

1 **Regulatory T cells prevent Th2 immune responses and pulmonary eosinophilia**
2 **during RSV infection in mice**

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4 Running title: Immune dysregulation in RSV bronchiolitis

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17 **Abstract**

18 During viral infection, inflammation and recovery are tightly controlled by competing pro-
19 inflammatory and regulatory immune pathways. Respiratory syncytial virus (RSV) is the
20 leading global cause of infantile bronchiolitis, which is associated with recurrent wheeze
21 and asthma diagnosis in later life. Th2 driven disease has been well described under
22 some conditions in RSV infected mice. In the present studies, we used the *Foxp3*^{DTR}
23 mice (that allow specific conditional depletion of Foxp3⁺ T cells) to investigate the
24 functional effects of regulatory T cells (Treg) during A2 strain RSV infection. Infected
25 Treg-depleted mice lost significantly more weight than wild-type mice, indicating
26 enhanced disease. This enhancement was characterized by increased cellularity in the
27 bronchoalveolar lavage (BAL) and a notable lung eosinophilia not seen in control mice.
28 This was accompanied by abundant CD4⁺ and CD8⁺ T cells exhibiting an activated
29 phenotype and induction of interleukin (IL)-13- and GATA3-expressing Th2-type CD4⁺ T
30 cells that remained present in the airways even 14 days after infection. Therefore, Treg
31 cells perform vital anti-inflammatory functions during RSV infection, suppressing
32 pathogenic T cell responses and inhibiting lung eosinophilia. These findings provide
33 additional evidence that dysregulation of normal immune responses to viral infection
34 may contribute to severe RSV disease.

35 Introduction

36 Viral lung infections lead to impairment of lung function by multiple mechanisms,
37 including the obstruction of airflow by inflammatory cells in the conducting airways,
38 immune infiltration of lung tissue and alveolar oedema (1,2). The safe elimination of
39 infection requires a precise control of inflammation, sufficient to control pathogen load
40 but limiting the severity of immunopathology. Improved understanding of the checks and
41 balances governing the immune response to infection may assist not only in
42 understanding the immune system, but also in developing new treatments and in
43 creating effective vaccines(3).

44 Human respiratory syncytial virus (RSV) infection is the leading cause of severe lower
45 respiratory tract disease, causing infantile bronchiolitis and significant morbidity and
46 mortality in the elderly (1,4-6). It is estimated that RSV infects 33.8 million children
47 under 5 years old each year, killing between 66,000 and 199,000 children globally.
48 Virtually all these deaths occur in developing countries (7,8); almost all children have
49 been infected at least once by the age of three (8). An over-exuberant immune
50 response to infection is thought to underlie the pathogenesis of bronchiolitis. Prevention
51 of RSV infection during infancy reduces the likelihood of recurrent wheezing in later life
52 (9), showing that RSV infection can lead to the development of asthma in some
53 children.

54 Regulatory T (Treg) cells are a subset of CD4⁺ T cells that specifically express the
55 forkhead box P3 (Foxp3) transcription factor and play an essential role in dampening
56 immune responses and preventing autoimmune disease (10-12). The importance of

57 Treg cells to immune homeostasis is illustrated by humans suffering from
58 immunodysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome
59 due to mutations in the *Foxp3* gene (13). This pathology is mimicked in *Foxp3*-deficient
60 *Scurfy* mice, which likewise suffer from a progressive and fatal spontaneous multi-organ
61 inflammatory disease (14,15).

62 Treg cells can regulate the response to several viral infections (16-23). During RSV
63 infection, Treg cells limit antigen-specific T cell responses, suppress inflammation and
64 may help to control viral replication (24-26). However, there is still uncertainty about
65 how Treg cells control the immune response during RSV, in part due to the use of
66 different strategies to deplete Treg cells *in vivo*. Several initial reports used the anti-
67 CD25 PC61 antibody, which binds and depletes cells expressing the high affinity IL-2
68 receptor alpha chain CD25 (24-26). While CD25 is highly expressed on *Foxp3*⁺ Treg
69 cells, this receptor is also expressed on activated T cells and therefore use of PC61
70 may additionally deplete non-Treg cells (27). More specific depletion strategies have
71 been developed using BAC transgenic and knock-in mice in which the *Foxp3* gene
72 locus is modified by insertion of a human diphtheria toxin receptor (DTR)-eGFP element
73 (28,29). Upon treatment with diphtheria toxin, the DTR-expressing *Foxp3*⁺ Treg cells
74 may be specifically killed while all other host cells are unaffected (32). Using the BAC
75 transgenic DERE mice, we have previously shown that Tregs require granzyme B
76 expression to cause functional regulation during RSV disease (30). We also have
77 shown that boosting Treg cells using the IL-2/IL-2R complex ameliorates aspects of
78 disease during primary RSV infection (30) and that RSV disease augmented by prior
79 vaccination with formalin inactivated vaccine (FI-RSV) is accompanied by the virtual

80 disappearance of Tregs from the airways (31). Importantly, recruitment of Treg cells into
81 the airways by administration of CCL17 and CCL22 intranasally ameliorates vaccine-
82 enhanced FI-RSV disease (31). Taken together, these studies suggest that Tregs
83 modulate the immune response to RSV infection and that severe RSV disease may be
84 caused by dysregulated immune responses to infection.

85 Previous studies used methods that partially or transiently deplete Tregs. In the case of
86 the DEREK mice, diphtheria toxin administration causes 95-98% depletion of Treg cells
87 by apoptosis, but this is transient due to the leaky nature of the BAC transgene; over
88 time, *Foxp3*⁺ Treg cells reappear that do not express the transgene and thus cannot be
89 depleted (32). Therefore, to examine the effects of more complete Treg cell depletion
90 during RSV infection, we used the *Foxp3*^{DTR} mouse model. The *Foxp3*^{DTR} mice are a
91 knock-in strain created using a targeting vector consisting of a human DTR fused to
92 GFP and containing an internal ribosome entry sequence (IRES) that inserts into the
93 3'untranslated region of the *Foxp3* gene locus. Similar to DEREK mice, both the DTR
94 and GFP are exclusively expressed in *Foxp3*⁺ Treg cells in *Foxp3*^{DTR} mice and
95 diphtheria toxin treatment in these mice specifically induces apoptosis of *Foxp3*⁺ Treg
96 cells (33).

97 Interestingly, although normal C57BL/6 mice are relatively resistant to RSV infection,
98 depletion of Tregs from the C57BL/6 background *Foxp3*^{DTR} mice caused marked weight
99 loss and enhanced cellular influx into the lungs and airways without affecting viral
100 clearance. Inflammatory T cell responses to RSV infection were enhanced, with
101 increased expression of GzmB and CD11a on CD4⁺ and CD8⁺ T cells. Most notably,
102 there was a marked and persistent eosinophilic response in the airways of the Treg-

103 depleted mice, indicative of a Th2-mediated pathology. This was associated with
 104 increased IL-13⁺ and IFN- γ ⁺ CD4⁺ T cell recruitment to the lungs and enhanced
 105 expression of the Th2-defining transcription factor Gata3 in the airways. Thus, we
 106 highlight novel functions for Treg cells in shaping the CD4⁺ effector cell response during
 107 RSV infection and promoting resolution of pathology.

108

109 **Materials and Methods**

110 **Mice, virus stocks and infection**

111 Six to ten week old C57BL/6 wild-type (purchased from Harlan, UK) and *Foxp3*^{DTR} mice
 112 were housed in specific-pathogen free conditions according to the UK Home Office
 113 guidelines.

114 Plaque-purified human RSV (A2 strain from ATCC) was grown to a high titer in HEp-2
 115 cells. Age-matched WT and *Foxp3*^{DTR} mice were lightly anaesthetized with isoflurane
 116 and challenged intranasally (i.n.) with a dose of 8 x 10⁵ focus-forming units (FFU) of
 117 RSV on day 0.

118 **Diphtheria toxin treatment**

119 *Foxp3*^{DTR} mice were injected with 0.75 μ g of DT (Diphtheria toxin, unnicked,
 120 *Corynebacterium diphtheria* from Merck Millipore, UK) in PBS intraperitoneally (i.p.) on
 121 days -1 and 3 during RSV infection to acutely deplete *Foxp3*⁺ Treg cells.

122 **Cell isolation and processing**

123 Mice were culled using a fatal dose (100-150 μ L) of pentobarbital injected i.p. according
 124 the UK home office guidelines. Bronchoalveolar lavage (BAL) cells were collected by
 125 inserting a syringe with a cannulated needle into the trachea and flushing 3 times with
 126 1ml of PBS supplemented with 12 mM Lidocaine powder (Sigma). Lung lobes were
 127 collected and digested with Collagenase XI (25 ug/ml, Sigma) using a gentleMACs cell
 128 dissociator (Miltenyi Biotech) according to the manufacturer's protocol. Spleen and lung
 129 cells were mashed through 100 μ M cell strainers to create single-cell suspensions. Total
 130 cell counts were determined by flow cytometry using Count Bright counting beads
 131 (Invitrogen) and dead cells were excluded by staining for 7-amino-actinomycin D (7-
 132 AAD, Sigma). To determine the cellular composition in the BAL, cells were transferred
 133 onto a microscope slide (Thermo Scientific, UK) using a Shandon Cytospin 3 Centrifuge
 134 and slides were stained with hematoxylin and eosin (H&E) (Reagent, Gamidor, UK).
 135 Cells were categorized as macrophages or monocytes, lymphocytes, neutrophils and
 136 eosinophils based on their morphology and size using the Axio Scope.A1 light
 137 microscope (Zeiss). Photographs of slides were taken using the AxioCam Erc 5s (Zeiss)
 138 at the 40X magnification.

139 **Real time quantitative PCR for viral load**

140 Total RNA was extracted from homogenized lung tissue using the Stat60-RNA
 141 extraction reagent (AMS Biotechnology Ltd., United Kingdom) and transcribed to cDNA
 142 using random hexamers and the Omniscript reverse transcriptase kit (Qiagen, United
 143 Kingdom). TaqMan real-time quantitative PCR was performed for RSV L-gene using
 144 primers and probes described previously (26) and the Quantitect Probe PCR Master

145 Mix (Qiagen). RSV L-gene copy numbers were normalized to 18S rRNA housekeeping
146 gene.

147 **Chemokine and cytokine detection:**

148 Eotaxin and IL-5 in the BAL were measured using a custom-made 10-plex Milliplex
149 MAP Mouse Cytokine/Chemokine kit and assayed according to the manufacturer's
150 instructions (Merck Millipore) and data was acquired on the Bio-plex 200 System (Bio-
151 Rad Laboratories, Hemel Hempstead, UK). The concentrations of cytokines and
152 chemokines were determined from a standard curve using the Bio-plex 6 software (Bio-
153 Rad Laboratories). Detection limits were 3.2 pg/ml (lower) and 2,000 pg/ml (upper) for
154 IL-5 and 3.2 pg/ml (lower) and 10,000 pg/ml (upper) for Eotaxin.

155 **Flow cytometry**

156 For flow cytometry analysis, dead cells were discriminated using LIVE/DEAD Fixable
157 Red Dead Cell Stain (Invitrogen) according to the manufacturer's instructions. Cells
158 were incubated in FACS Buffer (1X PBS containing 1% BSA and 5mM EDTA) with
159 antibody recognizing Fcγ II/III receptor (BD Biosciences) and then with the following
160 antibodies (purchased from BD unless otherwise stated: PE-Cy7- conjugated anti-CD3
161 (clone 145-2C11); V450-conjugated NKp46(CD335) (clone 29A1.4); APC H7 or APC
162 Cy7 conjugated anti-CD4 (clone GK1.5); AlexaFluor 700 conjugated anti-CD8a (clone
163 53-6.7); and PE conjugated anti-CD11a (clone 2D7). Intracellular staining of Foxp3, T-
164 bet, Gata3 and GzmB was performed using the Foxp3 staining kit (eBioscience)
165 according to the kit protocol and the following antibodies: AlexaFluor488-conjugated
166 anti-Foxp3 (clone FJK-16s), PE-conjugated anti-human/mouse T-bet (clone 4B10),

167 eFluor660-conjugated anti-human/mouse Gata3 (clone TWAJ) (all purchased from
168 eBioscience) and APC-conjugated anti-human GzmB (GB12, Invitrogen). Briefly, cells
169 were fixed for 30 mins in fixation buffer, washed with FACS buffer and then incubated in
170 permeabilization with antibodies for 1hr.

171 For intracellular detection of IFN- γ and IL-13, fresh cells were stimulated in 96-well
172 plates with 100ng/ml of PMA and 1 μ g/ml of Ionomycin or with 5 μ g/ml of M peptide (187-
173 195, NAITNAKII, Anaspec) in complete DMEM (DMEM medium supplemented with
174 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml
175 streptomycin). After 1h incubation, monensin (Golgi Stop, BD) was added. Cells were
176 incubated a further 3h, washed and stained for surface receptors. Intracellular cytokine
177 staining was performed using the Cytofix/Cytoperm kit (BD) according to manufacturer's
178 instructions using PerCP Cy5.5 conjugated anti-IFN- γ antibody (clone XMG1.2,
179 eBioscience) and PE-conjugated anti-IL-13 antibody (clone eBio13a, eBioscience).
180 Stimulation with the M-peptide was for 6h and monensin was added after 1h. Cells were
181 acquired on the LSR II or the Fortessa (BD) and data were analyzed using Flowjo
182 Software (Version 7.6.5). Cells were gated for live cells, singlets and lymphocytes and
183 then analyzed for indicated markers.

184 **Statistical analysis**

185 Results are presented as mean $\pm$ SEM. Statistical significance was determined using a
186 non-parametric, Mann-Whitney test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Values of $p < 0.05$
187 were considered significant (Prism software; Graph-Pad Software Inc.).

188 Results

189 Regulatory T cells limit and control inflammation after RSV infection

190 C57BL/6 mice can be infected with RSV, but are more resistant to RSV-induced illness
191 than the BALB/c mice used in most RSV studies (30). We infected wild type (WT)
192 C57BL/6 or *Foxp3*^{DTR} (DTR/DT) C57BL/6 mice with 8×10^5 FFU of intranasal human A2
193 RSV on day 0. *Foxp3*^{DTR} (DTR/DT) mice were depleted of *Foxp3*⁺ Treg cells by injection
194 of diphtheria toxin (DT) intraperitoneally (i.p) on days -1 and 3. Depletion was highly
195 effective, removing >90% of Treg cells from the lungs of *Foxp3*^{DTR} mice (DTR/DT/RSV)
196 on day 4 post infection compared to wild type (WT/RSV) controls (Fig. S1A). The
197 frequency of Tregs remained significantly reduced to day 8 in both the airways (BAL)
198 and lungs of depleted DTR/DT mice (Fig. S1B) although the total numbers of *Foxp3*⁺
199 Treg cells rebounded by day 8 post-infection in the airways (Fig S1C.). A significant loss
200 of Treg cells was also seen in uninfected Treg-depleted mice (DTR/DT) compared to
201 naïve wild-type (WT) controls (Fig. S1A and B).

202 Using weight loss as a measure of disease, we found that the depletion of Tregs
203 (DTR/DT/RSV) made mice more susceptible to disease, enhancing weight loss
204 beginning at day 7 and persisting out until day 14 post infection compared to wild type
205 controls (WT/RSV) (Fig. 1A). Pulmonary inflammation (assessed by determining total
206 cellular influx in the lungs and airways (BAL)) was significantly enhanced on day 8 post
207 infection in Treg-depleted DTR/DT/RSV mice compared to WT/RSV controls (Fig. 1B).
208 Furthermore, the inflammation persisted until at least day 14 post infection in Treg-
209 depleted mice.

210 One explanation for the enhanced pathology observed in the absence of Treg cells
 211 could be an inability to clear the virus. However, when RSV L gene was measured in
 212 the lungs of mice on day 4 (peak virus load) and day 8 (virus clearance) post infection,
 213 there was no detectable difference between WT and Treg-depleted mice (Fig. 1C).
 214 Instead, H&E staining of cytopins of the airway (BAL) cells showed a greater influx of
 215 eosinophils in the Treg-depleted DTR/DT/RSV but not in WT/RSV control mice (Fig.
 216 1D). Both eosinophils and neutrophils were detected in Treg-depleted mice until day 14
 217 post infection (Fig. 1D and E), indicative of an immune-mediated inflammation.
 218 Furthermore, this influx was associated with an increase in eotaxin levels in the BAL
 219 fluid of Treg-depleted mice compared to wild-type controls (Fig. 1F). Additionally, the
 220 Th2-associated cytokine interleukin (IL)-5 was only detectable in the airways of Treg-
 221 depleted mice and not WT mice following RSV infection (Fig. 1G). Together, these
 222 findings suggest that Treg cells are not essential for viral clearance but instead control
 223 the intensity and type of inflammatory response in the face of viral infection.

224 **T cell responses are enhanced in the absence of Tregs**

225 Loss of Treg cells resulted in enhanced weight loss, peaking at day 7 (Fig. 1A), which
 226 coincides with exuberant adaptive immune response. Therefore, we further
 227 characterized the T lymphocyte response in the lung and airways using flow cytometry.
 228 While there was a notable influx of both CD4⁺ and CD8⁺ T cells in the lungs and airways
 229 of WT mice infected with RSV compared to uninfected (DTR/DT) controls, both T cell
 230 subsets were significantly increased on day 8 post infection in the lungs and airways of
 231 Treg-depleted (DTR/DT/RSV) mice and T cell numbers remained heightened to day 14
 232 post infection (Fig. 2A and B). We further examined T cell activation by measuring

233 expression of granzyme B (GzmB), a marker of cytolytic activity, and CD11a (integrin
234 alpha L), a component of LFA-1 and a marker associated with anti-viral T cell responses
235 (34). The mean fluorescence intensity (MFI) of GzmB but not CD11a was increased on
236 both CD4⁺ and CD8⁺ T cells in the lungs of WT mice infected with RSV compared to
237 uninfected (DTR/DT) controls (Fig. 2C and D). However, expression of both activation
238 markers was even further enhanced on CD4⁺ and CD8⁺ T cells in the lungs of Treg-
239 depleted mice infected with RSV and this was significant at day 8 (for GzmB) or day 14
240 (for CD11a) post infection. Therefore, these data support that overactive and persistent
241 effector T cell responses contribute to immune pathology in Treg-depleted mice during
242 RSV infection.

243 **Antigen-specific CD8⁺ T cells are increased in the respiratory tract and spleen of**
244 **Treg-depleted mice following RSV challenge.**

245 Upon primary RSV challenge, there is a rapid expansion of antigen-specific CD8⁺ T
246 cells that contribute to effective viral clearance but may also lead to tissue pathology.
247 The predominant CD8⁺ T cell epitope induced by RSV challenge in C57BL/6 mice
248 recognizes the RSV M protein (34). Therefore, we quantified the M-peptide specific
249 CD8⁺T cell response in order to determine if increased antigen-specific T cell responses
250 could offer an explanation for the enhanced pathology with loss of Treg cells.

251 Lungs, BAL and spleens were isolated and cell homogenates were stimulated with RSV
252 M₁₈₇₋₁₉₅ peptide for 6hrs, following which IFN-γ expression was measured by
253 intracellular flow cytometry. As expected on day 8 post infection of WT mice, a clear
254 proportion of CD8⁺ T cells express IFN-γ in response to stimulation with M peptide and

255 the majority of these are localized to the airways (BAL; 16-26%) and lungs (11-20%)
 256 (Fig. 3A and B). There were a similar proportion of M peptide-specific CD8⁺ T cells in
 257 the BAL of control mice and Treg-depleted DTR mice despite the enhanced pathology
 258 in the Treg-depleted mice. However, the total numbers of M peptide-specific CD8⁺ T
 259 cells in Treg-depleted mice were increased in all of the tissues examined compared to
 260 WT mice (Fig. 3C), including the spleen, which suggests a less localized anti-viral T cell
 261 response in the absence of Treg cells.

262 We found that Treg-depleted RSV infected mice also develop a notable splenomegaly
 263 compared to WT mice, an effect persisting until day 14 post infection (Fig. 3D). The
 264 splenomegaly was characterized by expansion of both CD4⁺ and CD8⁺ T cells,
 265 providing evidence of systemic inflammatory T cell response (Figure 3E) after infection
 266 and Treg depletion (Fig. 3D and E). The weight loss in infected Treg-depleted mice
 267 therefore seems accounted for by enhanced local recruitment of inflammatory cells to
 268 the lung, an excessive RSV-specific CD8⁺ T cell response and evidence of systemic T
 269 cell activation.

270 **Enhanced Th2-type airway responses in Treg-depleted mice**

271 In addition to anti-viral CD8⁺ T cell responses, other factors might contribute to
 272 excessive pulmonary pathology in the Treg-depleted mice and thus we decided to
 273 further characterize the CD4⁺ T cell responses developing in the lung and airways
 274 following RSV challenge. To address this, we examined expression of Th1 and Th2-
 275 specifying cytokines and transcription factors using intracellular flow cytometry analysis
 276 of lung and BAL cells. Interestingly, at an early time point (day 4 post infection) there

277 was a significant increase in the proportion of CD4⁺ T cells expressing either IL-13 or
 278 IFN- γ in the lungs of Treg-depleted mice compared to WT controls, indicative of a mixed
 279 Th1 and Th2-type response (Fig. 4A and B). Additionally, expression of the Th2-
 280 associated transcription factor Gata3 in the lung cells on days 4 and 6 and in the BAL
 281 cells on day 14 (Fig. 4C and D), while the Th1-specifying transcription factor T-bet was
 282 enhanced on day 4 in the lungs only (Fig. 4E). By day 14, the majority (70%) of CD4⁺ T
 283 cells in the BAL expressed Gata3 while only a small proportion (3%) expressed T-bet in
 284 depleted mice (Fig. 4C-F). These findings correlate with the marked eosinophilia in the
 285 airways of Treg-depleted mice (Fig. 1D), also indicative of a Th2-type immune
 286 response.

287 Previous studies have shown that Foxp3⁺ Treg cells may also acquire expression of T
 288 helper cell-specifying transcription factors and this appears to be important for their
 289 suppressive function in different inflammatory settings (35-38). Therefore, we examined
 290 expression of T-bet and Gata3 on Foxp3⁺ Treg cells in the airways of WT and Treg-
 291 depleted mice following RSV infection. In WT mice, there was a clear proportion (10%)
 292 of CD4⁺ T cells in the BAL that co-expressed the Treg cell marker Foxp3 and the Th1
 293 cell marker T-bet at the peak of the adaptive response (days 6 and 8 post infection)
 294 (Fig. S2A). In the Treg depleted mice, there were very few Tregs at this time point (Fig.
 295 S2A). However, at day 14 post infection, Foxp3⁺ Treg cells reappeared in the airways of
 296 the Treg-depleted group and a high proportion (25%) of these cells co-expressed the
 297 Th2 marker Gata3 (Fig. S2B). In WT mice, the majority of Foxp3⁺ Treg cells left the
 298 airways by day 14 since pathology had resolved, and instead appeared at baseline
 299 proportions in the lungs (Fig. S2C). These findings render further support that Treg cells

300 function to limit effector T cell responses in the airways of RSV-challenged mice and
301 may switch their phenotype according to the type of inflammatory milieu.

302 Overall, this study provides further evidence for the essential role Treg cells play in
303 controlling inflammatory T cell responses and pathology in RSV infection. In particular,
304 we highlight novel roles for Treg cells in promoting resolution of disease by day 14 post
305 infection and preventing the persistence of Th2-type inflammation and eosinophilia.

306 **Discussion**

307 Pulmonary disease caused by RSV infection involves a complex interplay of
308 inflammatory and anti-inflammatory immune responses, tightly regulated by Foxp3⁺
309 Treg cells. We now provide evidence that Treg cells not only dampen overexuberant
310 and pathological T cell responses, but also limit Th2-type immune responses (e.g.
311 Gata3- and IL-13-expressing CD4⁺ T cells and eosinophilic influx into the airways).
312 Additionally, Treg cells suppress anti-viral CD8⁺ T cell responses and prevent systemic
313 manifestations in RSV infection. Our findings therefore support the concept that Tregs
314 play a central role in preventing pathology in viral bronchiolitis.

315 Perhaps the most exciting finding from our studies was that acute Treg cell depletion in
316 the *Foxp3*^{DTR} mice led to the appearance of a phased and mixed Th1/Th2-type immune
317 response following primary RSV infection. Previous studies have shown that the acute
318 loss of Treg cells alone can lead to a Th2-type immune response and pathology without
319 any additional antigenic stimulation (39) and in allergic inflammation, acute Treg
320 depletion at the time of sensitization (but not challenge) leads to an enhanced Th2 type
321 immune response and worsening of pathology (40-43). Mixed Th1/Th2-type immune
322 responses (including eosinophils, neutrophils and both IL-13- and IFN- γ -producing
323 CD4⁺ T cells) are also a feature of vaccine-enhanced RSV disease in mice and of
324 human bronchiolitis (44-46), indicating that dysregulation is the hallmark of RSV
325 disease.

326 We showed previously that RSV infected Treg-depleted DEREG mice (as well as mice
327 infected with RSV as neonates and rechallenged with RSV as adults) have a marked

328 eosinophilia associated with increased disease severity (30,47). Importantly, in the
329 Treg-depleted *Foxp3*^{DTR} mice, both the eosinophils and Th2 cells persist beyond the
330 peak of the anti-viral immune response and viral clearance, suggesting that they directly
331 contribute to the persistent pathology as reflected by delayed recovery from weight loss.
332 Thus, Treg cells not only drive an appropriate anti-viral response during RSV infection,
333 but also promote resolution of the disease.

334 Even more interesting was the observation that *Foxp3*⁺ Treg cells in the airways
335 acquired expression of Gata3, a marker of Th2 cells, in *Foxp3*^{DTR} mice at later time
336 points (day 14) following RSV infection. Previous studies have reported expression of
337 Gata3 by *Foxp3*⁺ Treg cells in mucosal sites like the intestine and skin and have shown
338 that expression of Gata3 is important for Treg cell-mediated suppression of
339 inflammation at these sites (38). In the FI-RSV vaccine model, Treg cells trafficking to
340 the airways is dependent on CCL17 and CCL22, chemokines typically associated with
341 Th2 cell responses and important in resolution of Th2-type inflammation (31). Similarly,
342 in the OVA model of allergic inflammation, expression of CCR4 (the receptor for CCL17
343 and CCL22) is important in the ability of Treg to suppress Th2 pathology (43). However,
344 mice tolerized to maternal OVA during their weaning period and then infected with RSV
345 displayed an excessive Th2-type pathology and this included expression of both Gata3
346 and Th2-type cytokines by *Foxp3*⁺ Treg cells, suggesting that these cells acquire
347 effector functions and contribute to worsened disease (48). Therefore, it would be
348 interesting in future studies to evaluate the role of these Gata3-expressing Treg cells in
349 RSV disease and to determine whether they maintain their suppressive function or are
350 converting to effector T cells that contribute to pathology.

351 Recent work has shown that induced (i) Treg cells (those generated in the tissues)
352 rather than thymically-derived natural (n) Treg cells are important for controlling Th2
353 responses and that specific depletion of iTreg cells by mutating a conserved non-coding
354 region in the *Foxp3* gene (*Foxp3*^{ΔCNS1}) resulted in pathology (49). Although we did not
355 distinguish between natural and induced Treg cells in our model, it would be interesting
356 in further studies to determine whether RSV infection in the *Foxp3*^{ΔCNS1} mouse model
357 results in a similar enhanced pathology or whether natural Tregs are capable of
358 controlling the immune response.

359 In RSV infection there is conflicting data concerning the role of Treg cells in regulating
360 the influx of antigen-specific CD8⁺ T cells in the lungs and previous studies demonstrate
361 that depletion of Treg cells can either result in an inhibited or enhanced antigen-specific
362 T cell response (24-26,30). We observed an enhanced number of peptide-specific
363 CD8⁺ T cells in the absence of Treg cells in all the tissues, including the spleen, after
364 RSV infection. This would suggest that Treg cells are important for controlling virus-
365 specific T cell responses not just in the airways but also systemically. Furthermore, the
366 excessive inflammatory milieu that develops in the Treg-depleted mice could directly
367 contribute to aberrant activation and homing of T effector cells to a variety of tissue sites
368 outside of the respiratory tract, thus prolonging disease resolution. In fact, previous work
369 has indicated that CD8⁺ T cells may be critical for controlling excessive Th2 responses
370 and eosinophilia in vaccine-enhanced RSV disease models (50,51). Therefore, the loss
371 of CD8⁺ T cells from the airways by day 14 post-infection, while the eosinophils and
372 CD4⁺ Th2 cells persisted, may further indicate a lack of control of these pathological
373 immune cells and contribute to the persistent disease in the respiratory tract.

374 Despite the enhanced anti-viral T cell response, we did not see any difference in viral
375 load in the lungs of WT and Treg-depleted mice and these findings contrast with
376 previous studies in our group using the DEREg model of acute Treg cell depletion (30).
377 Similar to the *Foxp3*^{DTR} model, DT treatment of DEREg mice is effective at short-term
378 systemic depletion of Foxp3⁺ Treg cells; however DEREg mice will not develop
379 systemic autoimmunity due to the gradual outgrowth of a suppressive Foxp3⁺gfp⁻
380 population (28). In contrast, *Foxp3*^{DTR} adult mice will succumb to a fatal autoimmunity
381 as early as 10 days after the first DT treatment if treatments are given every other day
382 (33). While experiments in this study involved only a short DT treatment course to avoid
383 complications from the systemic disease, it is certainly possible that the enhanced
384 inflammation that develops in the *Foxp3*^{DTR} mice could explain differences in antigen-
385 specific responses, pathological outcome and viral clearance compared to previous
386 studies using the DEREg mice or anti-CD25 treatment.

387 In conclusion, these studies provide further support for the concept that Tregs perform a
388 vital function in controlling the immune response during primary RSV infections. With
389 inadequate Treg responses, lung pathology is enhanced and prolonged, with an
390 immune phenotype similar to that seen in asthma. Although these effects may be
391 transient and only present in certain phases of disease, the long-term consequences of
392 Th2 'imprinting' of the respiratory mucosa (52) may account for the association between
393 severe infantile RSV infection and the development of wheezing disorders in later
394 childhood (9). If this is the case, strategies that boost Treg responses might not only
395 ameliorate disease but also result in long-term improvements in respiratory health.

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404

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589 **Figure Legends**

590

591 **Fig. 1. Regulatory T cells attenuate and modify responses to RSV infection.** Wild
 592 type (WT/RSV) or Treg-depleted *Foxp3*^{DTR} (DTR/DT/RSV) mice were infected with A2
 593 strain RSV intranasally on day 0. Uninfected wild type (WT) or Treg-depleted (DTR/DT)
 594 mice were used as controls. A) Illness was monitored by recording individual body
 595 weights daily and the mean ($\pm$ SEM) weight change for each group, measured as a
 596 percentage of the day 0 (initial) weight, is shown. Data are pooled from three
 597 experiments. B) Total cells in the lung and bronchoalveolar lavage (BAL) were
 598 quantified as a measure of inflammation. C) Expression of RSV L gene was quantified
 599 in the lung on days 4 and 8 post infection by qPCR. The dotted line shows the detection
 600 limit for the copy numbers of L-gene according to the standard curve. D) Numbers of
 601 eosinophils and neutrophils in the BAL were determined by performing H&E staining on
 602 cytopsin slides and quantifying numbers by differential cell counts. E) Representative
 603 BAL cytopsin from day 14 are shown with red arrows indicating eosinophils in Treg-
 604 depleted mice. BAL supernatants were analyzed for F) eotaxin and G) IL-5 protein
 605 levels using a multiplex kit. Data are representative of two or three independent
 606 experiments (n= 4-5 mice per group) unless otherwise indicated. WT/RSV vs
 607 DTR/DT/RSV **** p<0.0001 *** p<0.001 ** p<0.01 *p<0.05.

608 **Fig. 2. Treg depletion leads to enhanced activation and recruitment of T cells into**
 609 **the lungs and airways during RSV infection.** Following infection of wild type
 610 (WT/RSV) and Treg-depleted (DTR/DT/RSV) mice with RSV, whole lung and
 611 bronchoalveolar lavage (BAL) were harvested on days 4, 8 and 14 post-infection. A)

612 CD4⁺ and B) CD8⁺ T cells were quantified in the BAL and lung by flow cytometry
613 analysis. Expression (mean fluorescence intensity MFI) of activation markers including
614 GzmB and CD11a on both C) CD4⁺ and D) CD8⁺ T cells in the lung are shown. Data
615 are representative of two independent experiments (n=4-5 mice per group). WT/RSV vs
616 DTR/DT/RSV * p≤0.05, ** p≤0.01.

617 **Fig. 3. Peptide-specific CD8⁺ T cell responses are enhanced in the respiratory**
618 **tract and spleens of Treg-depleted mice during RSV infection.** Tissues were
619 harvested from wild type (WT/RSV) or Treg-depleted (DTR/DT/RSV) mice infected with
620 RSV at various days post infection. Following a 6h restimulation with the RSV M-
621 peptide, IFN-γ⁺ CD8⁺ T cells were quantified in the BAL, lung and spleen by FACS. A)
622 The frequency of CD8⁺ T cells expressing M peptide-specific IFN-γ among total CD8⁺ T
623 cells on day 8 post infection are shown with B) representative FACs plots for the various
624 tissues examined. C) Total numbers of M peptide-specific IFN-γ⁺ CD8⁺ T cells in all the
625 tissues from Treg-depleted or WT mice on day 8 post infection. Spleens were harvested
626 from RSV-infected WT or Treg-depleted mice on days 8 and 14 post infection. D) Total
627 spleen cells were quantified and E) total numbers of CD8⁺ and CD4⁺ T cells were
628 enumerated. Mice depleted of Treg cells (DTR/DT) but not infected with RSV were used
629 as controls. Data are representative of two or three independent experiments. *p<0.05
630 **p<0.01.

631 **Fig. 4. Regulatory T cells limit pathogenic Th2-type responses in the airways**
632 **following RSV infection.** Wild type (WT/RSV) and Treg-depleted (DTR/DT/RSV) mice
633 were challenged with RSV and tissues were harvested to examine CD4⁺ T cell
634 responses. A) The frequency of CD4⁺ T cells expressing cytokines IL-13 and IFN-γ

635 were quantified in the lung on day 4 post infection by flow cytometry and B)
 636 representative FACS plots are shown. C) The frequency of CD4⁺ T cells expressing the
 637 transcription factor Gata3 in the BAL and the lung at various days post infection with D)
 638 representative FACS plots from the BAL shown. E) The frequency of CD4⁺ T cells
 639 expressing the transcription factor T-bet in the BAL and lung at various days post
 640 infection with F) representative FACS plots from the BAL are shown. Data are
 641 representative of two independent experiments (n=3-4 mice per group). *p<0.05, ***
 642 p<0.001.

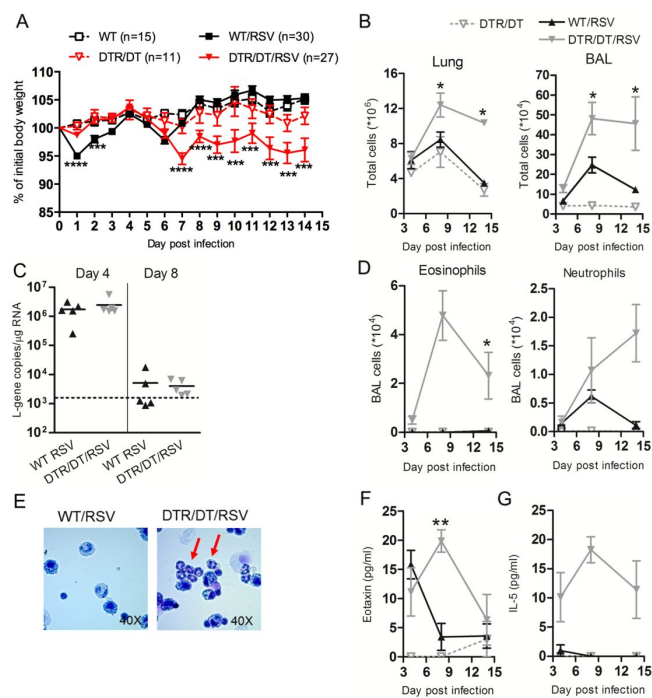


Fig. 1.

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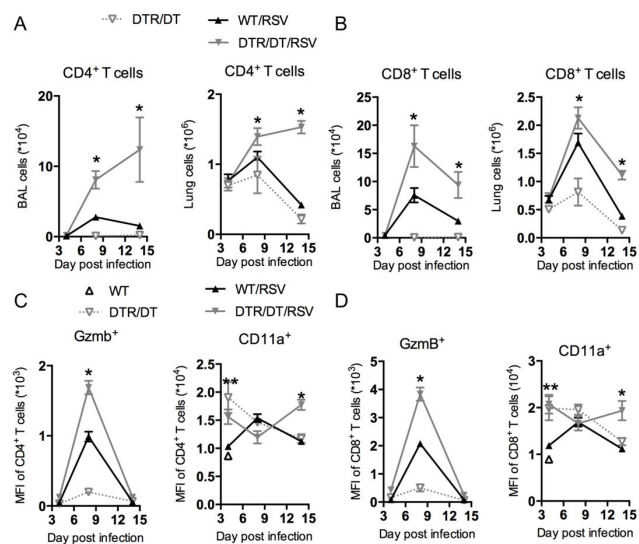


Fig. 2.

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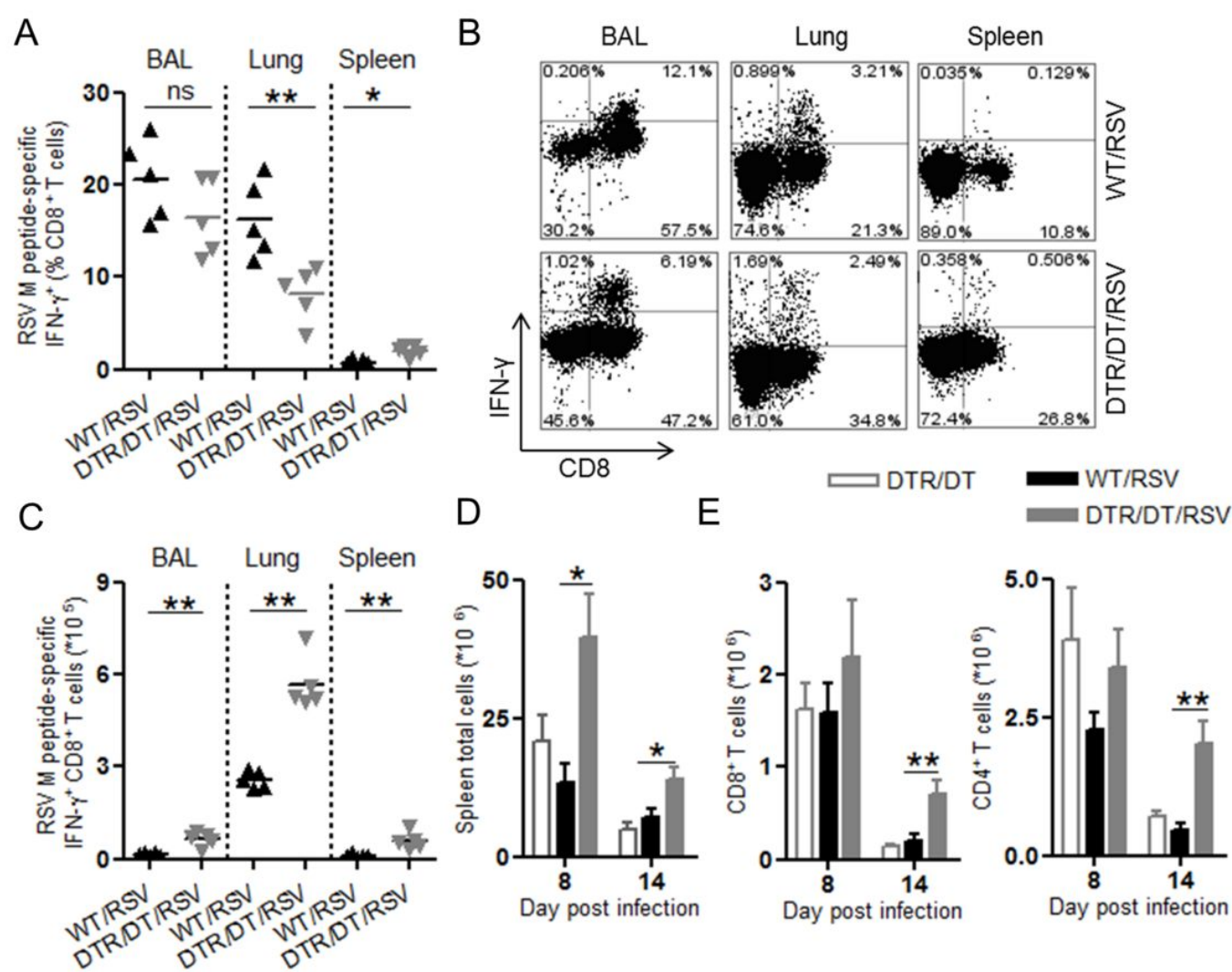


Fig. 3.

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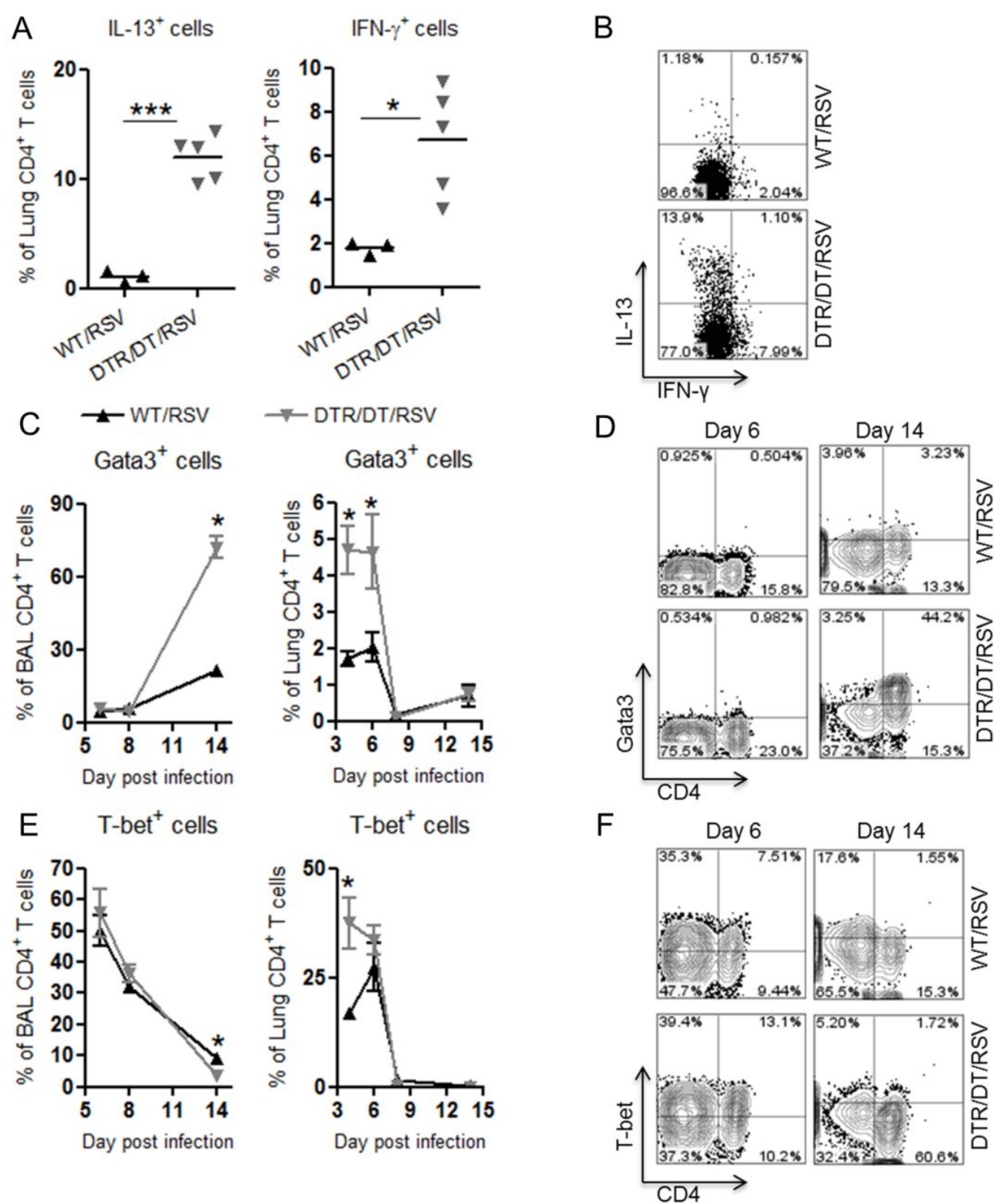


Fig. 4.

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