TNF deficiency dysregulates inflammatory cytokine production leading to lung pathology and death during respiratory poxvirus infection

Ma. Junaliah Tuazon Kels[1#], Esther Ng[1#], Zahrah Al Rumaih[1], Pratikshya Pandey[2], Sigrid R. Ruuls[3], Heinrich Korner[4], Timothy P. Newsome[5], Geeta Chaudhri[1,6##] and Gunasegaran Karupiah [1,2,7##]*

[1]Infection and Immunity Group, Department of Immunology, The John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia

[2]Viral Immunology and Immunopathology Group, Tasmanian School of Medicine, University of Tasmania, Hobart, TAS 7001, Australia

[3]Genmab, Uppsalalaan 15, 3584 CT Utrecht, The Netherlands

[4]Menzies Institute for Medical Research, University of Tasmania, Hobart, TAS 7000, Australia

[5]School of Life and Environmental Sciences, University of Sydney, Sydney, New South Wales 2006, Australia

[6]Research School of Population Health, The Australian National University, Canberra, ACT 2601, Australia

[7]Lead contact

[#] Authors contributed equally

[##] Equal senior authors

*Correspondence: Guna.Karupiah@utas.edu.au

20   **Abstract**

21   Excessive tumor necrosis factor (TNF) is known to cause significant pathology.

22   Paradoxically, deficiency in TNF (TNF$^{-/-}$) also caused significant pathology during

23   respiratory ectromelia virus (ECTV) infection, a surrogate mouse model for smallpox. TNF$^{-/-}$

24   mice succumbed to fulminant disease whereas wild-type mice, and those expressing only

25   transmembrane TNF, recovered. TNF deficiency did not affect viral load or leukocyte

26   recruitment but caused severe lung pathology and excessive production of the cytokines IL-

27   6, IL-10, TGF-β, and IFN-γ. Blockade of these cytokines reduced lung pathology

28   concomitant with induction of protein inhibitor of activated STAT3 (PIAS3) and/or

29   suppressor of cytokine signaling 3 (SOCS3), factors that inhibit STAT3 activation. Short-

30   term inhibition of STAT3 activation in ECTV-infected TNF$^{-/-}$ mice with an inhibitor reduced

31   lung pathology. TNF is essential for regulating inflammation and its deficiency exacerbates

32   ECTV infection as a consequence of significant lung pathology caused by dysregulation of

33   inflammatory cytokine production, in part via overactivation of STAT3.

34

35   INTRODUCTION

36   Tumor necrosis factor (TNF) plays essential roles in normal physiology (Uysal et al., 1997),

37   maintenance of immune homeostasis and acute inflammation (Arnett et al., 2001; Korner et

38   al., 1997; Marino et al., 1997; Papathanasiou et al., 2015; Pasparakis et al., 1996), and anti-

39   microbial defense (Bean et al., 1999; Domm et al., 2008; Kalliolias and Ivashkiv, 2016;

40   Wilhelm et al., 2001). It has also been implicated in many of the detrimental effects of

41   chronic inflammation in autoimmune conditions (Korner et al., 1995) and during infection

42   (Belisle et al., 2010; Szretter et al., 2007; Tracey et al., 1987). The antimicrobial property of

43   TNF has been underscored in patients receiving anti-TNF therapy to treat TNF-mediated

44   inflammatory diseases, which has been shown to result in reactivation of some bacterial,

45   parasitic, fungal and a limited number of viral infections (Domm et al., 2008; Garcia-

46   Gonzalez et al., 2012; Kalliolias and Ivashkiv, 2016; Saunders and Britton, 2007;

47   Subramaniam et al., 2013).

48   TNF is produced following activation of the nuclear factor-kappa B (NF-κB) inflammatory

49   pathway, often in response to stress or infection. It is expressed as a transmembrane protein

50   (mTNF), which is then cleaved by metalloproteinase enzymes to produce the secreted form,

51   sTNF (Black et al., 1997; Moss et al., 1997). Both forms of TNF can mediate effector

52   functions, but the relative contribution of each during viral infection is not known. TNF is

53   known to contribute to an exaggerated immune response leading to host tissue destruction

54   and immunopathology during some viral infections (Damjanovic et al., 2011; Peper and Van

55   Campen, 1995).

56   Some members of the orthopoxvirus (OPV) family, like variola virus (agent of smallpox),

57   monkeypox virus, cowpox virus and ectromelia virus (ECTV) encode several host response

58   modifiers including tumor necrosis factor receptor (TNFR) homologs, suggesting an

3

59   important role for TNF in protective immunity (Alcami, 2003; Alejo et al., 2009; Herbein

60   and O'Brien, 2000; Loparev et al., 1998; Rahman and McFadden, 2006; Seet et al., 2003).

61   ECTV is a mouse pathogen that causes mousepox, a disease very similar to smallpox, and

62   one of the best small-animal models available for investigating the pathogenesis of OPV

63   infections. Indeed, there are at least two lines of evidence indicating that TNF plays a crucial

64   role in protection and recovery of mice from OPV infections. First, TNF production is

65   strongly associated with resistance to ECTV infection in mice (Chaudhri et al., 2004).

66   Resistant mice like the wild-type (WT) C57BL/6 strain produce high levels of TNF, which

67   is associated with potent inflammatory and immune responses whereas the susceptible

68   BALB/c strain produces little TNF, associated with weak inflammatory and immune

69   responses. Second, the ECTV encoded viral TNFR (vTNFR) homolog, termed cytokine

70   response modifier D (CrmD), is known to modulate the host cytokine response (Alejo et al.,

71   2018). Infection of the ECTV-susceptible BALB/c strain with a CrmD deletion mutant virus

72   augmented inflammation, natural killer (NK) cell and cytotoxic T lymphocyte (CTL)

73   activities, resulting in effective control of virus replication and survival from an otherwise

74   lethal infection.

75   Excessive TNF production, via NF-κB activation, can induce the production of other pro-

76   inflammatory cytokines, including interleukin 6 (IL-6), which in turn can activate signal

77   transducer and activator of transcription 3 (STAT3) (Zhong et al., 1994). Both NF-κB and

78   STAT3 signaling pathways are closely intertwined and regulate an overlapping group of

79   target genes including those associated with inflammation (Goldstein et al., 2017; Kasembeli

80   et al., 2018; Ma et al., 2017; Oeckinghaus et al., 2011). STAT3 activation occurs through

81   phosphorylation to form phosphorylated STAT3 (pSTAT3), and its activity is regulated

82   through dephosphorylation by phosphatases (Hillmer et al., 2016; O'Shea et al., 2015) or by

83   inhibitors of STAT3, namely protein inhibitor of activated STAT3 (PIAS3) (Chung et al.,

84   1997; Shuai and Liu, 2005; Yagil et al., 2010) and suppressor of cytokine signaling 3

85   (SOCS3) (Babon et al., 2014; Carow and Rottenberg, 2014).

86   Using the mousepox model, we show here that TNF has no antiviral effects in C57BL/6 mice

87   but plays a key role in regulating inflammatory cytokine production and resolution of lung

88   inflammation during a respiratory infection. C57BL/6 WT mice and mice expressing only

89   the non-cleavable transmembrane form of TNF ($mTNF^{\Delta/\Delta}$) recovered from ECTV infection,

90   whereas TNF-deficient ($TNF^{-/-}$) mice succumbed with uniform mortality. TNF deficiency

91   resulted in dysregulated production of IL-6, IL-10, transforming growth factor beta (TGF-

92   β), and interferon gamma (IFN-γ) and caused significant lung pathology in virus-infected

93   mice. Cytokine blockade with monoclonal antibodies (mAb) significantly reduced lung

94   pathology contemporaneous with increased levels of PIAS3 and/or SOCS3 expression,

95   suggesting that excessive cytokine production in $TNF^{-/-}$ mice might be due to dysregulated

96   STAT3 activation. Indeed, short-term treatment of ECTV-infected $TNF^{-/-}$ mice with mAb

97   against IL-6, IL-10 receptor (IL-10R), TGF-β, or IFN-γ or an inhibitor of STAT3 activation

98   significantly reduced lung pathology. Long-term treatment with anti-IL-6 or anti-TGF-β but

99   not anti-IL-10R or STAT3 inhibitor resulted in the recovery of mice and effective virus

100   control. Our findings indicate that during respiratory OPV infection, TNF deficiency results

101   in dysregulated cytokine production, in part due to overactivation of STAT3 signaling,

102   causing massive lung pathology and death.

103   **RESULTS**

104   **mTNF is induced rapidly in WT mice and TNF deficiency exacerbates respiratory**

105   **ECTV infection independent of viral load or cell-mediated immunity**

106   In WT mice infected with ECTV through the intranasal (i.n.) route, TNF mRNA was

107   detectable in lungs at day 3 post-infection (p.i.), with levels still relatively high at day 12

108   (Figure S1A). In lung homogenates of WT mice, sTNF was detectable from about day 7 p.i.

109   (Figure S1B) whereas mTNF was detectable earlier but levels of both forms were highest at

110   day 12 p.i. (Figure S1C).

111   WT, TNF$^{-/-}$ and mTNF$^{\Delta/\Delta}$ mice were next infected i.n. with 25 plaque forming units (PFU)

112   of ECTV and assessed for clinical scores, weight loss, survival, viral load, NK cell and virus-

113   specific CTL responses.

114   Evaluation of the clinical presentation using a scoring system (described in Table S1)

115   indicated that TNF$^{-/-}$ animals fared significantly worse than WT or mTNF$^{\Delta/\Delta}$ mice beginning

116   at day 8 p.i. (Figure 1A). Mild clinical manifestations of infection were evident in all three

117   strains beginning at days 5 and 6 p.i., but only TNF$^{-/-}$ mice showed postural changes and

118   inactivity from day 7. All 3 strains exhibited piloerection, lacrimation, and nasal discharge

119   from day 8 p.i., however, only TNF$^{-/-}$ mice started to succumb to the infection, some with

120   apparent dyspnea. All strains lost weight from day 7 p.i., however, WT and mTNF$^{\Delta/\Delta}$ animals

121   started to recover from day 12 p.i. (Figure 1B). One mTNF$^{\Delta/\Delta}$ mouse died at day 9 whereas

122   all TNF$^{-/-}$ mice succumbed between days 9 and 11 p.i. (Figure 1C). In some experiments,

123   TNF$^{-/-}$ mice survived beyond 12 days but eventually succumbed to disease whereas WT and

124   mTNF$^{\Delta/\Delta}$ mice fully recovered.

125   Susceptibility to ECTV infection has been associated with uncontrolled virus replication in

126   all strains of mice studied to date (Chaudhri et al., 2004; Karupiah et al., 1993). Although

127   TNF$^{-/-}$ mice were susceptible to ECTV, they did not have increased viral load in the lung

128   (Figure 1D), liver (Figure 1E) or spleen (Figure 1F) compared to titers in either WT or

129   mTNF$^{\Delta/\Delta}$ animals at days 3, 5, 7 or 9 p.i. Conversely, mice deficient in IFN-α/β receptor

130   (IFN-α/βR$^{-/-}$) or IFN-γ (IFN-$\gamma^{-/-}$), strains known to rapidly succumb to mousepox due to

131   uncontrolled virus replication (Panchanathan et al., 2005), had significantly higher viral load

6

132   in lungs compared to WT or TNF$^{-/-}$ mice (Figure 1G). We investigated whether CrmD, the

133   vTNFR homolog encoded by ECTV, affected virus replication using the Naval strain of WT

134   (ECTV^{WT}) and CrmD mutant (ECTV$^{\Delta CrmD}$) viruses (Alejo et al., 2018). WT and TNF$^{-/-}$ mice

135   infected with either of the viruses had comparable viral loads in lungs, indicating that vTNFR

136   did not affect viral replication (Figure 1H).

137   Since early activation of NK cells and CTL responses are critical for virus control and

138   recovery from mousepox (Chaudhri et al., 2004; Delano and Brownstein, 1995; Karupiah et

139   al., 1996; Parker et al., 2007), we investigated the effect of TNF deficiency on these

140   responses. NK cell responses in the lungs (Figures S2A and S2B) and spleens (Figures S2C

141   and S2D) were similar in WT, mTNF$^{\Delta/\Delta}$, and TNF$^{-/-}$ strains. Likewise, TNF deficiency did

142   not affect the antiviral CTL responses in the lungs (Figure S2E) or spleen (Figure S2F). Thus,

143   unlike IFN-γ and IFN-α/β, TNF neither possesses antiviral activity nor influences NK cell

144   or CTL responses in C57BL/6 mice infected with ECTV (Chaudhri et al., 2004).

145   To exclude the possibility that the increased susceptibility of TNF$^{-/-}$ mice might be related to

146   the dysregulation of organogenesis and spatial organization of lymphoid tissue known to

147   occur during development (Korner et al., 1997; Neumann et al., 1996; Pasparakis et al., 1996;

148   Pasparakis et al., 1997), we used neutralizing anti-TNF mAb to treat ECTV-infected WT

149   mice. Compared to the control mAb-treated animals, mice treated with anti-TNF mAb

150   exhibited significantly higher clinical scores (Figure S3A) and 50% of the animals

151   succumbed by day 9 p.i. (Figure S3B). The histopathological scores were also higher in anti-

152   TNF mAb-treated animals (Figure S3C) but there were no differences in viral load between

153   the groups (Figure S3D). Representative lung histology sections from naïve animals and

154   from ECTV-infected mice treated with either control or anti-TNF mAb are shown in Figure

155   S3E. These results are consistent with those in TNF$^{-/-}$ mice and further confirmed that TNF

156   is critical for recovery of mice from ECTV infection but that the cytokine has no antiviral

157   effects.

Exacerbation of lung pathology in the absence of of TNF

159   An examination of lung histological sections indicated that there were minimal changes in

160   the lungs of WT and mTNF$^{\Delta/\Delta}$ mice on days 3, 5, and 7 p.i., whereas edema was visible at

161   day 7 in TNF$^{-/-}$ animals (Figure S4A). By day 9 p.i., edema and leukocyte extravasation were

162   seen in histological sections of all strains. Resolution of lung pathology was evident in WT

163   and mTNF$^{\Delta/\Delta}$ mice by day 11, but pathology worsened in TNF$^{-/-}$ mice. As the clinical,

164   virological, immunological, and histological responses to ECTV infection were similar in

165   WT and mTNF$^{\Delta/\Delta}$ mice (Figure 1, and Figures S2 and S4A), we undertook further analysis

166   of the response to ECTV infection only in WT and TNF$^{-/-}$ mice.

167   Examination of the gross anatomy of uninfected and virus-infected lungs indicated TNF$^{-/-}$

168   lungs were more congested, boggier, physically larger, and contained more and bigger

169   necrotic lesions compared to WT lungs (Figure 2A). The lung wet-to-dry weight ratio

170   allowed assessment of fluid extravasation into the lung interstitial space, and our results

171   indicated that TNF$^{-/-}$ lungs had significantly higher levels of fluid on days 7, 9, and 11 p.i.

172   (Figure 2B). Protein leakage is an indicator of increased vascular permeability, and TNF$^{-/-}$

173   lungs had significantly higher levels of protein in bronchoalveolar lavage fluid (BALF) at

174   day 11 p.i. compared to the WT lungs (Figure 2C).

175   Detailed microscopic examination of lung sections (Figures 2D and 2E) revealed that lung

176   pathology was more severe in virus-infected TNF$^{-/-}$ mice. There was intra-alveolar and

177   perivascular edema in both strains but in the TNF$^{-/-}$ group, the fluid extravasated to almost

178   the entire lobe leading to alveolar septal wall collapse by day 9. By day 11 p.i., resolution of

179   pathology was evident in WT lungs, wherein edema in the parenchyma started to clear, and

180 restoration of the bronchial epithelial architecture was evident. In contrast, alveolar septal

181 wall damage and edema in TNF$^{-/-}$ lungs continued to increase from day 9 to day 11 p.i.,

182 parenchymal edema worsened, and the bronchial epithelia were completely denuded. The

183 histopathological differences between the infected lungs of both mouse strains were semi-

184 quantified using a scoring system (described in Table S2). TNF$^{-/-}$ lungs had significantly

185 higher scores than WT lungs on days 9 and 11 p.i. (Figure 2F). In WT mice, the histological

186 scores were lower at day 11 compared to day 9, consistent with recovery of this strain from

187 ECTV infection. TNF deficiency resulted in significant lung tissue damage and pathology,

188 suggesting that TNF plays a role in anti-inflammatory processes.

189 **TNF is produced by many cell types and its deficiency does not affect leukocyte**

190 **recruitment to the lungs but results in dysregulated inflammatory cytokine production**

191 Cell types responsible for TNF production within the infected lungs were identified by

192 immunohistochemistry (Figures 3A and 3B), and these were observed in the bronchial

193 epithelium and alveoli where the virus antigen was also present (Figure S4B). Although

194 morphologically identified leukocyte subsets were present in both strains of mice, as

195 expected TNF protein was not detected in TNF$^{-/-}$ lungs (Figure 3B). Lungs from WT mice

196 showed that TNF protein was produced in response to infection by bronchial epithelial cells,

197 and by multiple leukocyte subsets (Figure 3A). Flow cytometric analysis of digested lungs

198 indicated that the total number of leukocytes (CD45^{+}; Figure S5A) or individual leukocyte

199 subsets (Figure 3C) were similar in both TNF$^{-/-}$ and WT strains. Histological analysis of

200 TNF$^{-/-}$ lungs suggested that there was a confluence of leukocytes in specific areas at day 11

201 p.i. (Figure 2E), but the total number of leukocytes within the lungs had decreased compared

202 to day 9 p.i. (Figures S5A-S5C). The consolidation of leukocytes in infected TNF$^{-/-}$ mice

203 was therefore not related to an increase in their numbers *per se* but may have been due to

204   dysregulated cytokine production. We, therefore, measured levels of some specific

205   inflammatory and regulatory cytokines in uninfected and virus-infected lung tissue.

206   TNF protein was not detected in TNF$^{-/-}$ animals whereas it was detectable in WT mice at day

207   9 p.i., and levels increased substantially by day 12 p.i. (Figure 3D). This was also reflected

208   by TNF mRNA expression in WT animals (Figure S5D). Levels of IFN-γ, IL-6, IL-10, and

209   TGF-β (Figures 3E-H) were significantly higher in lungs of TNF$^{-/-}$ mice compared to WT

210   animals at day 12 p.i. The higher levels of cytokine protein observed in the lungs of infected

211   TNF$^{-/-}$ mice were also reflected by increased mRNA levels of IFN-γ, IL-6, IL-10, and TGF-

212   β (Figures S5E-H). In summary, ECTV infection of TNF$^{-/-}$ mice resulted in increased

213   production of a number of inflammatory and regulatory cytokines with the potential to cause

214   lung damage.

215   **Blockade of specific cytokine function dampens lung pathology**

216   To ascertain whether the increased production of cytokines identified in Figure 3E-H

217   contributed to lung pathology, we reasoned that it might be possible to ameliorate the

218   pathology by neutralizing or blockading the function of each cytokine with specific mAb *in*

219   *vivo*. We used mAb against IFN-γ, IL-6, IL-10R or TGF-β to block cytokine function at day

220   7 p.i., co-incident with histological evidence of pulmonary edema (Figure S4A), and

221   sacrificed mice at day 9. A single dose of mAb administered at this time also potentially

222   minimized neutralization of cytokines known to play important antiviral roles early during

223   the course of ECTV infection (Chaudhri et al., 2004; Ivashkiv, 2010; Karupiah et al., 1993;

224   O'Gorman et al., 2010; Parker et al., 2007).

225   Treatment with control mAb (rat IgG) had minimal impact on disease outcome and, as

226   expected, the lung pathology in WT mice (Figures 4A and 4B) was not as severe as in TNF^{-}

227   $^{/-}$ mice (Figures 4K and 4L). Blockade of IFN-γ, IL-6, IL-10 or TGF-β function clearly

228   reduced lung pathology and edema, including the amelioration of damage to the epithelial

229   lining of bronchioles in both WT (Figures 4C-J) and TNF$^{-/-}$ (Figures 4M-T) mice. Despite

230   some improvement in lung pathology in mAb-treated WT mice, the histopathological scores

231   were not significantly different from the control mAb-treated group (Figure 4U), possibly

232   because WT mice did not produce high levels of these inflammatory cytokines (Figure 3)

233   and the lung pathology was not as severe as in the TNF$^{-/-}$ mice. Except for anti-IL-6

234   treatment, which reduced viral titers, mAb treatment did not affect viral load in WT mice

235   (Figure 4V).

236   In contrast, in TNF$^{-/-}$ mice, treatment with mAb to IFN-γ, (Figures 4M and 4N), IL-6 (Figures

237   4O and 4P), IL-10R (Figures 4Q and 4R), or TGF-β (Figures 4S and 4T) significantly

238   reduced histopathological scores (Figure 4W). The improved lung pathology in anti-cytokine

239   mAb-treated mice was not associated with a reduction in viral titers (Figure 4X). Although

240   not a consistent finding, ECTV titers in TNF$^{-/-}$ mice in this experiment were lower than in

241   the corresponding WT groups, further indicating that the worsened lung pathology in the

242   mutant strain was not related to the viral load.

243   ***In vivo* neutralization of cytokine function increases pSTAT3, PIAS3, and SOCS3 levels**

244   **in the lungs of infected mice**

245   Neutralization of the function of four different cytokines, i.e. IFN-γ, IL-6, IL-10 or TGF-

246   β led to the convergent result of dampening lung pathology (Figure 4), raising the possibility

247   that their activities may regulate a common cytokine-signaling pathway. It is known that

248   phosphorylation of STAT3 may be induced by cytokines like IL-6 (Zhong et al., 1994), TGF-

249   β (McGeachy et al., 2007; Yamamoto et al., 2001), IL-10 (Niemand et al., 2003) and IFN-γ

11

250   (Qing and Stark, 2004). We reasoned that cytokine blockade may have reduced STAT3

251   signaling by decreasing levels of pSTAT3 expression or through the induction of PIAS3 or

252   SOCS3.

253   Western blot analysis revealed that levels of the inactive form of STAT3 were similar in

254   uninfected or infected mice regardless of mouse strain or mAb treatment (Figure 5A).

255   Minimal levels of pSTAT3 were detected in uninfected animals, and they increased by about

256   2-fold in WT mice and about 5-fold in TNF$^{-/-}$ mice following infection (Figures 5A and 5B).

257   In WT mice, pSTAT3 levels increased further by about 2-fold with anti-IL-6 or anti-TGF-β

258   treatment, and higher than 3-fold following treatment with anti-IFN-γ or anti-IL-10R

259   (Figures 5A and 5B). On the other hand, in TNF$^{-/-}$ mice, the increases in pSTAT3 levels

260   above control IgG-treated groups were marginal.

261   The basal levels of PIAS3 were about 3-fold lower in TNF$^{-/-}$ mice compared with WT

262   animals (Figures 5A and 5C). Infection augmented PIAS3 levels in both strains, with further

263   increases noted after blockade of cytokine function (Figures 5A and 5C). In TNF$^{-/-}$ mice, the

264   PIAS3 band density increased by the largest magnitude following blockade of IL-6, IL-10R,

265   and TGF-β. The increase in PIAS3 levels may have contributed to dampening of pSTAT3

266   activity and as a consequence, reduction in lung pathology.

267   Naïve animals did not express SOCS3, and this protein was detectable at only low levels

268   after infection of both WT and TNF$^{-/-}$ mice (Figures 5A and 5D). Only treatment with mAb

269   against IL-6 resulted in significant increases in levels of SOCS3 (Figures 5A and 5D). It is

270   likely that the combined actions of increased levels of PIAS3 and SOCS3 in the anti-IL-6-

271   treated mice contributed to dampening of STAT3 activation and to effective amelioration of

272   lung pathology and survival of mice.

Inhibition of STAT3 activation ameliorates lung pathology

The preceding data suggested that it might be possible to dampen lung pathology through inhibition of STAT3 activation. Treatment with S3I-201, a selective STAT3 inhibitor that blocks STAT3 phosphorylation and dimerization (Chung et al., 1997), 7 days after ECTV infection resulted in a significant reduction in clinical scores in WT (Figure 6A) and TNF$^{-/-}$ (Figure 6B) mice, and lung histopathological scores in both strains at day 9 p.i. (Figure 6C). However, the treatment did not have any effect on viral load in either strain (Figure 6D). Microscopically, STAT3 inhibitor treatment improved lung pathology in both WT (Figure 6E) and TNF$^{-/-}$ (Figure 6F) lungs, and the effects were similar to blockade of specific cytokines (Figure 4). These results indicate that dysregulated inflammatory cytokine production and generation of lung immunopathology in the absence of TNF function is associated, at least in part, with hyperactivation of STAT3.

Treatment with S3I-201 affected the levels of expression of a number of inflammatory cytokines. In WT animals, which are able to recover from infection without significant lung pathology, inhibitor treatment resulted in significant increases in the levels of mRNA for TNF (Figure 6G), IL-10 (Figure 6H), TGF-β (Figure 6I), IFN-γ (Figure 6J), IL-1α (Figure 6K), IL-1β (Figure 6L), and IL-12p40 (Figure 6M). In sharp contrast, in TNF$^{-/-}$ mice, which exhibit severe lung pathology and succumb to infection, treatment with the STAT3 inhibitor resulted in a significant reduction in the levels of some cytokine mRNA transcripts compared to the mock-treated group. A reduction in mRNA levels was observed for IL-10 (Figure 6H), IFN-γ (Figure 6J), and IL-12p40 (Figure 6M). Although levels of mRNA for IL-1β (Figure 6L) and IL-6 (Figure 6N) were also reduced compared to the mock-treated group, they were not statistically significant. Crucially, STAT3 inhibitor treatment reduced mRNA levels of a number of cytokines in TNF$^{-/-}$ mice down to those observed in mock-treated WT animals.

297   Therefore, the effect of STAT3 inhibition on the expression of mRNA transcripts for

298   inflammatory cytokines is dictated by the presence or absence of TNF.

299   **TNF$^{-/-}$ mice recover from ECTV infection after extended treatment with anti-IL-6 or**

300   **TGF-β**

301   Short-term treatment with anti-cytokine mAb or STAT3 inhibitor significantly reduced lung

302   pathology in TNF$^{-/-}$ animals. It was thus of interest to determine whether prolonged treatment

303   with these agents would allow TNF$^{-/-}$ mice to recover from an otherwise lethal infection.

304   ECTV-infected TNF$^{-/-}$ mice were treated with either a control mAb or mAb against IL-6, IL-

305   10R or TGF-β beginning at day 7 p.i., and every 2 days thereafter until day 20 p.i. A separate

306   ECTV-infected group was treated with the STAT3 inhibitor from day 7 p.i. and every day

307   thereafter until day 20 p.i. All animals were monitored for 22 days. For virus load and

308   histopathological scores, organs were collected from animals that were moribund (and

309   euthanized for ethical reasons) or found dead before day 22 p.i., and those that were

310   sacrificed on day 22. Therefore, statistical analysis was not performed for virus load and

311   histopathological scores.

312   Control mAb- and anti-IL-10R-treated mice exhibited 100% mortality by days 10-13 p.i.,

313   whereas animals treated with anti-IL-6 or anti-TGF-β survived the infection (Figure 7A).

314   Four of 5 animals treated with the STAT3 inhibitor died between days 11-18, and one animal

315   survived until the day of sacrifice. Extended treatment with anti-IL-10R did not improve

316   weight loss whereas all animals treated with anti-IL-6 or anti-TGF-β showed improvements

317   in body weights from day 13 (Figure 7B) and clinical scores (Figure 7C) within 1-2 days

318   after initiation of treatment. The one STAT3 inhibitor-treated mouse that survived gained

319   weight from day 19 p.i. (Figure 7B) in parallel with a reduction in clinical scores (Figure

320   7C). Viral load in lungs of control mAb-, anti-IL-10R- or STAT3 inhibitor-treated mice were

14

321   very high compared to titers in mice treated with anti-IL-6 or anti-TGF-β, which were close

322   to the limit of detection (Figure 7D). Viral load in the one mouse treated with the STAT3

323   inhibitor that survived was also below the limit of detection.

324   Histologically, only prolonged treatment with anti-IL-6 reduced lung pathology (Figure 7E).

325   All control mAb-, anti-TGF-β- or anti-IL-10R-treated mice and 4 of 5 STAT3 inhibitor-

326   treated mice had significant lung pathology as revealed by the histopathological scores

327   (Figure 7E). The finding with anti-TGF-β treatment was surprising as even with this level of

328   pathology, the viral load was close to the limit of detection, the clinical scores were close to

329   baseline and the animals regained their loss in body weights.

330   The results indicate that the immune dysregulation and pulmonary pathology in TNF$^{-/-}$ mice

331   following infection with ECTV were due to excessive production of some inflammatory and

332   regulatory cytokines and that blockade of those cytokines can protect mice from an otherwise

333   lethal infection.

334   **DISCUSSION**

335   TNF plays fundamental roles in homeostasis, acute inflammation and antimicrobial defense

336   but excessive or chronic production of the cytokine can cause serious pathology and

337   mortality. Paradoxically, our results have shown that TNF deficiency in mice also results in

338   significant pathology and death during mousepox, a surrogate model for smallpox. We also

339   established that mTNF alone was necessary and sufficient to protect against lung pathology

340   in ECTV infected mice. The immunopathology was not due to excessive leukocyte

341   infiltration into the lungs as numbers and phenotypes of leukocytes present in virus-infected

342   WT and TNF$^{-/-}$ groups were not different. The numbers and magnitude of the cytolytic

343   activities of two key cell populations, i.e., NK and CD8^{+} T cells, present in the lungs and

15

344 spleens of infected animals were not also affected in the absence of TNF. Both NK cell and

345 CD8^{+} T cell-mediated CTL responses are essential for early virus control and recovery of

346 mice from ECTV infection (Chaudhri et al., 2006; Chaudhri et al., 2004; Delano and

347 Brownstein, 1995; Fang et al., 2008; Fang and Sigal, 2005; Karupiah et al., 1996; Parker et

348 al., 2007) and TNF can influence proliferation and effector functions of CD8^{+} T cells

349 (Damjanovic et al., 2011; Peper and Van Campen, 1995; Scheurich et al., 1987). The absence

350 of any impact of TNF deficiency on NK cell and CTL responses is also consistent with a

351 lack of any effect on viral load since deficiencies in either effector cell type can significantly

352 augment viral load.

353 The antimicrobial activity of TNF is well-documented for bacterial, fungal and some viral

354 pathogens. In humans, treatment with TNF blocking agents can exacerbate some viral

355 infections (Murdaca et al., 2015; Vigano et al., 2012) but not others like influenza A (Shale

356 et al., 2010). In mice, TNF deficiency did not have any effects on influenza A virus titers

357 (Damjanovic et al., 2011; Peper and Van Campen, 1995), but exacerbated illness and

358 heightened lung immunopathology. Our results with the ECTV model are consistent with

359 findings in the influenza A virus model. The current study indicates that TNF, unlike IFN-

360 α/β and IFN-γ (Chaudhri et al., 2004; Karupiah et al., 1993; Panchanathan et al., 2005), has

361 no antiviral activity against ECTV in C57BL/6 mice but is necessary for regulation of

362 inflammation.

363 WT C57BL/6 mice produced high levels of TNF during the resolution stage (day 12 p.i.) of

364 ECTV infection. In the absence of TNF, levels of IFN-γ, IL-6, IL-10 and TGF-β were

365 significantly higher than in WT mice. Both IL-6 and IFN-γ are considered pro-inflammatory

366 cytokines with the capacity to cause tissue damage (Rincon, 2012; Schroder et al., 2004). On

367 the other hand, TGF-β and IL-10 are known to regulate the immune response but they can

16

368   also mediate pro-inflammatory activities (Sanjabi et al., 2009). IL-10 can regulate immunity

369   by limiting inflammation to prevent tissue damage but high levels of the cytokine can itself

370   cause chronic inflammation and immunopathology (Couper et al., 2008). The short-term

371   cytokine neutralization experiments provided direct evidence that the exaggerated lung

372   tissue destruction in the absence of TNF was due to dysregulated production of IFN-γ, IL-6,

373   IL-10 and TGF-β. The timing of cytokine neutralization *in vivo, i.e.* co-incident with

374   generation of pulmonary edema and lung pathology, was crucial as some of these cytokines

375   also play very important protective roles during the early stages of the infection. In particular,

376   IFN-γ and IL-6 are critical for ECTV control very early during the course of infection

377   (Chaudhri et al., 2004; Ivashkiv, 2010; Karupiah et al., 1993; O'Gorman et al., 2010). TNF

378   thus plays a critical role in the suppression of immunopathology at the resolution stage of

379   infection through regulation of inflammatory/ regulatory cytokine production.

380   The finding that dampening of IFN-γ, IL-6, IL-10 or TGF-β reduced lung pathology

381   suggested at least two, non-mutually exclusive, possibilities. First, IFN-γ, IL-6, IL-10 and

382   TGF-β can phosphorylate and activate STAT3 (McGeachy et al., 2007; Niemand et al.,

383   2003; Qing and Stark, 2004; Yamamoto et al., 2001; Zhong et al., 1994), potentially

384   involving a common cytokine-signaling pathway. Second, the different signaling pathways

385   activated by each of these cytokines can cross-regulate each other. For example, STAT3 and

386   NF-κB can regulate the expression of inflammatory genes in a cooperative manner

387   (Oeckinghaus et al., 2011). TNF can activate STAT3 by induction of IL-6 through NF-κB

388   activation (Zhong et al., 1994). However, TNF can also inhibit IL-6-mediated STAT3

389   activation in macrophages through the recruitment of SOCS3 (Bode et al., 2003). In our

390   present study, the anti-inflammatory role of TNF during ECTV infection was evidenced by

391   low levels of PIAS3 in the lungs of TNF$^{-/-}$ mice compared to the WT animals.

392   PIAS3 not only negatively regulates STAT3 but may also physically interact with p65

393   subunit of NF-κB, thereby inhibiting the latter's activity (Jang et al., 2004). PIAS3 is a small

394   ubiquitin-like modifier (SUMO) E3 ligase that belongs to a family of STAT signaling

395   regulators, and the main cellular inhibitor of STAT3 (Chung et al., 1997). Levels of PIAS3

396   expression are directly correlated with inhibition of STAT3 DNA-binding and

397   transcriptional activity (Dabir et al., 2009). Hence, the increased levels of pSTAT3 in the

398   anti-cytokine mAb-treated mice was likely inhibited by the correspondingly increased levels

399   of PIAS3. IL-6 blockade also induced high levels of SOCS3 and the combined actions of

400   PIAS3 and SOCS3 in this group of animals likely contributed to effective control of lung

401   pathology and viral load.

402   Dysregulated or prolonged activation of STAT3 can lead to severe pathologic outcomes and

403   disease (Grivennikov and Karin, 2008). Our data indicates that overactivation of the STAT3

404   signaling pathway, at least in part, contributed to the exacerbated immunopathology in TNF-

405   deficient mice and that lung pathology could be ameliorated by short-term inhibition of

406   STAT3 activation. Nonetheless, long-term treatment with the inhibitor was ineffective in

407   reducing lung pathology or overcoming morbidity and mortality. Such an outcome is likely

408   due to the possibility that some cytokines or factors produced through the STAT3 signaling

409   pathway may be directly or indirectly essential for down-regulating inflammation or

410   involved in lung tissue repair during the resolution phase of the infection. A number of

411   cytokine transcripts, including TGF-β, were down-regulated by STAT3 inhibitor treatment

412   in TNF$^{-/-}$ mice. TGF-β is critical for tissue repair and although long-term treatment of TNF^{-}

413    $^{/-}$ mice with anti-TGF-β protected ECTV-infected mice from death, the lung pathology was

414   not resolved fully at day 22 p.i. Additionally, prolonged anti-IL-10 treatment, unlike short-

415   term treatment, was not protective as this cytokine is also important for regulation of the

416   inflammatory response.

417   The outbred human population exhibited varying degrees of susceptibility to smallpox

418   (Fenner, 1988) and similarly, inbred strains of mice are either resistant or susceptible to

419   mousepox. ECTV-resistant mice like the C57BL/6 strain generate a strong inflammatory

420   response and produce high levels of IL-2, IFN-γ and TNF very early in infection (Chaudhri

421   et al., 2004). In these animals, a polarized type I cytokine response is not only closely

422   associated with induction of robust cell-mediated immunity but also generation of potent

423   antibody responses, both of which are critical for recovery from infection (Chaudhri et al.,

424   2006; Fang and Sigal, 2005). In contrast, ECTV-susceptible strains such as BALB/c mice

425   produce significantly lower levels of these cytokines, associated with very weak

426   inflammatory responses and cell-mediated immunity (Chaudhri et al., 2004). In the current

427   study, we have found that TNF deficiency in ECTV-resistant mice results in significant lung

428   pathology and death during a respiratory ECTV infection. The increased susceptibility of

429   mice was clearly not due to an increase in viral load. These results might appear to contradict

430   with a recent report that TNF plays an important antiviral role in the recovery of the ECTV-

431   susceptible BALB/c mice (Alejo et al., 2018). In that study, infection of the BALB/c strain

432   with the CrmD deletion mutant virus augmented inflammation and cell-mediated immunity,

433   resulting in effective virus control and complete recovery from an otherwise lethal infection.

434   There are at least two reasons for the observed differences in the outcomes of infection with

435   the the mutant virus in the two strains of mice and these are discussed below.

436   First, the potent immune response generated by C57BL/6 mice can largely overcome the

437   effects of the various host response modifiers that ECTV encodes, including CrmD.

438   C57BL/6 mice produce significantly higher levels of TNF than BALB/c mice in response to

439   WT ECTV infection (Chaudhri et al., 2004). Our prediction is that in C57BL/6 mice infected

440   with the CrmD deletion mutant virus, we would expect to see further increases in TNF levels.

441   That is indeed the case as C57BL/6 mice develop significant lung pathology due to excessive

19

442   TNF production, an overexuberant inflammatory response in the lung and succumb to

443   mousepox (see accompanying manuscript). Because BALB/c mice produce low levels of

444   TNF, CrmD is likely to have neutralized the cytokine, resulting in poor quality of the

445   inflammatory and cell-mediated immune responses (Alejo et al., 2018). Second, we used the

446   i.n. route of inoculation in our studies with C57BL/6 mice whereas Alcami's group (Alejo

447   et al., 2018) used the subcutaneous route. ECTV can be naturally transmitted via the

448   subcutaneous route through scratches or bites from infected animals as well as via the

449   respiratory route through aerosols. Both routes of virus transmission result in systemic

450   infection, but the i.n. route is more sensitive, with virus replicating to high titers in the lungs,

451   and makes the resistant C57BL/6 strain succumb to mousepox at low doses of virus.

452   Curiously, we did not see any significant differences in the susceptibility of WT and TNF$^{-/-}$

453   C57BL/6 mice inoculated with varying doses of ECTV through the subcutaneous route (data

454   not shown). That finding suggested that a requirement for TNF to protect against ECTV

455   infection in C57BL/6 mice, specifically to regulate the local inflammatory response, depends

456   on the route through which virus is transmitted to the host.

457   In summary, TNF has no direct antiviral activity against ECTV but is necessary to regulate

458   lung inflammation. Respiratory ECTV infection of TNF$^{-/-}$ mice caused lethal lung pathology

459   due to dysregulation and excessive production of some specific cytokines and overactivation

460   of STAT3. Short-term blockade of any of these cytokines with mAb or inhibition of STAT3

461   activation ameliorated lung pathology in ECTV-infected mice. Our data indicates that

462   targeting specific cytokines or cytokine signaling pathways to reduce or ameliorate lung

463   inflammation during a viral infection is possible but that the timing and duration of the

464   interventive measure will be critical.

465

Acknowledgements

Author contributions

Conceptualization, M.J.T.K., E.N, G.C. and G.K.; Methodology, M.J.T.K., E.N., Z.A.R., P.P., G.C. and G.K.; Investigation, M.J.T.K., E.N., Z.A.R., T.P.N.; Writing – Original Draft, M.J.T.K., G.C. and G.K.; Writing – Review & Editing, M.J.T.K., E.N, H.K., T.P.N., G.C. and G.K.; Funding Acquisition, G.C. and G.K.; Resources, S.R.R., H.K., G.C. and G.K.; Supervision, G.C. and G.K.

Declaration of Interests

All authors declare no conflict of interests.

REFERENCES

488   Alcami, A. (2003). Viral mimicry of cytokines, chemokines and their receptors. Nat Rev

490   Immunol. 3(1), 36-50.

491   Alejo, A., Ruiz-Arguello, M.B., Pontejo, S.M., Fernandez de Marco, M.D.M., Saraiva, M.,

492   Hernaez, B., and Alcami, A. (2018). Chemokines cooperate with TNF to provide protective

493   anti-viral immunity and to enhance inflammation. Nat Commun. 9(1), 1790.

494   Alejo, A., Saraiva, M., Ruiz-Arguello, M.B., Viejo-Borbolla, A., de Marco, M.F., Salguero,

495   F.J., and Alcami, A. (2009). A method for the generation of ectromelia virus (ECTV)

496   recombinants: in vivo analysis of ECTV vCD30 deletion mutants. PLoS One. 4(4), e5175.

497   Arnett, H.A., Mason, J., Marino, M., Suzuki, K., Matsushima, G.K., and Ting, J.P. (2001).

498   TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. Nat

499   Neurosci. 4(11), 1116-1122.

500   Babon, J.J., Varghese, L.N., and Nicola, N.A. (2014). Inhibition of IL-6 family cytokines by

501   SOCS3. Semin Immunol. 26(1), 13-19.

502   Bean, A.G., Roach, D.R., Briscoe, H., France, M.P., Korner, H., Sedgwick, J.D., and Britton,

503   W.J. (1999). Structural deficiencies in granuloma formation in TNF gene-targeted mice

504   underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection,

505   which is not compensated for by lymphotoxin. J Immunol. 162(6), 3504-3511.

506   Belisle, S.E., Tisoncik, J.R., Korth, M.J., Carter, V.S., Proll, S.C., Swayne, D.E., Pantin-

507   Jackwood, M., Tumpey, T.M., and Katze, M.G. (2010). Genomic profiling of tumor necrosis

508   factor alpha (TNF-alpha) receptor and interleukin-1 receptor knockout mice reveals a link

509 between TNF-alpha signaling and increased severity of 1918 pandemic influenza virus

510 infection. J Virol. 84(24), 12576-12588.

511 Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner,

512 B.J., Stocking, K.L., Reddy, P., Srinivasan, S., et al. (1997). A metalloproteinase disintegrin

513 that releases tumour-necrosis factor-α from cells. Nature. 385, 729.

514 Bode, J.G., Schweigart, J., Kehrmann, J., Ehlting, C., Schaper, F., Heinrich, P.C., and

515 Haussinger, D. (2003). TNF-alpha induces tyrosine phosphorylation and recruitment of the

516 Src homology protein-tyrosine phosphatase 2 to the gp130 signal-transducing subunit of the

517 IL-6 receptor complex. J Immunol. 171(1), 257-266.

518 Carow, B., and Rottenberg, M.E. (2014). SOCS3, a Major Regulator of Infection and

519 Inflammation. Front Immunol. 5, 58.

520 Chaudhri, G., Kaladimou, G., Pandey, P., and Karupiah, G. (2018). Propagation and

521 Purification of Ectromelia Virus. Curr Protoc Microbiol. 51(1), e65.

522 Chaudhri, G., Panchanathan, V., Bluethmann, H., and Karupiah, G. (2006). Obligatory

523 requirement for antibody in recovery from a primary poxvirus infection. J Virol. 80(13),

524 6339-6344.

525 Chaudhri, G., Panchanathan, V., Buller, R.M., van den Eertwegh, A.J., Claassen, E., Zhou,

526 J., de Chazal, R., Laman, J.D., and Karupiah, G. (2004). Polarized type 1 cytokine response

527 and cell-mediated immunity determine genetic resistance to mousepox. Proc Natl Acad Sci

528 U S A. 101(24), 9057-9062.

529 Chung, C.D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., and Shuai, K. (1997). Specific

530 Inhibition of Stat3 Signal Transduction by PIAS3. Science. 278(5344), 1803-1805.

531   Couper, K.N., Blount, D.G., and Riley, E.M. (2008). IL-10: the master regulator of immunity

532   to infection. J Immunol. 180(9), 5771-5777.

533   Dabir, S., Kluge, A., and Dowlati, A. (2009). The Association and Nuclear Translocation of

534   the PIAS3-STAT3 Complex Is Ligand and Time Dependent. Mol Cancer Res. 7(11), 1854-

535   1860.

536   Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A., and Stewart, T.A. (1993).

537   Multiple defects of immune cell function in mice with disrupted interferon-gamma genes.

538   Science. 259(5102), 1739-1742.

539   Damjanovic, D., Divangahi, M., Kugathasan, K., Small, C.L., Zganiacz, A., Brown, E.G.,

540   Hogaboam, C.M., Gauldie, J., and Xing, Z. (2011). Negative regulation of lung inflammation

541   and immunopathology by TNF-alpha during acute influenza infection. Am J Pathol. 179(6),

542   2963-2976.

543   Delano, M.L., and Brownstein, D.G. (1995). Innate resistance to lethal mousepox is

544   genetically linked to the NK gene complex on chromosome 6 and correlates with early

545   restriction of virus replication by cells with an NK phenotype. J Virol. 69(9), 5875-5877.

546   Domm, S., Cinatl, J., and Mrowietz, U. (2008). The impact of treatment with tumour necrosis

547   factor-alpha antagonists on the course of chronic viral infections: a review of the literature.

548   Br J Dermatol. 159(6), 1217-1228.

549   Fang, M., Lanier, L.L., and Sigal, L.J. (2008). A role for NKG2D in NK cell-mediated

550   resistance to poxvirus disease. PLoS Pathog. 4(2), e30.

551   Fang, M., and Sigal, L.J. (2005). Antibodies and CD8+ T cells are complementary and

552   essential for natural resistance to a highly lethal cytopathic virus. J Immunol. 175(10), 6829-

553   6836.

554   Fenner, F. (1988). Smallpox and its eradication (World Health Organization: Geneva).

555   Garcia-Gonzalez, E., Guidelli, G.M., Bardelli, M., and Maggio, R. (2012). Mucocutaneous

556   leishmaniasis in a patient treated with anti-TNF-α therapy. Rheumatology. 51(8), 1517-

557   1518.

558   Goldstein, I., Paakinaho, V., Baek, S., Sung, M.H., and Hager, G.L. (2017). Synergistic gene

559   expression during the acute phase response is characterized by transcription factor assisted

560   loading. Nat Commun. 8(1), 1849.

561   Grivennikov, S., and Karin, M. (2008). Autocrine IL-6 signaling: a key event in

562   tumorigenesis? Cancer Cell. 13(1), 7-9.

563   Herbein, G., and O'Brien, W.A. (2000). Tumor necrosis factor (TNF)-alpha and TNF

564   receptors in viral pathogenesis. Proc Soc Exp Biol Med. 223(3), 241-257.

565   Hillmer, E.J., Zhang, H., Li, H.S., and Watowich, S.S. (2016). STAT3 signaling in immunity.

566   Cytokine Growth Factor Rev. 31, 1-15.

567   Ivashkiv, L.B. (2010). STAT activation during viral infection in vivo: where's the interferon?

568   Cell Host Microbe. 8(2), 132-135.

569   Jang, H.D., Yoon, K., Shin, Y.J., Kim, J., and Lee, S.Y. (2004). PIAS3 suppresses NF-

570   kappaB-mediated transcription by interacting with the p65/RelA subunit. J Biol Chem.

571   279(23), 24873-24880.

572   Kalliolias, G.D., and Ivashkiv, L.B. (2016). TNF biology, pathogenic mechanisms and
573   emerging therapeutic strategies. Nat Rev Rheumatol. 12(1), 49-62.

574   Karupiah, G., Buller, R.M., Van Rooijen, N., Duarte, C.J., and Chen, J. (1996). Different
575   roles for CD4+ and CD8+ T lymphocytes and macrophage subsets in the control of a
576   generalized virus infection. J Virol. 70(12), 8301-8309.

577   Karupiah, G., Coupar, B.E., Andrew, M.E., Boyle, D.B., Phillips, S.M., Mullbacher, A.,
578   Blanden, R.V., and Ramshaw, I.A. (1990). Elevated natural killer cell responses in mice
579   infected with recombinant vaccinia virus encoding murine IL-2. J Immunol. 144(1), 290-
580   298.

581   Karupiah, G., Fredrickson, T.N., Holmes, K.L., Khairallah, L.H., and Buller, R.M. (1993).
582   Importance of interferons in recovery from mousepox. J Virol. 67(7), 4214-4226.

583   Karupiah, G., Woodhams, C.E., Blanden, R.V., and Ramshaw, I.A. (1991). Immunobiology
584   of infection with recombinant vaccinia virus encoding murine IL-2. Mechanisms of rapid
585   viral clearance in immunocompetent mice. J Immunol. 147(12), 4327-4332.

586   Kasembeli, M.M., Bharadwaj, U., Robinson, P., and Tweardy, D.J. (2018). Contribution of
587   STAT3 to Inflammatory and Fibrotic Diseases and Prospects for its Targeting for Treatment.
588   Int J Mol Sci. 19(8).

589   Korner, H., Cook, M., Riminton, D.S., Lemckert, F.A., Hoek, R.M., Ledermann, B.,
590   Kontgen, F., Fazekas de St Groth, B., and Sedgwick, J.D. (1997). Distinct roles for
591   lymphotoxin-alpha and tumor necrosis factor in organogenesis and spatial organization of
592   lymphoid tissue. Eur J Immunol. 27(10), 2600-2609.

593 Korner, H., Goodsall, A.L., Lemckert, F.A., Scallon, B.J., Ghrayeb, J., Ford, A.L., and
594 Sedgwick, J.D. (1995). Unimpaired autoreactive T-cell traffic within the central nervous
595 system during tumor necrosis factor receptor-mediated inhibition of experimental
596 autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 92(24), 11066-11070.

597 Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using
598 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25(4), 402-408.

599 Loparev, V.N., Parsons, J.M., Knight, J.C., Panus, J.F., Ray, C.A., Buller, R.M., Pickup,
600 D.J., and Esposito, J.J. (1998). A third distinct tumor necrosis factor receptor of
601 orthopoxviruses. Proc Natl Acad Sci U S A. 95(7), 3786-3791.

602 Ma, J.F., Sanchez, B.J., Hall, D.T., Tremblay, A.K., Di Marco, S., and Gallouzi, I.E. (2017).
603 STAT3 promotes IFNgamma/TNFalpha-induced muscle wasting in an NF-kappaB-
604 dependent and IL-6-independent manner. EMBO Mol Med. 9(5), 622-637.

605 Marino, M.W., Dunn, A., Grail, D., Inglese, M., Noguchi, Y., Richards, E., Jungbluth, A.,
606 Wada, H., Moore, M., Williamson, B., et al. (1997). Characterization of tumor necrosis
607 factor-deficient mice. Proc Natl Acad Sci U S A. 94(15), 8093-8098.

608 McGeachy, M.J., Bak-Jensen, K.S., Chen, Y., Tato, C.M., Blumenschein, W., McClanahan,
609 T., and Cua, D.J. (2007). TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T
610 cells and restrain T(H)-17 cell-mediated pathology. Nat Immunol. 8(12), 1390-1397.

611 Moss, M.L., Jin, S.L.C., Milla, M.E., Burkhart, W., Carter, H.L., Chen, W.-J., Clay, W.C.,
612 Didsbury, J.R., Hassler, D., Hoffman, C.R., et al. (1997). Cloning of a disintegrin
613 metalloproteinase that processes precursor tumour-necrosis factor-α. Nature. 385, 733.

614 Muller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., and Aguet,

615 M. (1994). Functional role of type I and type II interferons in antiviral defense. Science.

616 264(5167), 1918-1921.

617 Murdaca, G., Spano, F., Contatore, M., Guastalla, A., Penza, E., Magnani, O., and Puppo, F.

618 (2015). Infection risk associated with anti-TNF-alpha agents: a review. Expert Opin Drug

619 Saf. 14(4), 571-582.

620 Neumann, B., Machleidt, T., Lifka, A., Pfeffer, K., Vestweber, D., Mak, T.W., Holzmann,

621 B., and Kronke, M. (1996). Crucial role of 55-kilodalton TNF receptor in TNF-induced

622 adhesion molecule expression and leukocyte organ infiltration. J Immunol. 156(4), 1587-

623 1593.

624 Niemand, C., Nimmesgern, A., Haan, S., Fischer, P., Schaper, F., Rossaint, R., Heinrich,

625 P.C., and Müller-Newen, G. (2003). Activation of STAT3 by IL-6 and IL-10 in Primary

626 Human Macrophages Is Differentially Modulated by Suppressor of Cytokine Signaling 3. J

627 Immunol. 170(6), 3263-3272.

628 O'Gorman, W.E., Sampath, P., Simonds, E.F., Sikorski, R., O'Malley, M., Krutzik, P.O.,

629 Chen, H., Panchanathan, V., Chaudhri, G., Karupiah, G., et al. (2010). Alternate mechanisms

630 of initial pattern recognition drive differential immune responses to related poxviruses. Cell

631 Host Microbe. 8(2), 174-185.

632 O'Shea, J.J., Schwartz, D.M., Villarino, A.V., Gadina, M., McInnes, I.B., and Laurence, A.

633 (2015). The JAK-STAT pathway: impact on human disease and therapeutic intervention.

634 Annu Rev Med. 66, 311-328.

635 Oeckinghaus, A., Hayden, M.S., and Ghosh, S. (2011). Crosstalk in NF-[kappa]B signaling

636 pathways. Nat Immunol. 12(8), 695-708.

637 Panchanathan, V., Chaudhri, G., and Karupiah, G. (2005). Interferon function is not required

638 for recovery from a secondary poxvirus infection. Proc Natl Acad Sci U S A. 102(36),

639 12921-12926.

640 Papathanasiou, S., Rickelt, S., Soriano, M.E., Schips, T.G., Maier, H.J., Davos, C.H., Varela,

641 A., Kaklamanis, L., Mann, D.L., and Capetanaki, Y. (2015). Tumor necrosis factor-alpha

642 confers cardioprotection through ectopic expression of keratins K8 and K18. Nat Med. 21(9),

643 1076-1084.

644 Parker, A.K., Parker, S., Yokoyama, W.M., Corbett, J.A., and Buller, R.M.L. (2007).

645 Induction of natural killer cell responses by ectromelia virus controls infection. J Virol.

646 81(8), 4070-4079.

647 Pasparakis, M., Alexopoulou, L., Episkopou, V., and Kollias, G. (1996). Immune and

648 inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha

649 in the formation of primary B cell follicles, follicular dendritic cell networks and germinal

650 centers, and in the maturation of the humoral immune response. J Exp Med. 184(4), 1397-

651 1411.

652 Pasparakis, M., Alexopoulou, L., Grell, M., Pfizenmaier, K., Bluethmann, H., and Kollias,

653 G. (1997). Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is

654 defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its

655 55-kDa receptor. Proc Natl Acad Sci U S A. 94(12), 6319-6323.

656   Peper, R.L., and Van Campen, H. (1995). Tumor necrosis factor as a mediator of

657   inflammation in influenza A viral pneumonia. Microb Pathog. 19(3), 175-183.

658   Qing, Y., and Stark, G.R. (2004). Alternative activation of STAT1 and STAT3 in response

659   to interferon-gamma. J Biol Chem. 279(40), 41679-41685.

660   Rahman, M.M., and McFadden, G. (2006). Modulation of tumor necrosis factor by microbial

661   pathogens. PLoS Pathog. 2(2), e4.

662   Rincon, M. (2012). Interleukin-6: from an inflammatory marker to a target for inflammatory

663   diseases. Trends Immunol. 33(11), 571-577.

664   Ruuls, S.R., Hoek, R.M., Ngo, V.N., McNeil, T., Lucian, L.A., Janatpour, M.J., Korner, H.,

665   Scheerens, H., Hessel, E.M., Cyster, J.G., et al. (2001). Membrane-bound TNF supports

666   secondary lymphoid organ structure but is subservient to secreted TNF in driving

667   autoimmune inflammation. Immunity. 15(4), 533-543.

668   Sanjabi, S., Zenewicz, L.A., Kamanaka, M., and Flavell, R.A. (2009). Anti-inflammatory

669   and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity.

670   Curr Opin Pharmacol. 9(4), 447-453.

671   Saunders, B.M., and Britton, W.J. (2007). Life and death in the granuloma:

672   immunopathology of tuberculosis. Immunol Cell Biol. 85(2), 103-111.

673   Scheurich, P., Thoma, B., Ucer, U., and Pfizenmaier, K. (1987). Immunoregulatory activity

674   of recombinant human tumor necrosis factor (TNF)-alpha: induction of TNF receptors on

675   human T cells and TNF-alpha-mediated enhancement of T cell responses. J Immunol.

676   138(6), 1786-1790.

677   Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an

678   overview of signals, mechanisms and functions. J Leukoc Biol. 75(2), 163-189.

679   Seet, B.T., Johnston, J.B., Brunetti, C.R., Barrett, J.W., Everett, H., Cameron, C., Sypula, J.,

680   Nazarian, S.H., Lucas, A., and McFadden, G. (2003). Poxviruses and immune evasion. Annu

681   Rev Immunol. 21, 377-423.

682   Shale, M., Czub, M., Kaplan, G.G., Panaccione, R., and Ghosh, S. (2010). Anti-tumor

683   necrosis factor therapy and influenza: keeping it in perspective. Therap Adv Gastroenterol.

684   3(3), 173-177.

685   Shuai, K., and Liu, B. (2005). Regulation of gene-activation pathways by PIAS proteins in

686   the immune system. Nat Rev Immunol. 5(8), 593-605.

687   Subramaniam, K., D'Rozario, J., and Pavli, P. (2013). Lymphoma and other

688   lymphoproliferative disorders in inflammatory bowel disease: A review. J Gastroen Hepatol.

689   28(1), 24-30.

690   Szretter, K.J., Gangappa, S., Lu, X., Smith, C., Shieh, W.J., Zaki, S.R., Sambhara, S.,

691   Tumpey, T.M., and Katz, J.M. (2007). Role of host cytokine responses in the pathogenesis

692   of avian H5N1 influenza viruses in mice. J Virol. 81(6), 2736-2744.

693   Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F., and

694   Cerami, A. (1987). Anti-cachectin/TNF monoclonal antibodies prevent septic shock during

695   lethal bacteraemia. Nature. 330(6149), 662-664.

696   Uysal, K.T., Wiesbrock, S.M., Marino, M.W., and Hotamisligil, G.S. (1997). Protection

697   from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature.

698   389(6651), 610-614.

699   Vigano, M., Degasperi, E., Aghemo, A., Lampertico, P., and Colombo, M. (2012). Anti-

700   TNF drugs in patients with hepatitis B or C virus infection: safety and clinical management.

701   Expert Opin Biol Ther. 12(2), 193-207.

702   Wilhelm, P., Ritter, U., Labbow, S., Donhauser, N., Rollinghoff, M., Bogdan, C., and

703   Korner, H. (2001). Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF. J

704   Immunol. 166(6), 4012-4019.

705   Yagil, Z., Nechushtan, H., Kay, G., Yang, C.M., Kemeny, D.M., and Razin, E. (2010). The

706   enigma of the role of protein inhibitor of activated STAT3 (PIAS3) in the immune response.

707   Trends Immunol. 31(5), 199-204.

708   Yamamoto, T., Matsuda, T., Muraguchi, A., Miyazono, K., and Kawabata, M. (2001). Cross-

709   talk between IL-6 and TGF-beta signaling in hepatoma cells. FEBS Lett. 492(3), 247-253.

710   Zhong, Z., Wen, Z., and Darnell, J. (1994). Stat3: a STAT family member activated by

711   tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science.

712   264(5155), 95-98.

713

714 **Figure Legends**

715 **Figure 1. TNF is critical for recovery of mice from ECTV infection but is not required**

716 **for control of virus replication.** Groups of WT, TNF$^{-/-}$ and mTNF$^{\Delta/\Delta}$ mice were infected

717 i.n. with 25 PFU ECTV. (A) Clinical scores (criteria detailed in Table S1), (B) weights and

718 (C) survival were monitored during the course of infection. Additional groups of mice were

719 infected with ECTV and 5 mice from each strain were sacrificed on the indicated days p.i.

720 and viral load in (D) lungs, (E) livers, and (F) spleens were determined by plaque assay. (G)

721 Lung viral load in groups of 5 WT, TNF$^{-/-}$, IFN-α/βR$^{-/-}$ and IFN-$\gamma^{-/-}$ ECTV-infected mice at

722 day 5 p.i. determined by plaque assay. (H) Lung viral load in groups of 5 WT and 5 TNF$^{-/-}$

723 mice infected with either ECTV^{WT}or ECTV$^{\Delta CrmD}$ at day 7 p.i. Data are expressed as means

724 $\pm$ SEM. Statistical analysis was undertaken as follows: for (A), clinical scores for TNF$^{-/-}$ and

725 mTNF$^{\Delta/\Delta}$ mice on each day p.i. were compared to WT mice on that day using two-tailed

726 Mann-Whitney test; for (B) weights for TNF$^{-/-}$ and mTNF$^{\Delta/\Delta}$ mice on each day p.i. were

727 compared to WT mice using multiple unpaired t-tests and the Holm-Sidak's correction for

728 multiple comparisons; for (C) survival curves were compared using Log-rank (Mantel-Cox)

729 statistical test; and for (D)-(H), log transformed viral loads were analyzed by two-way

730 ANOVA with Holm-Sidak's correction for multiple comparison and the broken line

731 corresponds to the limit of detection in plaque assays. *, p $\leq$ 0.05; **, p $\leq$ 0.01; ***, p $\leq$

732 0.001; ****, p $\leq$ 0.0001. Results shown are representative of 3 independent experiments for

733 (A)-(F), 1 experiment for (G) and 2 experiments for (H). See also Table S1.

734 **Figure 2. TNF deficiency in mice during ECTV infection exacerbates lung pathology.**

735 Groups of WT and TNF$^{-/-}$ mice were infected with ECTV. Five mice from each group were

736 sacrificed on days 7, 9, and 11 p.i, in addition to 5 uninfected mice of both genotypes. (A)

737 Gross morphology of lungs from naïve WT and TNF$^{-/-}$ mice is shown in comparison to lungs

738   from day 11 p.i. Areas of consolidation and necrosis on the surface of the lungs are marked

739   with arrows. (B) Fluid extravasation in the lungs was measured by the lung wet-to-dry weight

740   ratios on the days indicated, and (C) protein concentration in BALF of naïve mice (day 0)

741   and day 11 p.i. Histological changes in naïve and ECTV-infected (D) WT and (E) TNF$^{-/-}$

742   mice at days 9 and 11 p.i. Slides were examined on all fields at 400x magnification and the

743   bars shown correspond to 100 μm. Parenchymal edema, perivascular edema (thick arrows)

744   and bronchial epithelial necrosis (thin arrows) are shown. (F) Lung histopathological scores

745   on days 0, 9 and 11 p.i. using a scoring system described in Table S2. Data are expressed as

746   means ± SEM. For (B), (C) and (F), statistical analysis was undertaken using two-way

747   ANOVA followed by Holm-Sidak's multiple comparisons test. Results are representative of

748   3 separate experiments. See also Figure S3 and Table S2.

749   **Figure 3. TNF deficiency does not affect lung leukocyte recruitment but results in**

750   **increased production of specific inflammatory and regulatory cytokines.**

751   Immunohistochemistry of lung sections for TNF expression by bronchial epithelial cells and

752   leukocyte subsets in ECTV-infected (A) WT and (B) TNF$^{-/-}$ mice on day 9 p.i. Slides were

753   examined at 1000x magnification. Arrows point to bronchial ephithelial cells and different

754   cell types in the alveoli and bars correspond to 50 μm. (C) Flow cytometry analysis of

755   digested lungs from naïve and ECTV-infected WT and TNF$^{-/-}$ mice at day 9 p.i. Leukocyte

756   subsets were identified by CD45.2 expression and one or more specific phenotypic markers

757   as shown. Concentrations of (D) TNF, (E) IFN-γ, (F) IL-6, and (G) IL-10 in lung

758   homogenates determined by Cytokine Bead Array. (H) TGF-β concentrations measured by

759   ELISA. For (C)-(H), data are expressed as means ± SEM and were analyzed by two-way

760   ANOVA followed by Holm-Sidak's multiple comparisons test. Results are representative of

761   3 separate experiments with 5 animals per group. See also Figures S1B and S5 and Table

762   S2. Data shown in panel 3D is the same as that shown in Figure S1B.

763   **Figure 4. Blockade of TNF, IFN-γ, IL-6, IL-10R or TGF-β with mAb dampens lung**

764   **pathology.** Groups of WT (A-J) and TNF$^{-/-}$ (K-T) ECTV-infected mice were treated on day

765   7 p.i. with isotype control rat IgG mAb (A, B, K, L), or specific mAb against IFN-γ (C, D,

766   M, N), IL-6 (E, F, O, P), IL-10R (G, H, Q, R), or TGF-β (I, J, S, T) at 500 µg i.p. and

767   sacrificed on day 9 p.i., lungs were collected, fixed, sectioned, H&E-stained and examined

768   on all fields at 400x magnification. Perivascular edema (thick arrows) and bronchial epithelia

769   (thin arrows) are shown. Bars correspond to 100 µm. In separate experiments, groups of WT

770   (U-V) and TNF$^{-/-}$ (W-X) mice were infected with ECTV and treated with mAb as above.

771   Histopathological scores and viral load in WT mice (U and V) and TNF$^{-/-}$ mice (W and X)

772   at day 9 p.i. For panels V and X, the broken line correspond to the limit of virus detection.

773   Data are expressed as means ± SEM and analyzed using unpaired t-test for (U) and (W). For

774   (V) and (X), data were log transformed and analyzed by two-way ANOVA with Holm-

775   Sidak's correction for multiple comparison. Results are representative of 2 separate

776   experiments with 5 animals per group.

777   **Figure 5. Short-term cytokine blockade increases levels of pSTAT3, PIAS3 and SOCS3**

778   **expression in ECTV-infected lungs.** Groups of 5 WT and 5 TNF$^{-/-}$ ECTV-infected mice

779   were treated on day 7 p.i. with control rat IgG, or mAb against IFN-γ (α-IFN-γ), IL-6 (α-IL-

780   6), IL-10R (α-IL-10R) or TGF-β (α-TGF-β) at 500 µg i.p. Lungs were collected on day 9

781   p.i. and homogenized for detection of STAT3, pSTAT3 (tyrosine-705 phosphorylation),

782   PIAS3 and SOCS3 protein levels (A) by Western blot analysis. Band densities (B, C and D)

783   were quantified using the ThermoScientific Pierce MYImageAnalysis Software. Analysis

784   was done for individual mice and results shown are representative of one mouse per

785   treatment group.

786

787 **Figure 6. STAT3 inhibition dampens ECTV infection induced lung pathology and**

788 **modulates inflammatory cytokine responses but does not affect virus load.** Groups of 5

789 WT and 5 TNF$^{-/-}$ ECTV-infected mice were treated on days 7 and 8 p.i. with 5 mg/kg of the

790 STAT3 inhibitor, S3I-201, and mock-treated groups were given inhibitor diluent. Clinical

791 scores of (A) WT and (B) TNF$^{-/-}$ mice based on the clinical scoring criteria (Table S1). (C)

792 Lung histopathological scores and (D) viral load at day 9 p.i. (E, F). Representative lung

793 sections are shown at 400x magnification and bars correspond to 100 μm. Levels of

794 expression of mRNA transcripts for (G) TNF, (H) IL-10, (I) TGF-β, (J) IFN-γ, (K) IL-1α,

795 (L) IL-1β, (M) IL-12p40 and (N) IL-6. Data are expressed as means ± SEM and analyzed

796 using Mann-Whitney tests for (A)-(C). For (D) data were log transformed and analyzed by

797 two-way ANOVA, with Holm-Sidak's correction for multiple comparison and the broken

798 line corresponds to the limit of virus detection. For (G)-(N), data were analyzed by two-way

799 ANOVA with Holm-Sidak's correction for multiple comparison. Results are representative

800 of 2 separate experiments.

801 **Figure 7. Long-term treatment with anti-IL-6 or anti-TGF-β protects TNF$^{-/-}$ mice from**

802 **lethal disease.** A group of 5 TNF$^{-/-}$ ECTV-infected mice were treated every day from days

803 7-21 p.i. with 5 mg/kg STAT3 inhibitor and other groups were treated with control rat IgG

804 mAb, anti-IL-6, anti-IL-10R, or anti-TGF-β at 500 μg i.p. every 2 days from day 7 until day

805 21 p.i., and monitored until day 22. (A) Survival, (B) weights and (C) clinical scores were

806 monitored during the course of infection. (D) Lung viral load and (E) histopathological

807 scores of lung H&E sections for organs collected from animals that were moribund and

808 euthanized for ethical reasons or found dead before day 22 p.i., and those that were sacrificed

809 on day 22. Data are expressed as means ± SEM. For (A), (B) and (C), data for STAT3

810 inhibitor- or anti-cytokine mAb-treated mice were compared with control mAb-treated mice

811 for each day. Survival curves (A) were compared using Log-rank (Mantel-Cox) statistical

36

812   test. Weights (B) were compared by multiple unpaired t-tests and the Holm-Sidak's

813   correction for multiple comparisons. Clinical scores (C) were compared by the two-tailed

814   Mann-Whitney test. For (D), viral load data were log transformed and the broken line

815   corresponds to the limit of detection in the plaque assay. Statistical analysis was not

816   performed for viral load and histopathological scores. Results are representative of 2 separate

817   experiments.

818   **Methods**

819   **Mice**

820   Six- to twelve-week old, female WT, $TNF^{-/-}$ (Korner et al., 1997), $mTNF^{\Delta/\Delta}$ (Ruuls et al.,

821   2001), $IFN-\alpha/\beta R^{-/-}$ (Muller et al., 1994) and $IFN-\gamma^{-/-}$ (Dalton et al., 1993) mice on a C57BL/6

822   background were bred under specific pathogen-free conditions at the Australian Phenomics

823   Facility, Australian National University, Canberra, Australia. Animal experiments were

824   performed in accordance with protocols approved by the ANU Animal Ethics and

825   Experimentation Committee (Protocol numbers A2011/011 and A2014/018).

826   **Cell lines and viruses**

827   BS-C-1 cells (ATCC No. CCL-26), MC57G (ATCC No. CRL-2295) and YAC-1 (ATCC

828   No. TIB-160) were cultured in Eagle's minimum essential medium (EMEM) supplemented

829   with 2 mM L-glutamine (Sigma Aldrich), antibiotics (penicillin, 120 μg/mL, streptomycin,

830   200 μg/mL and neomycin sulphate), 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

831   acid (HEPES) and 10% fetal calf serum (FCS).

832   The Moscow strain of ECTV (ATCC No. VR-1374) was used to infect mice in all

833   experiments except for data shown in Figure 1H, for which the Naval strain of WT ECTV

834   ($ECTV^{WT}$) and the CrmD deletion mutant ($ECTV^{\Delta CrmD}$) (Alejo et al., 2018) were used.

835   Viruses were propagated in BS-C-1 cells, semi-purified using a sucrose cushion and single-

836   use aliquots were stored at -80ºC as described elsewhere in detail (Chaudhri et al., 2018).

837   **Virus infection, animal weights, clinical scores and treatments**

838   Tribromoethanol-anesthetized mice (160-240 mg/kg i.p.) were infected through the i.n. route

839   with 25 PFU of ECTV in 30 µL PBS. Clinical manifestation of disease during ECTV

840   infection was assessed using the scoring system (Table S1) with 5 clinical parameters. In

841   some experiments, mice were administered i.p. with STAT3 inhibitor VI, S3I-201 (Merck

842   Millipore cat. no. 573102) at 5 mg/kg, or neutralizing/blocking mAb against IFN-γ (R4.6A2,

843   rat IgG$_1$, cat. no. BE0054), IL-6 (clone MP5-20F3, rat IgG$_1$, cat. no. BE0046), IL-10R

844   (1B1.3A, rat IgG$_1$, cat. no. BE0050), TGF-β (1D11.16.18, mouse IgG$_1$, cat. no. BE0057) or

845   isotype control mAb (HRPN, rat IgG$_1$, cat. no. BE0088) at 500 µg on day 7 p.i. Purified mAb

846   were purchased from Bio X Cell. Mice were individually ear-tagged, weighed and clinical

847   scores recorded daily on a scale from 0 to 3 for each criterion, with a maximum score of 15.

848   For ethical reasons, mice that were severely moribund and/or lost $\geq$ 25% of the original body

849   weight were euthanized and considered dead the following day.

850   **Histology and microscopic assessment of lung pathology**

851   The left lobe of lung tissue was removed and fixed in 10% neutral-buffered formaldehyde at

852   room temperature for 24 h, embedded in paraffin, cut in 6 µm sagittal sections and stained

853   with hematoxylin and eosin (H&E) for analysis with a bright field microscope (Olympus IX

854   71). A semi-quantitative scoring system for lung pathology was developed (Table S2) with

855   6 pathologic parameters. Individual slides were scored in a single-blinded fashion on a scale

856   from 0 to 4 for each criterion, with a maximum score of 24.

Lung total protein concentration and wet-to-dry weight ratio

Total protein concentration in bronchoalveolar lavage fluid (BALF) were measured by the Bradford Assay using Protein Assay Dye Reagent Concentrate (Bio-Rad) according to the manufacturer's protocol. For the quantification of pulmonary edema, the left lung lobe was removed and immediately wrapped in a pre-weighed aluminium foil, weighed (wet lung weight) and dried in an oven at 80ºC for 72 h. The weights (oven-dried lung weight) of samples were recorded after the 72-h period to determine wet-to-dry weight ratio (wet lung weight / oven-dried lung weight).

Flow cytometric analysis of lung leukocytes

Individual lung tissue samples were cut into small pieces and digested in EMEM containing 4 μg DNAse I, grade II and 4 mg collagenase A (Roche Applied Science) and placed in a 37ºC water bath for 1 h. Undigested tissue was further processed by passing through a 19-G needle attached to a syringe and then filtered through a 70 μm nylon mesh. Cells were washed with Hank's balanced salt solution and red blood cells lysed with red cell lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) for 1 min. Cells were next washed and resuspended in PBS containing 2% FCS. For labelling, FcγII/III receptors (FcγR) were first blocked with anti- FcγR mAb (clone 2.4G2), the cells were washed, and stained with CD45.2-FITC (to identify leukocytes) and cell subset specific fluorochrome-conjugated mAb (all were obtained from BD Biosciences and Biolegend). Data were acquired on a BD LSR Fortessa flow cytometer with BD FACS Diva software and analyzed using FlowJo Software version 9.5 (Tree Star, Inc).

Determination of cytokine protein levels

878

879 The levels of IFN-γ, IL-6, IL-10 and TNF in the lung homogenates were measured using the

880 Mouse Inflammation Cytokine Bead Array Kit (BD Biosciences) according the

881 manufacturer's protocol. Acquisition of data was done using a BD FACSCalibur flow

882 cytometer with BD Cell Quest software. Data were analyzed using FlowJo Software version

883 9.5. TGF-β levels were measured using capture ELISA kits (Biolegend), performed

884 according to the manufacturer's protocol. Optical density was measured at 450 nm with

885 SOFTmax Pro software (Molecular Devices Corp).

SDS PAGE and Western blot analysis

886

887 Lung homogenates and a pre-stained recombinant protein ladder (Bio-Rad) were run in a

888 12% SDS-polyacrylamide gel and were transferred to a nitrocellulose membrane (Bio-Rad).

889 After blocking with 1 M glycine, 5% w/v dry skim milk blocking solution, the membrane

890 was incubated with primary antibody (STAT3, polyclonal rabbit IgG, cat. no. 9132; pSTAT3

891 (Tyr705), polyclonal rabbit IgG, cat. no. 9131; PIAS3, polyclonal rabbit IgG, cat. no. 4164,

892 all from Cell Signaling Technology; or SOCS3, Abcam, cat. no. ab16030). The

893 immunoreactive proteins were visualized with horseradish peroxidase (HRP)-conjugated

894 secondary antibody (polyclonal goat anti-rabbit IgG, Santa Cruz Biotechnology, cat.no. sc-

895 2004) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific)

896 in a chemiluminescent imager (ImageQuant LAS 4000, GE Healthcare Life Sciences).

Immunohistochemistry of lung sections

897

898 Immunohistochemistry was performed on formaldehyde-fixed and paraffin-embedded lung

899 sections. Samples were deparaffinized and rehydrated through different concentrations of

900 xylene and ethanol, followed by antigen retrieval in Tris/EDTA Buffer. The sections were

901 incubated in primary TNF antibody (MP6-XT3, rat IgG_1) and secondary HRP-conjugated

40

902   goat anti-rat IgG (Santa Cruz Biotechnology, cat. no. sc-52619 and sc-2006, respectively).

903   The reaction was developed using a 3,3'-diaminobenzidine substrate buffer and

904   counterstained with H&E for analysis with a bright field microscope (Olympus IX 71).

905

906   **RNA extraction**

907   Lung tissue from individual mice were homogenized in 1 mL of TRIsure reagent with

908   TissueLyser II (Qiagen, cat. no. 853000) and as per the manufacturer's instructions and

909   samples were centrifuged for 15 min at 12,000 x g at 4°C to remove cellular debris. In the

910   final step, RNA pellets were air-dried and resuspended in 30-50 µL RNAse-free water and

911   incubated for 10 min at 60°C to ensure they were dissolved. RNA concentration was

912   determined using a Nanodrop ND-1000 spectrophotometer. Samples were stored at -80°C

913   until cDNA synthesis.

914

915   **cDNA generation**

916   Tetro cDNA Synthesis Kit (Bioline, cat. no. BIO- 65043) was used to synthesize single-

917   stranded cDNA from 1 µg RNA in 12 µL RNAse-free water following the manufacturer's

918   instructions. Briefly, the priming premix was prepared on ice by mixing Oligo $(dT)_{18}$ primer

919   mix to a final concentration of 0.5 µg/µL, 10mM dNTP mix, 5x RT Buffer, 10 units/µL

920   RiboSafe RNase Inhibitor and Tetro Reverse Transcriptase (200 unit/µL). To each sample,

921   8 µl of the mastermix was added and incubated at 45°C for 30 min. The reaction was

922   terminated by incubating samples at 85°C for 5 min then chilled on ice. Samples were diluted

923   with 180 µL RNAse free water and stored at -20°C until further use.

924

925   **Quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)**

926 For each gene of interest, a real-time PCR reaction mixture was prepared containing 10 μL

927 of SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, cat. no. 172-5265), 1 μL each of 5

928 μM gene-specific forward and reverse primer pair, and 6 μL of RNAse-free water. 2 μL of

929 the appropriate cDNA template and 18 μL of the reaction mixture were added to the wells

930 of iQ 96-well semi-skirted PCR Plate (Bio-Rad, cat. no. 223-9441). Amplification was

931 achieved using Bio-Rad iQ5 Real-time PCR Detection System. Relative changes in gene

932 expression was determined using delta/delta cycle threshold ($2^{-\Delta\Delta CT}$) analysis method (Livak

933 and Schmittgen, 2001). *Ubiquitin C* (*UBC*) was used as the internal control. Results are

934 reported as the fold difference relative to a calibrator cDNA (naïve mice) prepared in parallel

935 with the experimental cDNAs.

936 **^{51}Chromium (^{51}Cr) release cytotoxic assays**

937 CTL and NK cell responses were measured by the ^{51}Cr release assay as described previously

938 (Karupiah et al., 1990; Karupiah et al., 1991). Briefly, for CTL assays, 6 x 10^6 MC57G target

939 cells that were either uninfected or infected with ECTV (10 PFU/cell) were labelled with

940 20 μCi ^{51}Cr ($Na_2^{51}CrO_4$) and incubated in a humidified atmosphere of 5% CO_2 and 95% air

941 for 1 h. Cells were washed three times and resuspended in complete medium and 2 x 10^4

942 cells were added into wells of U-bottom 96-well plates. Effector cells (single cell

943 suspensions of digested lungs or splenocytes) were added to wells with target cells in

944 triplicate to obtain effector cell:target cell ratios of 100:1, 33:1, 11:1 and 3.7:1. Control wells

945 included target cells only (spontaneous release) or target cells plus 1% Triton X (maximum

946 release). After incubation for 6 h in a humidified atmosphere of 5% CO_2 and 95% air, the

947 96-well plates were centrifuged at 300 x *g* for 5 min, and 25 μl of supernatant from each well

948 was transferred to a corresponding well in Luma-96 plate (Perkin-Elmer, Boston, USA).

949 Plates were air dried overnight and radioactive counts per minute (cpm) were measured using

42

950 a TopCount NXT^{TM} microplate scintillation counter (Packard Bioscience Company, Meriden

951 CT, USA). Percent specific lysis was calculated from the mean cpm of triplicate wells, using

952 the following equation:

953
954 $$\% \text{ specific lysis} = 100 \times \frac{\text{Test sample release (cpm) - spontaneous release (cpm)}}{\text{Maximum release (cpm) - spontaneous release (cpm)}}$$
955

956 Virus-specific CTL activity was determined by subtracting % specific lysis values of

957 uninfected targets from infected targets.

958 For NK cell responses, YAC-1 target cells were used without the need for infection with

959 ECTV. YAC-1 cells were labelled with ^{51}Cr as described for MC57G cells. Effector and

960 target cells were incubated for 4 h after which supernatants were collected to determine cpm

961 as described for the CTL assay.

962 **Statistical analysis**

963 Statistical analyses of experimental data, as indicated, were performed using GraphPad

964 Prism 8 (GraphPad Software, Inc.). A p value of <0.05 was taken to be significant, *, p ≤

965 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001. Log-rank (Mantel-Cox) test, two-tailed

966 Mann-Whitney test, unpaired t-test with Holm-Sidak's correction and two-way ANOVA

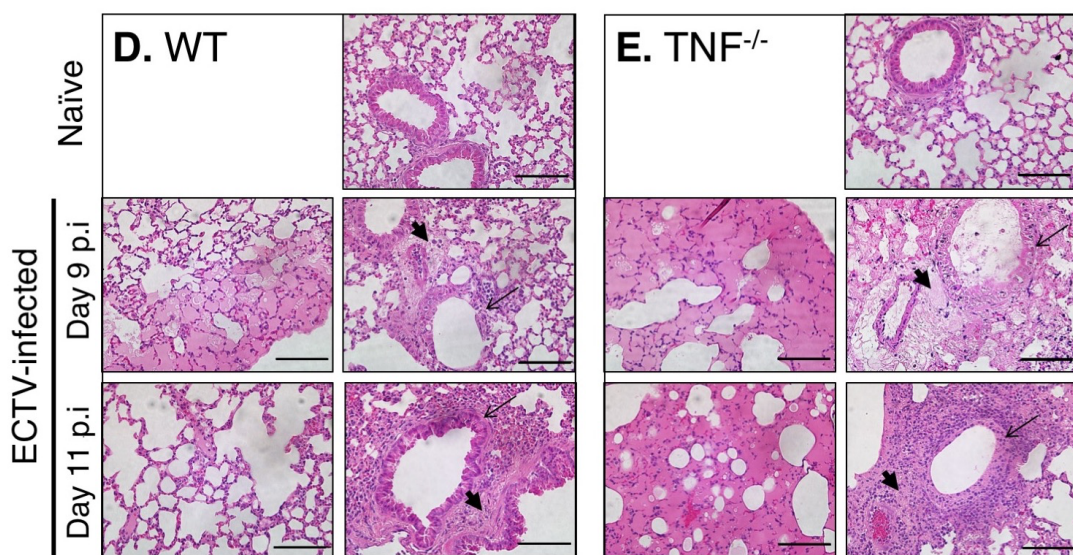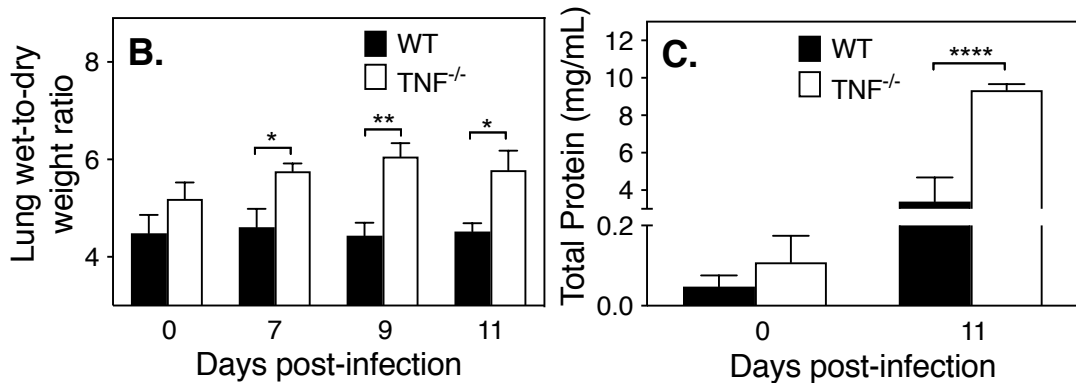967 with Holm-Sidak's correction for multiple comparison were used to compare results.

Figure 1

A. Figure 2

WT TNF$^{-/-}$ WT TNF$^{-/-}$

Naïve (uninfected)   ECTV-infected day 11 p.i.

B. Lung wet-to-dry weight ratio — WT, TNF$^{-/-}$; Days post-infection: 0, 7, 9, 11; * ** *

C. Total Protein (mg/mL) — WT, TNF$^{-/-}$; Days post-infection: 0, 11; ****

D. WT — Naïve, Day 9 p.i, Day 11 p.i (ECTV-infected)

E. TNF$^{-/-}$ — Naïve, Day 9 p.i, Day 11 p.i (ECTV-infected)

F. Histopathologic Score — WT, TNF$^{-/-}$; Days post-infection: 0, 9, 11; ** ****

A. WT

B. TNF⁻ᐟ⁻

Bronchial epithelium   Macrophages   Neutrophils   Monocytes   Lymphocytes

C.

Cell Count / Lungs

WT Naïve   WT Infected
TNF⁻ᐟ⁻ Naïve   TNF⁻ᐟ⁻ Infected

CD3⁺   CD3⁺CD4⁺   CD3⁺CD8⁺   CD3⁺TCR γδ   CD45R⁺   CD11c⁺   F4/80⁺   NK1.1⁺   CD11bʰⁱ Ly6cʰⁱ   CD11bʰⁱ Ly6cˡᵒʷ   CD4⁺ Foxp3⁺

D. TNF   ***
Concentration (pg/ml ×100)
WT
TNF⁻ᐟ⁻
Days post-infection

E. IFN-γ   *
WT
TNF⁻ᐟ⁻
Days post-infection

F. IL-6   *
WT
TNF⁻ᐟ⁻
Days post-infection

G. IL-10   ****
Concentration (pg/ml)
WT
TNF⁻ᐟ⁻
Days post-infection

H. TGF-β   ****
WT
TNF⁻ᐟ⁻
Days post-infection

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7