presence of Met-RANTES treatment was mild.

531 **Figure 6: Contribution of $V\alpha 14/NKT$ cells to hepatic $\gamma\delta T$ and CD8(+) T cell activation.** (a) C57BL6 WT
532 mice, IFN- γ KO mice and $\gamma\delta T$ KO mice were all infected with AdLacZ and hepatic CCL5 production was
533 determined 6 days later. Data is presented as mean \pm SEM of 5-6 mice/group; * $P \leq 0.05$ vs. WT mice. (b-c)
534 WT and $J\alpha 18^{-/-}$ mice (which are $V\alpha 14/NKT$ cell deficient) were infected with AdLacZ for 1 or 6 days for the
535 determination of intracellular IFN- γ production by infiltrating hepatic $\gamma\delta T$ cells and CD8(+) T cells. Dotted line
536 denotes numbers of cells in the liver of in uninfected WT mice. All results are shown as mean \pm SEM of 5-6
537 mice per group; * $P \leq 0.05$ vs. WT mice.

538

539 **FIGURE 1**

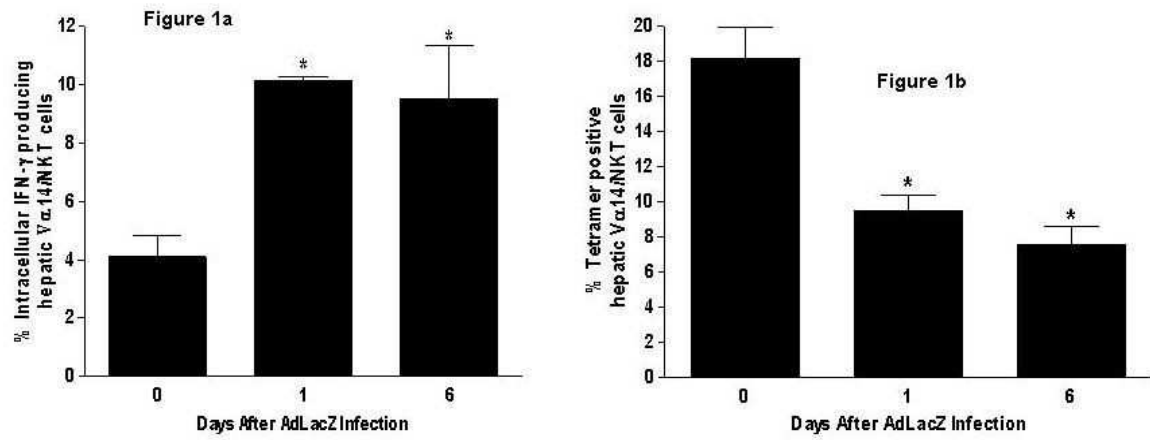
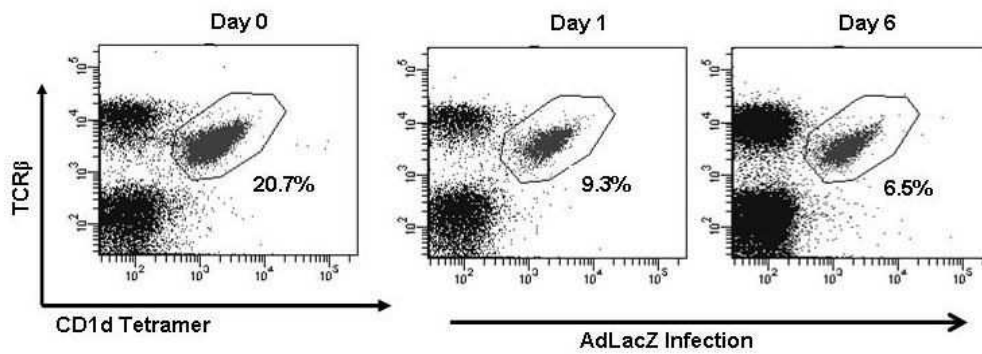
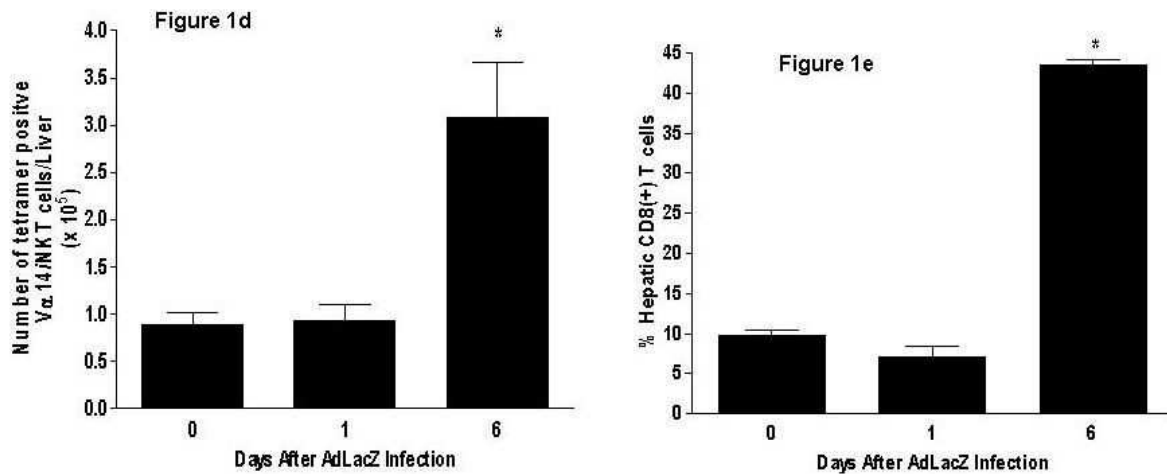


Figure 1c

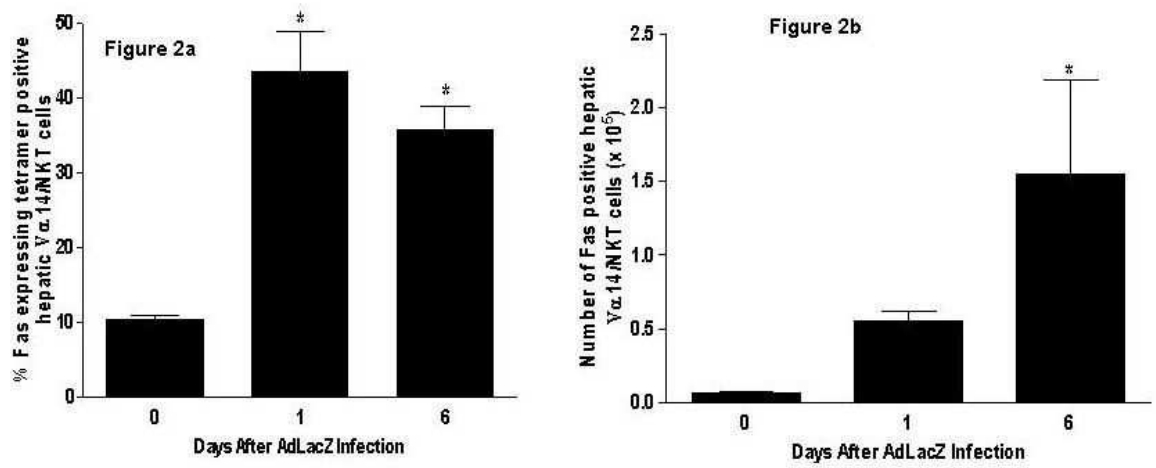


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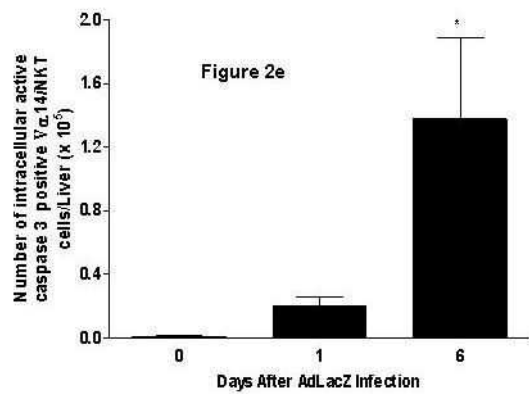
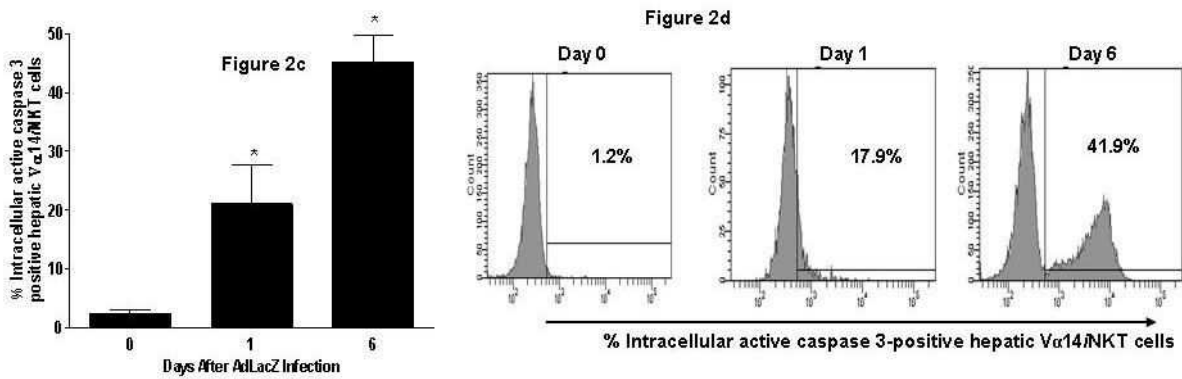
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542 **FIGURE 2**



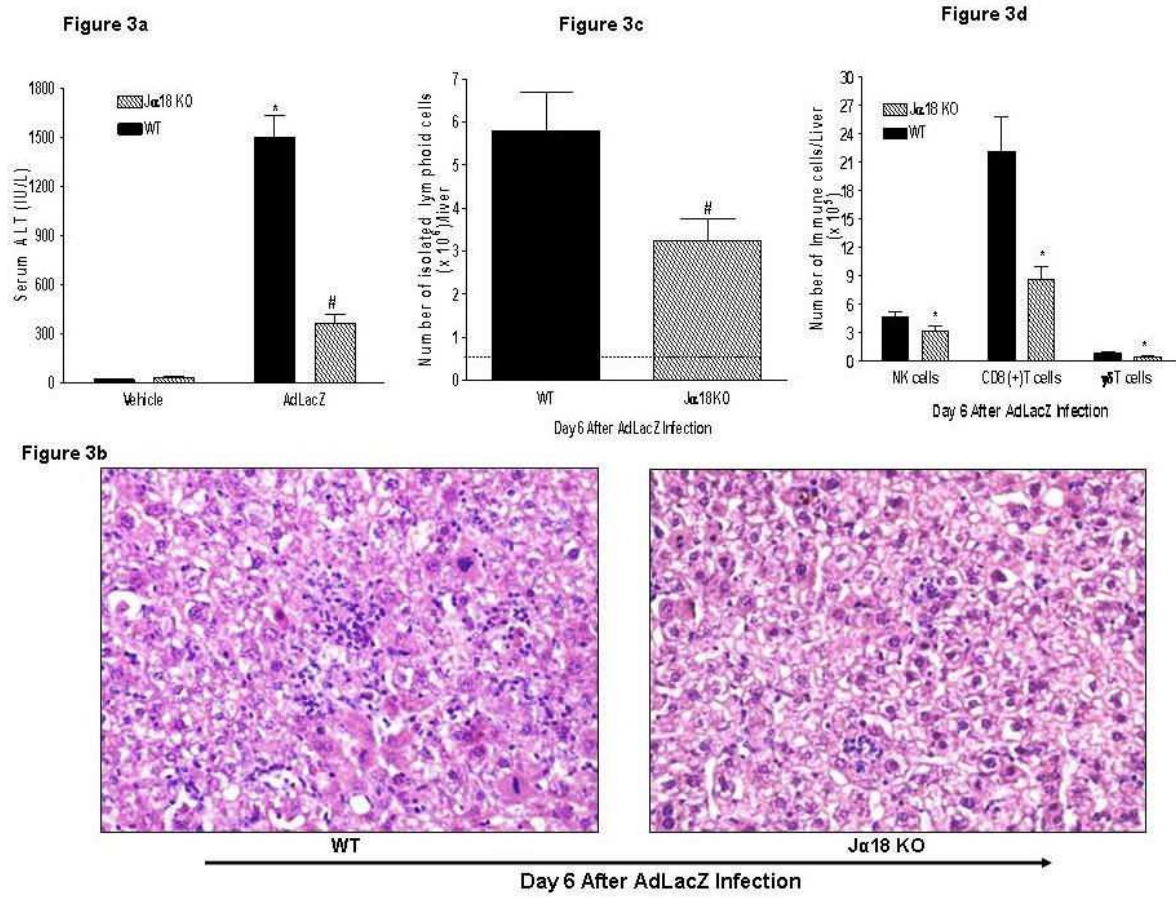
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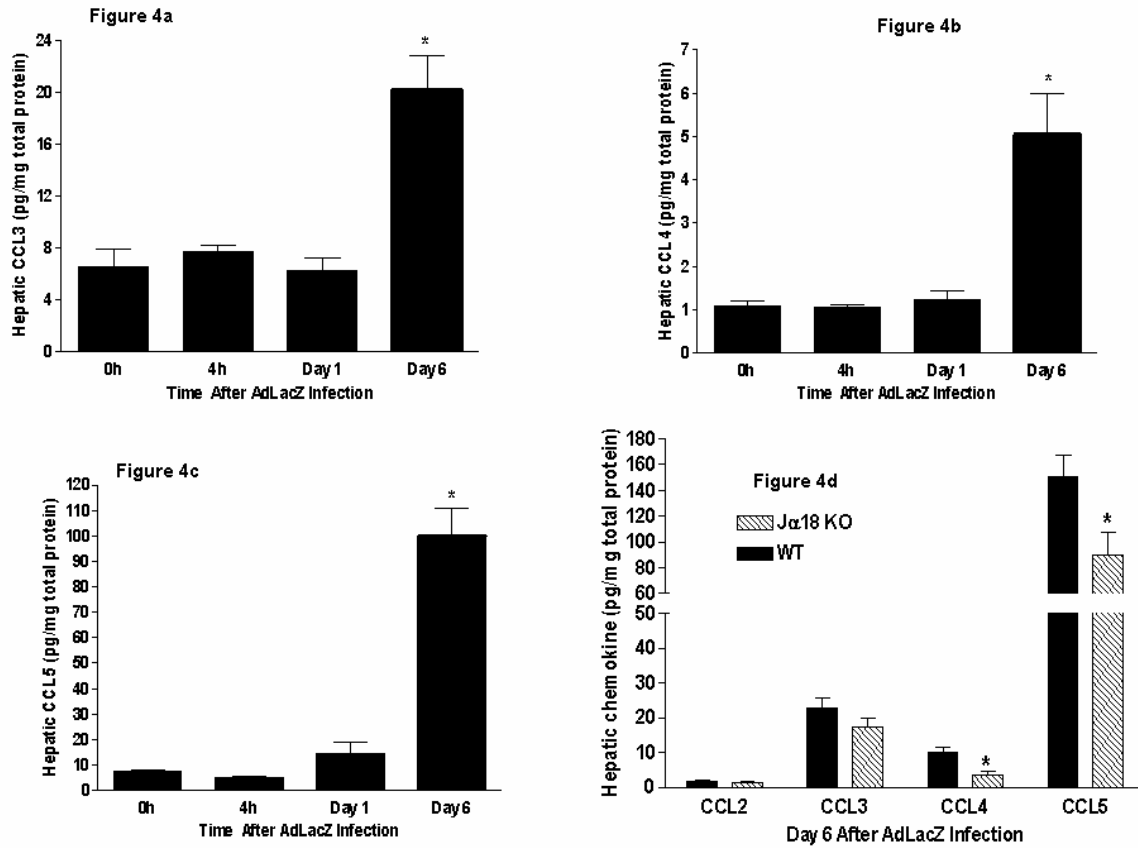
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546 **FIGURE 3**



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550 **FIGURE 4**



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554 **FIGURE 5**

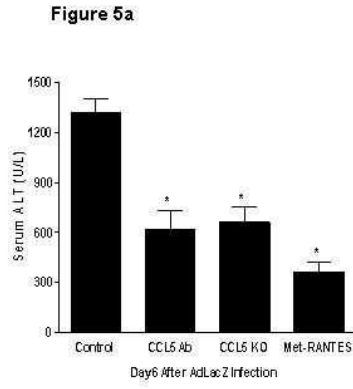
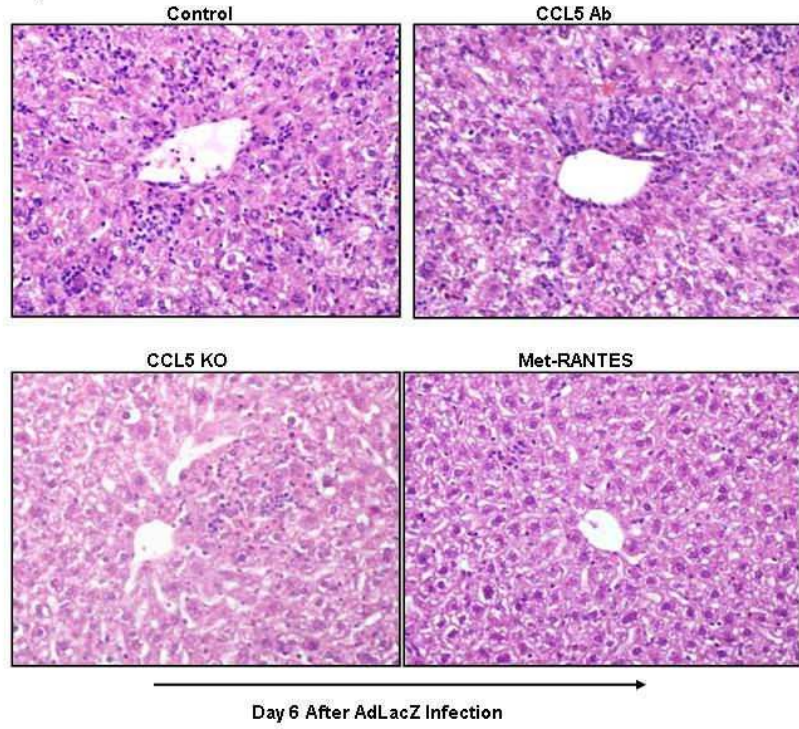


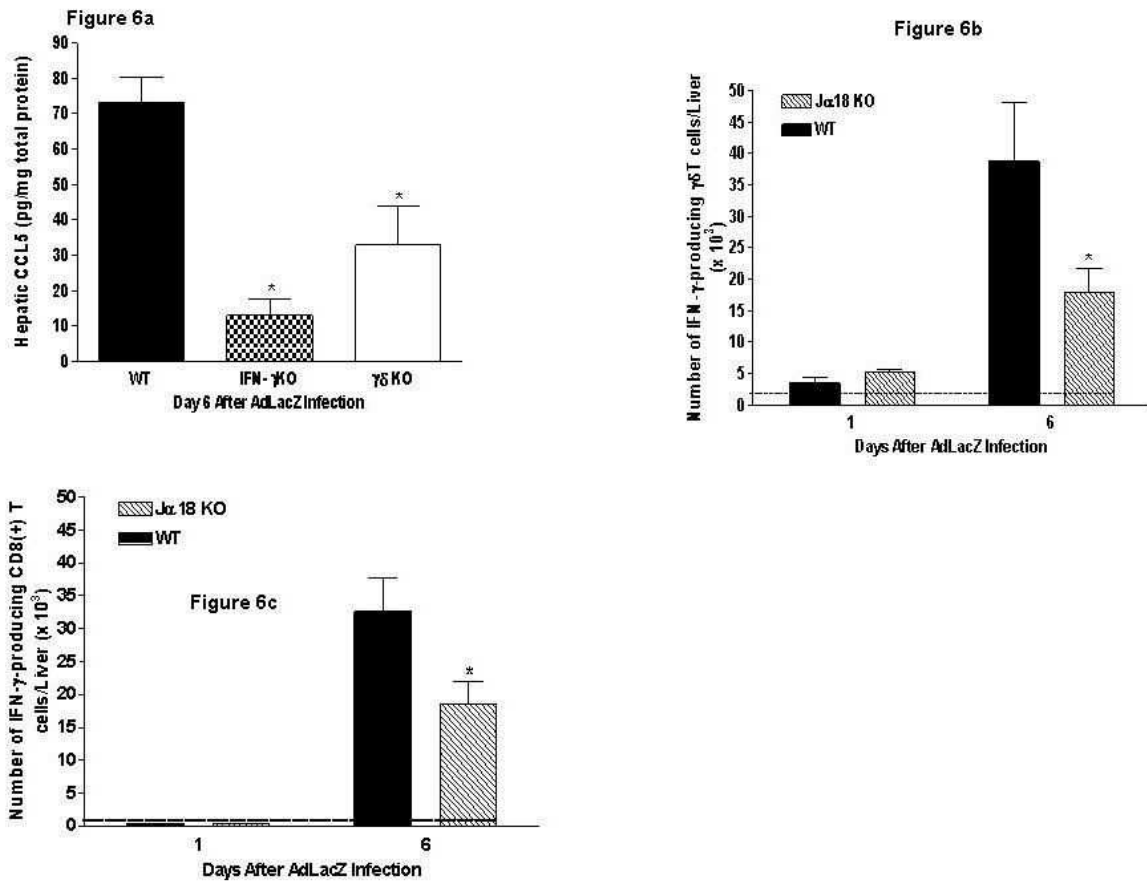
Figure 5b



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557 **FIGURE 6**



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560 **TABLE I**

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562 **Table I: Effects of CCL5 deficiency on hepatic chemokine production**

AdLacZ Infection	CCL3 (pg/mg total protein)	CCL4 (pg/mg total protein)
WT mice	17.31 ± 3.35	7.42 ± 1.06
CCL5 KO mice	3.35 ± 0.36*	2.71 ± 0.22*

563 WT and CCL5 KO mice were infected with AdLacZ for 6 days. Perfused livers were processed for the
 564 determination of chemokine protein levels using the Bioplex Assay. Data is presented as mean ± SEM with 6-
 565 9 mice per group; *p≤0.05 versus WT.

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