The role of APRIL in immunity and tumorigenicity
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CHAPTER 5

APRIL affects antibody responses and early leukocyte infiltration, but not influenza A viral control.

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Submitted
ABSTRACT

A Proliferation Inducing Ligand (APRIL) is implicated in the regulation of class switch recombination to IgA in T-independent B cell responses. Since B cells play an important role in the immunity to influenza A virus and resistance against the virus is partly controlled by T-independent IgA B cell responses, we studied the role of APRIL during an influenza A infection in vivo. APRIL transgenic, wildtype and APRIL deficient mice were intranasally infected with a non-lethal dose of a mouse adapted strain of influenza A. Compared to wildtype mice, APRIL deficient mice showed a two-fold reduction in the amount of macrophages in the lungs and a tendency towards decreased granulocyte influx in the early leukocyte recruitment phase. Although the T cell immune response against influenza was unaffected, APRIL Tg mice showed prolonged influenza-specific IgM production and differential class switching. Unexpectedly, the IgA B cell response was completely T helper cell dependent and also not affected by the absence or presence of APRIL. In addition, viral clearance and recovery from the infection was not influenced by APRIL. Combined these results indicate that APRIL affects specific aspects of the anti-influenza response, but plays a limited role in disease recovery.
INTRODUCTION

Influenza A is a single stranded RNA virus that can cause upper respiratory tract infections and can lead to serious morbidity and mortality as a consequence of secondary pneumonia [1, 2]. Protection against respiratory virus infections is accomplished by a combination of innate and adaptive defense mechanisms [3-5]. Considerable redundancy in the antiviral defense against influenza A virus exists as mice lacking IFNα/β receptor [6], CD4+ T [7], CD8+ T cells [8] or B cells [9] are able to resolve a moderate infection. Despite this redundancy, antibody production by influenza specific B cells coincides with viral clearance and improves removal of influenza A virus [5, 10-13]. Class switch recombination (CSR) plays an important role in protection against and recovery from influenza infection. Different antibody classes mediate different effector functions at distinct locations in influenza A virus infections. IgA is mainly involved in preventing viral attachment and neutralising the virus at the mucosal epithelium of the upper respiratory tract, whereas IgG isotypes are responsible for neutralising the virus at a later stage in the lungs [14-20]. In contrast to common belief, B cells can produce isotype-switched antibodies in response to a number of different bacterial and viral infections, without help from CD4+ T cells [21-23]. In the case of influenza there is also clear evidence for such thymus independent (TI) antiviral B cell responses [7, 24-27]. APRIL (A Proliferation Inducing Ligand) is a member of the TNF family of ligands, which is involved in CSR of TI B cell responses. In agreement, APRIL secretion by human DCs and intestinal epithelial cells leads to CSR of B cells to IgG and IgA [28-30] and APRIL plays a similar role in mice. Mice expressing human APRIL under the lck-distal promoter have elevated IgM, IgG [31] and IgA (GH – Immunology and Cell Biology 2008, in press) B cell responses after NP-Ficoll immunisation, a typical TI type 2 antigen. Furthermore these mice show increased B-1 B cell numbers with increased levels of natural IgA antibodies upon ageing [31, 32]. Two viable APRIL deficient mouse strains exist [33, 34]. The first generated by the group of Ashkenazi has no apparent phenotype and shows no difference in B cell responses after immunisations with TNP-KLH and Streptococcus pneumoniae. Also, no apparent differences in natural antibodies were observed in this study [33]. The APRIL deficient mice described by Castigli et al. have significantly decreased levels of serum IgA. In addition, both IgM and IgA serum antibody responses against the TI type 1 antigen NP-LPS, but not against NP-Ficoll, are impaired in these mice [34]. Recently, inducible nitric oxide synthase (iNOS) deficient mice were found to be impaired in TI IgA CSR via iNOS dependent regulation of APRIL and/or BAFF (B cell activating factor belonging to the TNF family) production of dendritic cells (DCs) [35]. Together, these observations indicate that the physiological role of APRIL in B cell responses seems to mainly impinge on the regulation of IgA
antibody production in TI responses. Based upon their location it is possible to distinguish two forms of IgA; serum IgA, which is monomeric or polymeric in human and mice respectively and secretory IgA (sIgA), which is polymeric. In addition, humans have two subclasses of IgA. Serum IgA is mainly of the IgA1 subtype, whereas sIgA is IgA2 [36]. Serum IgA can function as an inflammatory antibody. Interaction of antigen-antibody complexes with Fcα receptor on human myeloid cells facilitates antigen presentation and triggers a variety of effector mechanisms, such as antibody-dependent T cell-mediated cytotoxicity and phagocytosis [37-40]. sIgA is induced by intestinal dendritic and epithelial cells as a consequence of constant antigenic stimulation by the intestinal flora in a TI manner via APRIL and/or BAFF [29, 35, 41, 42]. sIgA has a critical role in the mucosal immune system, as it is constitutively released into the lumen to prevent binding of pathogens (including viruses) to epithelial cells [36]. Two additional functions for mucosal IgA have been proposed. IgA may be able to neutralise pathogens directly within epithelial cells and transport antigens that have crossed the epithelial cell barrier back to the lumen [43-45]. Since (1) B cells play such an important role in the immunity to influenza A virus, (2) resistance against the virus is dependent on TI IgA B cell responses and (3) APRIL plays an important role in regulating TI B cell responses, we decided to study the role of APRIL during influenza A virus infection in vivo.

MATERIALS AND METHODS

Animals
6-8 weeks old APRIL transgenic mice, wildtype littermates (WT) [31], and APRIL deficient mice [33], all on a C57BL/6 background were used in the experiments. Mice were bred in the animal facility of the Academic Medical Center. All experiments were approved by the institutional animal ethical committee.

Viral infection
Influenza infection was performed as described [46]. Briefly, mice were anesthetised using isoflurane inhalation (Abbott Laboratories, Kent, UK), weighed and inoculated intranasally with 1400 viral copies of influenza A/PR/8/34 (ATCC VR-95, Rockville, MD, USA) in 50 μL phosphate-buffered saline (PBS).

Flow cytometry
Single cell suspensions were prepared by homogenising lung, spleen and draining
lymph node on nylon mesh sieves. Erythrocytes were lysed by resuspending the cells in cold ACK lysing buffer and incubating the suspension at 4°C for 5 minutes. The cells were then washed twice with FACS staining buffer (PBS with 0.5% (w/v) bovine serum albumin and 0.02% Na-azide) and subsequently counted in a Coulter counter. Cells were stained with CD69 fitc (H1.2F3), Gr-1 PE (RB6-8C5), CD4 PerCP (RM4-5), CD8 APC (53-6.7) (all from BD Biosciences), F4/80 APC (A3-1) (from Serotec, Oxford, UK) and PE-labeled influenza specific MHC class I tetramers against the PR/8 influenza virus nucleoprotein epitope ASNENMETM (Sanquin, Amsterdam, The Netherlands) for 30 minutes at 4°C or at room temperature (for tetramer stainings). FACS analysis was performed on a FACS Canto II with FACS Diva software. CBA analysis was performed on a FACS Calibur with Cell Quest Software (all from BD Biosciences).

Cytokine assays
Lung homogenates were lysed with an equal volume of lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% (v/v) Triton X-100, 20 ng/mL pepstatin A, 20 ng/mL leupeptin and 20 ng/mL aprotinin at pH 7.4) for 30 minutes on ice, followed by centrifugation at 680 x g for 10 minutes. Supernatants were stored at –20°C until analysis. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-12p70, monocyte chemoattractant protein (MCP)-1 and interferon (IFN)-γ were measured in 1:5 diluted supernatants using the cytometric bead array (CBA) mouse inflammation kit (BD Biosciences, San Jose, CA, USA).

Immunoglobulin ELISA
Virus-specific Ig titers were determined in serum and lung supernatants. Plates were coated with 100 μL of live influenza virus (containing 10E5 TCID50/mL) in sodium carbonate buffer (pH 9.6) overnight at 4ºC. The wells were blocked with PBS containing 0.05% Tween-20 and 1% BSA at 1 hour at 37°C and incubated with diluted sera and supernatants in PBS containing 0.05% Tween-20 and 1% BSA for 2 hours at room temperature. HRP-conjugated isotype-specific antibodies (all from Southern Biotech, Birmingham, AL) were used as revealing antibodies. The plates were developed with 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 0.015% H2O2 (both from Sigma Aldrich, Zwijndrecht, the Netherlands) and the optical density at 405 nm was measured.

CD4+ T cell depletion
CD4+ T cell depletion was performed by intraperitoneal (IP) administration of 150 μg depleting CD4 antibody (clone GK1.5, Bioceros B.V., Utrecht, The Netherlands), 3 and 1 day prior to virus inoculation. To assure complete CD4+ T cell depletion during the
whole experiment, 75 μg GK1.5 was administered IP every 4 days thereafter.

**Determination of viral load**
Mice were anesthetised by intraperitoneal injection of Hypnorm (Janssen Pharmaceutics, Beerse, Belgium) and midazolam (Roche, Mijdrecht, The Netherlands), weighed and sacrificed by heart puncture. The lungs were harvested, weighed and homogenised on a nylon mesh sieve (BD Falcon, Oxford, UK) with a syringe plunger using 2 mL PBS. RNA was extracted from 100 μL of lung homogenate using 1 mL Trizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer’s protocol. RNA extraction was followed by cDNA synthesis using a random hexamer cDNA synthesis kit (Applera, Foster city, CA, USA) and quantitative real-time polymerase chain reaction (PCR) analysis [47].

**Statistical analysis**
Data are expressed as mean ± SEM. The Mann-Whitney U and student T-test were used to determine whether differences between experimental groups were significant. P < 0.05 was considered significant.

**RESULTS**

**Early leukocyte recruitment**
APRIL transgenic, APRIL deficient and WT mice were inoculated intranasally with a non-lethal dose of a mouse adapted strain of influenza A virus, PR/8/34. We first determined the magnitude and composition of the early leukocyte infiltrate in the lungs. Therefore we determined the number of granulocytes and macrophages in the lung single cell suspensions at day 3. Granulocytes and macrophages were identified on basis of expression of the granulocyte antigen Gr-1 and the macrophage marker F4/80. Granulocytes were defined as Gr-1hi and F4/80lo and macrophages as Gr-1lo and F4/80hi. No differences in the number of these leukocyte subsets were observed between APRIL Tg and WT mice. APRIL deficient mice however showed a significant decrease in the number of macrophages in the lung compared to WT mice and a tendency towards decreased numbers of granulocytes (Table 1). Importantly, no differences in the number of macrophages and granulocytes between WT and APRIL deficient mice were observed in unimmunised mice (data not shown). Concurring with the decreased number of infiltrating cells, we found significantly lower levels of IL-6 and MCP-1 in the lungs of APRIL deficient mice at day 3 (Figure 1).
T cell response
To investigate the role of APRIL in the cellular immune response to influenza A, the percentage of CD4+ T cells and CD8+ influenza tetramer+ T cells and the expression of the early activation marker CD69 on CD8+ T cells was determined in spleen and lung on day 3 and in spleen, lung and draining lymph node on day 10 and day 15 after infection. There was no significant difference in the percentage of CD4+, CD8+ T cells and CD8+ influenza tetramer+ T cells in the spleen, lung and draining lymph node between the different strains at the various time points (Table 2). The kinetics of CD69 expression on CD8+ T cells differed between the different compartments. CD69 expression on CD8+ T cells in spleen and draining lymph node was high on day 3 and/or 10 and decreased on day 15, whereas CD69 expression on CD8+ T cells in the lungs remained high over time. No difference in CD69 expression between the different strains in the different compartments were detected, except for a small decrease in the percentage of CD69-positive CD8+ T cells on day 10 in the spleen of APRIL deficient mice compared to WT mice (Figure 2a). Analysis of IL-12p70 and IFN-γ, cytokines mainly implicated in T cell responses, showed similar expression levels in all mouse strains (Figure 2b). Together, these data indicate that APRIL does not affect the T cell response to influenza A.

Figure 1. Decreased lung IL-6 and MCP-1 levels in APRIL deficient mice. Pulmonary cytokine and chemokine levels from APRIL Tg (black bars), WT (grey bars) and APRIL deficient mice (white bars) on day 3 after infection with influenza A. Data are mean ± SEM, n=7 mice per group. The asterix indicates statistical difference (Student's T test; IL-6, p=0.0447; MCP-1, p=0.0245) of APRIL deficient mice versus WT.
Humoral immune response

Immunoglobulins play an important role in the prevention of virus entry and virus neutralisation. Since antiviral antibodies in general and influenza A antibodies in particular can be produced in part in a TI fashion, APRIL likely plays a role in the humoral immune response against influenza A. At day 3, 10 and 15, influenza specific IgM, IgG and IgA levels were determined in the serum (Figure 3a and 3b) and lung supernatants (Figure 3c and 3d). There was a clear increase, followed by a decrease of IgM in both serum and lungs in the different mouse strains (Figure 3a and 3c). The serum and lung IgM levels of the APRIL transgenic mice were significantly higher compared to the IgM levels in the WT on day 15. The influenza specific IgM in the serum was already increased in the APRIL Tg mice at day 0 and this is in line with the increased natural antibody lev-

Figure 2. No difference in CD69 expression on CD8+ T cells and T cell cytokines. A. CD69 expression on CD8+ T cells in APRIL Tg (solid lines), WT (striped lines) and APRIL deficient mice (dotted lines) 3, 10 and 15 days after infection with influenza A. Data are mean ± SEM, n=7 mice per group. The asterix indicates statistical difference (Mann-Whitney U test, p=0.0175) of APRIL deficient mice versus WT. B. IL-12p70 and IFN-γ expression in the lungs of APRIL Tg (black bars), WT (grey bars) and APRIL deficient mice (white bars) on day 3, 10 and 15 after infection with influenza A. Data are mean ± SEM, n=7 mice per group.
Figure 3. Prolonged influenza specific IgM response in APRIL Tg and differential class switch recombination. A. Influenza specific IgM levels in unimmunised mice and on day 3, 10 and 15 after influenza A infection in serum of APRIL Tg (black circles or bars), WT (grey circles or bars) and APRIL deficient mice (white circles or bars). ΔIgM relative to influenza specific IgM levels of unimmunised mice are also shown (mean ± SEM, n=7 mice per group). Black lines are group averages and statistical significance (Mann-Whitney U test) versus WT is indicated with the P-value. B. Influenza specific total IgG, IgG1, IgG2a, IgG2b and IgG3 levels in unimmunised mice and on day 3, 10 and 15 after influenza A infection in serum of APRIL Tg, WT and APRIL deficient mice. Black lines are group averages and statistical significance (Mann-Whitney U test) versus WT is indicated with the P-value. C. Influenza specific IgM, total IgG, IgG1, IgG2a, IgG2b and IgG3 levels on day 3, 10 and 15 after influenza A infection in lungs of APRIL Tg, WT and APRIL deficient mice. Black lines are group averages and statistical significance (Mann-Whitney U test) versus WT is indicated with the P-value. D. Influenza specific IgA levels on day 3, 10 and 15 after influenza A infection in lungs of APRIL Tg, WT and APRIL deficient mice.
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However, the data clearly reveal that APRIL Tg mice had a considerably prolonged IgM response, as evidenced by analysis of the IgM levels above background (ΔIgM). At day 15, the IgM levels in serum remained high in the APRIL Tg mice, whereas the WT and APRIL deficient mice were already down to baseline (Figure 3a). Influenza specific IgG was barely detectable on day 0 and day 3 in both serum and lungs, but clearly induced at the later time points. No significant differences in total IgG were observed between the groups at these time points. However, closer analysis of the different IgG subtypes did reveal some differences. IgG2a (lung) and IgG2b (serum and lung) were slightly enhanced in the APRIL Tg mice on day 10 compared to WT. On day 15, IgG1 (lung and serum) and IgG2a (lung) were slightly lower in the APRIL Tg and APRIL deficient mice compared to WT. The relevance of this differential CSR to IgG subtypes is not yet clear. As APRIL has previously been shown to mainly affect IgA CSR in vivo [28-30, 34, 35], IgA titers were also determined. Influenza specific IgA could be detected in the lungs, but not in the serum, and increased over time. Despite the reported role of APRIL, IgA levels in the lungs between the different mouse strains did not differ (Figure 3d).
T cell dependency of B cell response
As APRIL mediates TI IgA production, these findings suggested that the influenza A IgA responses are not induced in a T-independent fashion. To confirm this, we studied the effect of CD4+ T cell depletion on the influenza specific IgA response. APRIL transgenic, WT and APRIL deficient mice were either treated with a depleting anti-CD4 antibody (GK1.5) or not treated, and inoculated with influenza A virus. 15 days after inoculation, the influenza specific IgA levels in the lung were determined. Compared to their non-depleted counterparts, CD4+-depleted APRIL transgenic, WT and APRIL deficient mice showed an almost complete reduction of antiviral IgA antibodies (Figure 4). The IgA responses in the different groups and the reduction to nearly baseline IgA levels after CD4+ T cell depletion shows that the induction of IgA during influenza A infection is for the most part dependent on CD4+ T cell help.

Body weights and viral loads
To determine whether the differences we observed in the early leukocyte response, in IgM production and the slight effects in CSR affected the clinical course of the disease, body weight and viral loads in lung supernatants were measured at day 3, 10 and 15 (Figure 5a). The body weights of the different strains showed a similar decline, reached the lowest point at day 10 and had recovered at day 15. The body weights between the different groups did not differ. The viral titers peaked at day 3, were reduced at day 10 and undetectable at day 15 and were comparable between the different strains (Figure 5b). In conclusion, we conclude that despite its role in the humoral response and the

![Graph](image-url)

Figure 4. Influenza specific IgA levels in lungs of APRIL Tg (black bars), WT (grey bars) and APRIL deficient mice (white bars) on day 15, with or without prior depletion of CD4+ T cells and with or without influenza A immunisation (indicated by + and -). Data are mean ± SEM, n=6 mice per group, statistical significance (Mann-Whitney U test) versus WT is indicated with the P-value.
clear effect of APRIL deficiency on macrophage numbers in the lungs, APRIL does not affect the clinical course of influenza A infection in mice.

DISCUSSION

B cell responses are an important part of the defense mechanism against respiratory viruses, such as influenza A virus. By the process of CSR, B cells expand their ability to adequately respond to viral infections. In agreement, IgG2a antibodies, which are often induced during viral infection, are superior in fixing complement and binding to Fc receptors on macrophages and NK cells [15] and IgA antibodies have the unique capacity to act against viruses in the lumen of the respiratory tract [36] before the actual infection. Since APRIL plays an important role in CSR during TI B cell responses [28-35] and antiviral antibodies can be produced in a TI fashion [7, 22-27], we studied the role of APRIL during influenza A virus infection. In an earlier report by Roque et al. [48], the effect of TACI-Ig, a receptor antagonist of APRIL and BAFF, on the immune response to influenza was studied. The TACI-Ig-treated mice showed a dose-dependent decrease in B cells and production of influenza-specific IgM and IgG but had no differences in survival or viral clearance. In the present study, we decided to specifically study the role of APRIL deficiency and overexpression during an influenza A virus infection.

The early phase of an influenza A infection is characterised by the extravasation of small numbers of blood-derived granulocytes, followed by large quantities of blood macrophages into the infected lung [49, 50]. A significant reduction in the number of macrophages in the lungs, APRIL does not affect the clinical course of influenza A infection in mice.

![Graph](image_url)

**Figure 5.** Similar body weights and lung viral loads. Body weight (A) and viral load (B) in APRIL Tg (solid squares and lines, black bars), WT (solid triangles up and striped lines, grey bars) and APRIL deficient mice (solid triangles down and dotted lines, white bars) 3, 10 and 15 days after infection with influenza A. (A) Body weights are relative to day 0. (B) Viral load is expressed as viral RNA copies per gram lung. N.D. means not detected. Data are mean ± SEM, n=7 mice per group.
rophages was detected in the early phase of the immune response against influenza A in the lungs of APRIL deficient mice. The decreased number of macrophages could point to impaired recruitment or impaired survival of this cell population. Macrophages are responsible for the production of inflammatory mediators, such as IL-6 and MIP-1α, and CCR2 is a major regulator of induced macrophage migration to infected lungs [51]. The significantly lower IL-6 levels in APRIL deficient mice could thus be the result of decreased macrophage numbers in the lung and the observed decreased levels of MCP-1, the ligand of CCR2, could be instrumental in impaired recruitment of this cell type to the lung. The decreased macrophage numbers did however not affect the viral loads in the lungs and the clinical course of the disease in the APRIL deficient mice. This is not surprising, since granulocytes and not macrophages are mainly responsible for protection in the early phase of the infection and selective depletion of macrophages does not influence the viral titer after influenza A infection [52]. Moreover, CCR2-deficient mice, which have significant defects in macrophage recruitment, even show decreased mortality after influenza A infection [51]. Even though, APRIL deficient mice show a tendency towards reduced granulocyte infiltration, it is clear from the resulting anti-viral response that this does not affect the course of the infection.

Analysis of the influenza specific antibody response in the APRIL Tg mice showed a clear effect on IgM production. These mice have increased levels of natural IgM antibodies, due to increased B-1 B cell activity [31]. This concurs with the increased influenza specific IgM titers in unimmunised mice observed in this study. In addition, the IgM titers in these mice remained high throughout the B cell response. Both B-1 and B-2 B cells play a role in the immune response against influenza infection in mice and B-1 B cell derived natural antibodies promote subsequent B-2 B cell IgG2a responses [53-56]. IgG2a is the predominant IgG isotype produced in response to virus infection [57] and the increased levels of influenza specific IgG2a in the lungs of APRIL Tg at day 10 thus suggest a positive effect of IgM. The significantly lower IgG2a levels in the lungs of APRIL Tg mice on day 15 can be the result of increased kinetics of this response. The increased levels of influenza specific IgG2b in the lungs of APRIL Tg at day 10 point at a similar positive effect on this Ig subtype.

As mentioned previously, APRIL is implicated in regulating IgA CSR in TI B cell responses and B cells produce isotype-switched antibodies in response to viral infections. Despite these implications, APRIL overexpression or deficiency did not affect the influenza specific IgA response. Furthermore, the IgA response against influenza A was completely dependent on T cells, indicating that it is not mediated by dendritic cell-derived APRIL or BAFF. APRIL therefore has no effect on viral clearance via the production of IgA. Combined these observations provide evidence for a role of APRIL in early macrophage recruitment and IgM production, but that viral control occurs independent of this TNF family member.
APRIL affects antibody responses and early leukocyte infiltration, but not influenza A viral control.

ACKNOWLEDGEMENTS

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Table 1. Decreased numbers of macrophages in lung of APRIL deficient mice. Total number of granulocytes and macrophages in lung at 3 days after influenza A infection.

<table>
<thead>
<tr>
<th>Absolute numbers *10E6</th>
<th>APRIL Tg</th>
<th>WT</th>
<th>APRIL deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>54.0</td>
<td>41.8</td>
<td>44.4</td>
</tr>
<tr>
<td>Macrophages</td>
<td>8.53</td>
<td>5.68</td>
<td>11.0</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>87.1</td>
<td>35.9</td>
<td>47.5</td>
</tr>
<tr>
<td>Macrophages</td>
<td>6.46</td>
<td>4.40</td>
<td>4.93</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>18.4</td>
<td>40.7</td>
<td>37.2</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.23</td>
<td>1.38</td>
<td>3.28</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>44.2</td>
<td>44.2</td>
<td>51.1</td>
</tr>
<tr>
<td>Macrophages</td>
<td>3.51</td>
<td>3.14</td>
<td>3.97</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>57.0</td>
<td>35.6</td>
<td>26.3</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.96</td>
<td>5.43</td>
<td>3.80</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>14.8</td>
<td>13.8</td>
<td>45.5</td>
</tr>
<tr>
<td>Macrophages</td>
<td>3.28</td>
<td>1.69</td>
<td>2.13</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>29.6</td>
<td>26.8</td>
<td>24.1</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.16</td>
<td>4.99</td>
<td>4.99</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>36.6</td>
<td>21.5</td>
<td>21.5</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1.85</td>
<td>6.29</td>
<td>6.29</td>
</tr>
</tbody>
</table>

Average (± s.d.)

- **Granulocytes** (± s.d.): 40.9 (± 11.4) 5.83 (± 2.91) 44.4 (± 22.3) 4.89 (± 1.28) 29.6 (± 13.4) 2.16 (± 0.63)

P-value compared to WT

- Granulocytes: 1.00 0.80 0.32 0.012
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Table 2. No differences in T cell recruitment. Percentage of CD4⁺, CD8⁺ and CD8⁺ influenza tetramer⁺ T cells in spleen, lung and draining lymph nodes (dLN) after influenza A infection.

<table>
<thead>
<tr>
<th></th>
<th>APRIL Tg</th>
<th>WT</th>
<th>APRIL deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>CD4⁺</td>
</tr>
<tr>
<td>Average percentage (± s.d.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>17.1 (2.7)</td>
<td>7.8 (2.3)</td>
<td>0.087 (0.057)</td>
</tr>
<tr>
<td>Day 10</td>
<td>14.7 (2.2)</td>
<td>10.3 (1.9)</td>
<td>7.5 (2.1)</td>
</tr>
<tr>
<td>Day 15</td>
<td>23.2 (3.3)</td>
<td>12.8 (1.6)</td>
<td>2.3 (1.0)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>0.15 (0.07)</td>
<td>0.093 (0.022)</td>
<td>0.18 (0.09)</td>
</tr>
<tr>
<td>Day 10</td>
<td>7.6 (2.6)</td>
<td>34.7 (6.6)</td>
<td>20.6 (10.3)</td>
</tr>
<tr>
<td>Day 15</td>
<td>0.30 (0.13)</td>
<td>0.50 (0.23)</td>
<td>12.1 (9.7)</td>
</tr>
<tr>
<td>dLN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>21.2 (9.0)</td>
<td>5.3 (1.8)</td>
<td>3.2 (2.1)</td>
</tr>
<tr>
<td>Day 15</td>
<td>25.3 (4.2)</td>
<td>8.5 (1.8)</td>
<td>1.2 (0.4)</td>
</tr>
</tbody>
</table>
19 Liew, F. Y., Russell, S. M., Appleyard, G., Brand, C. M. and Beale, J. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than


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