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The nature of innate and adaptive interleukin-17A responses in sham or bacterial inoculation

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Summary

Streptococcus pyogenes is the causative agent of numerous diseases ranging from benign infections (pharyngitis and impetigo) to severe infections associated with high mortality (necrotizing fasciitis and bacterial sepsis). As with other bacterial infections, there is considerable interest in characterizing the contribution of interleukin-17A (IL-17A) responses to protective immunity. We here show significant _il17a_ up-regulation by quantitative real-time PCR in secondary lymphoid organs, correlating with increased protein levels in the serum within a short time of _S. pyogenes_ infection. However, our data offer an important caveat to studies of IL-17A responsiveness following antigen inoculation, because enhanced levels of IL-17A were also detected in the serum of sham-infected mice, indicating that inoculation trauma alone can stimulate the production of this cytokine. This highlights the potency and speed of innate IL-17A immune responses after inoculation and the importance of proper and appropriate controls in comparative analysis of immune responses observed during microbial infection.

Keywords: bacterial immunity; infection; interleukin-17A; natural killer cells; *Streptococcus pyogenes*; T helper type 17 cells

Introduction

Interleukin-17A (IL-17A) was first described as a pro-inflammatory cytokine at the end of the twentieth century.[1] Much interest in this cytokine has been generated by the description in humans and mice of a discrete T helper type 17 (Th17) subset (IL-17A-producing CD4$^+$ T cells), initially believed to be critical for experimental allergic encephalomyelitis and then linked to other autoimmune diseases as well as to responses to infection.[2,3] Although much attention has centred on its production by T cells (CD8$^+$, CD4$^+$ and $\gamma\delta$ subsets), it is also secreted by other innate populations such as neutrophils, natural killer (NK) cells and invariant natural killer T (iNKT) cells.[4]

The precise role of IL-17A in responses to infection remains controversial. Most studies have focused on a physiological role of Th17 cells in defence against bacteria.[5] Production of IL-17A by CD4$^+$ T cells during *Streptococcus pneumoniae* or *Klebsiella pneumoniae* infection has been shown to have a protective effect in mice through the rapid recruitment of neutrophils that aid bacterial clearance.[6,7] Such a role has also been confirmed by antibody-mediated IL-17A or CD4$^+$ T-cell depletion experiments, which showed reversal of the recruitment of neutrophils and monocytes to the mucosal surface leading to increased bacterial colonization and poor survival outcome.[6] Over-expression of IL-17A in the pulmonary compartments has been shown to be beneficial, enhancing survival after lethal challenge with *K. pneumoniae*.[8]

Although a role for Th17 cells during infection has been demonstrated, innate immune cells such as $\gamma\delta$ T, NK and NKT cells also produce IL-17A in the face of infection and are considered to constitute the first line of host defence, acting before adaptive immunity can be initiated.[9–11] Interleukin-17A is secreted by $\gamma\delta$ T cells during *Escherichia coli* infection: upon cytokine depletion, decreased neutrophil recruitment to the site of infection is observed, resulting in impaired microbial clearance and increased bacterial burden.[12] A similar pattern of response by $\gamma\delta$ T cells has been described in *Listeria monocytogenes* and *Salmonella enterica* serovar enteritidis infection models.[13,14] The NK cells are an early source of IL-17A during toxoplasmosis and depletion of this immune population with anti-asialo-GM1 decreased serum IL-17A levels.[11] Taken together, these studies provide evidence for the

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protective role of IL-17A in the early immune response to serious bacterial infection.

However, production of IL-17A during infection has not always been correlated with protection. In the murine caecal ligation puncture model of polymicrobial sepsis, IL-17A from $\gamma\delta$ T cells was detected, but depletion of this cytokine led to a decrease in bacteraemia and a reduction in systemic pro-inflammatory cytokines [tumour necrosis factor-α (TNF-α), IL-1β and IL-6] and chemokines.[15] This offers a somewhat conflicting view suggesting that the consequence of IL-17A release in different disease models varies, depending on the nature and magnitude of the infection, conferring different survival outcomes.

Streptococcus pyogenes is a Group A Streptococcus (GAS),[16] the causative agent of diverse diseases, ranging from non-invasive ('strep throat', impetigo) to severely invasive (necrotizing fasciitis and bacterial sepsis). Many symptoms of sepsis have been attributed to a so-called 'cytokine storm', classically characterized by an excessive release of pro-inflammatory cytokines (TNF-α and IL-1β) leading to heightened systemic inflammatory responses observed in patients.[17–19] However, this may be an over-simplified view because administration of anti-cytokine antibodies, either in the clinical setting or in murine models, does not offer protection from toxic shock.[20]

Streptococcus pyogenes has a wide array of virulence factors implicated in pathogenesis and immune evasion, the most widely studied being the superantigens[21–23] and M proteins.[24,25] To date, only a handful of papers have been published relating to aspects of IL-17A release during *S. pyogenes* infection. Purified superantigens from *S. pyogenes* and superantigen-contaminated preparations of peptidoglycan are potent inducers of IL-17A from T cells.[26] Patients with GAS infections including streptococcal toxic shock syndrome show elevated levels of $\gamma\delta$ T cells, though IL-17A release was not analysed.[27] Tonsil cultures from patients with recurrent GAS-associated tonsillitis can be stimulated with heat-killed M1 serotype GAS to produce IL-17A together with transforming growth factor-β, which suggests the possible differentiation of Th17 cells.[28]

With a potential role for adaptive Th17 cells during *S. pyogenes* infection previously reported, the innate source of IL-17A has often been overlooked. We sought to clarify the contribution of IL-17A from innate cell types during GAS infection. Using a murine model of acute sepsis, we demonstrated rapid up-regulation of both *il17a* transcript and serum IL-17A levels in *S. pyogenes*-infected mice. To our surprise, we also detected IL-17A responses in sham-treated animals; with NK and CD4$^+$ T cells representing the main producers of this pro-inflammatory cytokine in both PBS-treated and GAS-treated mice. This study provides evidence that early IL-17A responses are initiated by tissue damage and trauma caused by the route of inoculation or by bacterial infection. Th17 responses are also observed within hours, suggesting that the CD4/Th17 cells are closer to the innate/adaptive interface than previously recognized and play a more essential role in the acute response to trauma and infection.

Materials and methods

Mice

HLA-DQ8.Aβ^0 transgenic mice, used for their heightened sensitivity to streptococcal superantigen and GAS infection,[29] were bred on-site and maintained in accordance with UK Home Office guidelines. Female mice used in the experiments were aged between 10 and 21 weeks, and age-matched in any given infection experiment.

Acute sepsis infection model

Streptococcus pyogenes (NCTC8198) was cultured in Todd–Hewitt broth (Oxoid, Basingstoke, UK) overnight at 37° in 5% CO_2. The next day, the culture was washed three times in sterile PBS by centrifugation at 3000 ***g***, for 15 min at 4° to remove contaminating broth and resuspended in injection-grade sterile saline. To study IL-17A responses during the time–course of infection, 50 µl bacterial suspension or sterile saline (as sham control) was given via the intramuscular (i.m.) route into the right thigh of female mice at $t = 0$ hr. The bacterial inoculum was quantified by serial plating on to columbia blood agar plates (Oxoid) and was between 10^8 and 10^9 colony-forming units per dose. Sterile saline inoculum was also plated out and no bacterial contamination was detected after 24 hr of incubation. At per-determined time-points post-infection, groups of 10 infected mice or groups of five sham-treated mice were killed for analysis (carried out as part of two independent experiments). Tissue was also obtained from five non-inoculated naive female mice for normalization of quantitative reverse transcription (qRT-) PCR data. For *ex vivo* IL-17A cytokine flow cytometry, female mice were inoculated with *S. pyogenes* ($n = 4$) or PBS ($n = 3$) as described above, and killed at 24 hr along with untreated naive mice ($n = 3$).

Quantitative RT-PCR

Spleen and inguinal draining lymph nodes were harvested from naive, sham-infected or infected mice at 4, 8, 12 or 24 hr and stored in RNAlater™ RNA Stabilisation Reagent (Qiagen, Crawley, UK) at −80° until ready for use. Total RNA was extracted using the acid phenol method with TRIzol (Invitrogen, Paisley, UK) as per the manufacturer's instructions before resuspension in RNase-free water containing RNaseOUT Recombinant

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Ribonuclease Inhibitor (Invitrogen). Concentration of RNA and the nucleic acid : protein ratio were analysed using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). One microgram of total RNA was reverse transcribed into cDNA using Superscript® III Reverse Transcriptase (Invitrogen). A qRT-PCR was run on cDNA samples in triplicates using in-house-designed target-gene-specific primers and hydrolysis probes (Table 1) on an MX3000P real-time PCR thermocycler (Agilent Technologies, Inc., Santa Clara, CA). Reactions were performed in 20 µl total volume with a thermal profile of 50° for 2 min, followed by 10 min at 95° and then 50 cycles of 15 seconds at 95°, 15 seconds at 60° and 15 seconds at 72°. The fold changes in gene expression levels, relative to unimmunized naive samples, were normalized to two tissue-specific reference genes and calculated based on the $\Delta\Delta C_T$ method,[30] along with differential amplification efficiencies and randomization statistical analysis using the Relative Expression Software Tool (rest) (Qiagen, Hilden, Germany).[31]

ELISA

Blood from naive, infected or sham-infected mice was collected by cardiac puncture. Serum IL-17A concentrations were determined by sandwich ELISA (MABTECH AB, Nacka Strand, Sweden) and optical densities of samples against an IL-17A standard curve were measured using an ELISA plate reader (µQuant BIO-Tek Instruments, Inc., Winooski, VT) and KC Junior software.

Ex vivo intracellular IL-17A flow cytometry

Female mice inoculated with *S. pyogenes* or with PBS or left untreated (naive) were given 50 µg brefeldin A (BFA; Sigma-Aldrich, Poole, UK) or PBS via an intraperitoneal (i.p.) route in a volume of 100 µl at 20 hr to stop the release of cytokines from cells in the last 4 hr of infection.[32] Mice were killed at 24 hr and inguinal draining lymph nodes and spleens were harvested and homogenized into a single cell suspension in PBS containing 10% fetal calf serum using cell strainers (BD, Oxford, UK). Cells were washed twice in cold PBS (10% fetal calf serum) before blocking with Fc Block (eBioscience, San Diego, CA) for 10 min on ice. Surface staining was carried out with anti-mouse CD3 V500-conjugated, anti-mouse T-cell receptor-$\gamma\delta$ phycoerythrin-conjugated, anti-CD4 allophycocyanin-H7-conjugated and anti-mouse NK1.1 phycoerythrin-Cy7-conjugated (all BD) for 20 min at 4°. Cells were washed and resuspended in 1× Fix/Perm solution (eBioscience) for 30 min at 4° before being washed twice in 1× permeabilization buffer (eBioscience) and intracellular IL-17A was stained using AlexaFluor 647-conjugated anti-mouse IL-17A (BD) for 30 min at 4° before washing and fixation in 1% paraformaldehyde. The IL-17A Fluorescence Minus One (FMO) controls were used to determine positive populations (Fig. S1) and samples were run on a BD FACSAria II™ flow sorter (BD, Mountain View, CA) and analysed using FlowJo software (Treestar, Ashland, OR).

Statistical analysis

Statistical analysis of qRT-PCR data was performed using rest software. Data are presented as mean ± SEM. Any significant differences between treatment groups for ELISA and *ex vivo* IL-17A flow cytometry were determined with a Kruskal–Wallis significance test using Graphpad prism 4.0 software (Graphpad Inc., La Jolla, CA).

Results

Rapid induction of *il17a* expression after inoculation

Female HLA-DQ8.Aβ^0 transgenic mice were used in this study because of their increased susceptibility to *S. pyogenes* infection and superantigen sensitivity.[29] Female mice received either *S. pyogenes* ($n = 10$ per time-point) or PBS ($n = 5$ per time-point) using the intramuscular

Table 1. Quantitative real-time reverse transcription-PCR primers and hydrolysis probes

Gene	Sense primer	Anti-sense primer	6FAM-probe-TAMRA
b2m	CTACTGGGATCGAGACATTGTGAT	TGTGTACATTGCTATTTCTTTCTGC	TGCTCTGAAGATTCATTTGAACCTGCT
gapdh	GAGAAACCTGCCAAGTGTGATGAC	AGACAACCTGGTCCTCAGTGTAG	TCAAGAAGGTGGTGAAGCAGGCATC
tfrc	AATGGTAACTTAGACCCAGTGGAG	ATTAGCATGGACCAGTTTACCAGA	TCCCGAGGGTTATGTGGCATTCAGT
tbp	CAGTGCCCAGCATCACTATTT	GCATCCTCTGAATATCTCCTTAGAA	CATGGTGTGAAGATAACCCAGAACA
il17a	CTGTGTGTGTGATGCTGTTGCT	AAGGGAGTTAAAGACTTTGAGGTTG	AGCTCAGCGTGTCCAAACACTGAGG
rorc	GTCTGCAAGTCCTTCCGAGAG	ATCTCCCACATTGACTTCTG	CTGCGACTGGAGGACCTTCTACGGC
il6	GTTCCTCTCTGCAAGAGACTTCC	GTATCCTCTGTGAAGTCTCCTCTCC	CTTGGGACTGATGCTGGTGACAACC
tgfb	ATGTTCTTCAATACGTCAGACATTC	TTGCTATATTTCTGGTAGAGTTCCA	GCAGAGCTGCGCTTGCAGAGATTAA

Sequences of sense, anti-sense and probes used for quantification of fold changes in gene expression. *b2m*, *gapdh*, *tfrc* and *tbp* were used as tissue-specific reference genes to normalize the fold change in *il17a*, *rorc*, *il6 and tgfb* expression.

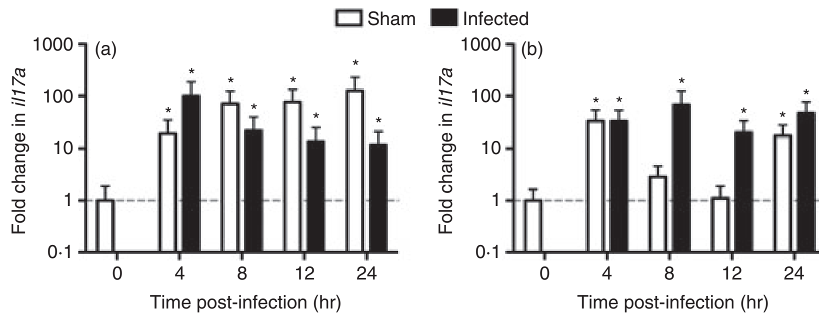

Figure 1. Fold change in expression of *il17a* after *Streptococcus pyogenes* infection or sham infection. HLA-DQ8.Aβ^0 mice were injected intramuscularly with $1{\cdot}1 \times 10^9$ colony-forming units *S. pyogenes* (n = 10 per time-point) or PBS (n = 5 per time-point) at t = 0 hr. Draining lymph nodes (a) or spleens (b) were taken at t = 0, 4, 8, 12 and 24 hr for quantitative real-time reverse transcription-PCR. Sham and infected samples were normalized to naive uninfected mice (n = 5) and to two tissue-specific reference genes. Fold change in *il17a* expression was determined using REST software and any significant differences (*P < 0·05) relative to naive samples are indicated.

route to mimic acute septic infection and were killed at defined time-points. Naive, untreated mice (n = 5 females) were also killed, one at each time-point, and pooled together to form the normalization group (t = 0). From qRT-PCR analysis, it was observed that there was rapid up-regulation in *il17a* expression in the draining lymph nodes after GAS infection, peaking at 4 hr, then slowly declining and remaining steady from 12 to 24 hr post-infection compared with unimmunized mice (Fig. 1a). Interestingly, sham infection with sterile saline also produced swift expression of *il17a* in the lymph nodes and expression remained higher than in infected samples even 24 hr after treatment (Fig. 1a). Increased transcription of *il17a* was also observed in the spleen (Fig. 1b) but with no significant difference between sham-treated and naive mice at 8 and 12 hr post-treatment. This suggested a return to basal levels of *il17a* expression in the spleen at the intermediate time-points, whereas *S. pyogenes*-infected mice displayed more sustained up-regulation of *il17a* transcription throughout the time–course of infection (Fig. 1b). Hence, inoculation with either saline or *S. pyogenes* induced a dramatic increase in *il17a* expression in the secondary lymphoid organs analysed.

Elevated IL-17A levels in the serum were detected post-inoculation

The rapid up-regulation in *il17a* gene expression was matched by serum IL-17A concentrations. Serum IL-17A was markedly elevated 4 hr after infection with *S. pyogenes*, but was reduced at 8 hr before rising again during the later stages of infection. The data were in contrast to those for naive controls (t = 0 hr), which displayed low background levels of IL-17A in the serum (Fig. 2). Sham infection with saline resulted in strong production of IL-17A, which followed a similar bimodal pattern to GAS-infected samples (Fig. 2). Taken together, inoculation with either saline or *S. pyogenes* resulted in elevated levels of IL-17A in the serum.

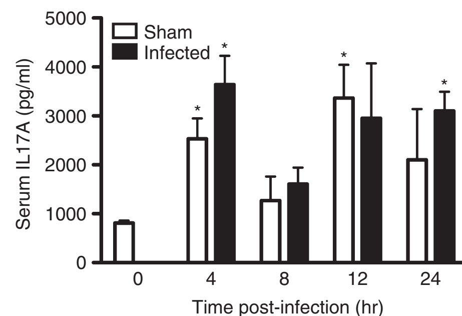

Figure 2. Serum interleukin-17A (IL-17A) released after *Streptococcus pyogenes* infection or sham infection. HLA-DQ8.Aβ^0 mice were injected intramuscularly with $1{\cdot}1 \times 10^9$ colony-forming units *S. pyogenes* (n = 6 per time-point) or PBS (n = 3 per time-point) or naive (n = 4) at t = 0 hr. Serum was collected from mice at t = 0, 4, 8, 12 and 24 hr by cardiac puncture for serum IL-17A analysis by ELISA. Any significant differences between sham or infected samples compared with naive mice are shown above (*P < 0·05). No significant differences were found between time-matched sham or infected samples).

Effects of inoculation on *rorc* gene expression

Quantitative RT-PCR analysis was also carried out on the draining inguinal lymph node and spleen tissue to analyse any changes in *rorc*, a hallmark transcription marker of Th17 cells, to determine the contribution of this cell type during immunization.[33] Paradoxically, this transcript was significantly down-regulated, both in sham-infected and *S. pyogenes*-infected lymph node samples (Fig. 3a) relative to naive uninfected controls, despite an increase in *il17a* transcription (Fig. 1a). This suggested that increased IL-17A in draining lymph nodes may not be the result of rapid induction of Th17 cells, but may be produced from some other cell-type. In contrast to changes in the draining lymph node, transcription of *rorc* increased in the

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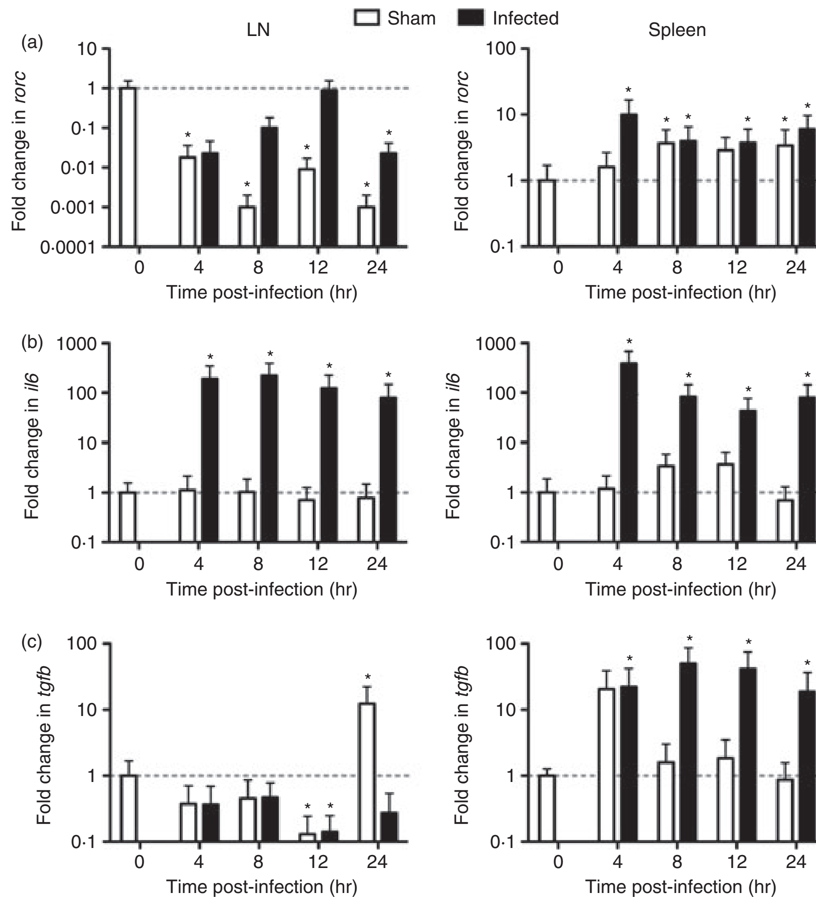

Figure 3. Fold change in expression of *rorc* during *Streptococcus pyogenes* infection or sham infection. HLA-DQ8.Aβ^0 mice were injected intramuscularly with $1{\cdot}1 \times 10^9$ colony-forming units *S. pyogenes* ($n = 10$ per time-point) or PBS ($n = 5$ per time-point) at $t = 0$ hr. Draining lymph nodes and spleens were taken at $t = 0$, 4, 8, 12 and 24 hr for quantitative real-time reverse transcription-PCR analysis. Fold change in *rorc* (a), *il6* (b) and *tgfb* (c) expressions was determined using REST software and any significant differences (*$P < 0{\cdot}05$) relative to naive samples are indicated.

spleen following both GAS infection and sham infection (Fig. 3a). These changes in the spleen may represent a delayed, systemic Th17 response to GAS infection or injection trauma and could contribute to the released IL-17A in the serum as observed in Fig. 2.

The qRT-PCR analysis of fold changes in *il6* and *tgfb* expression between GAS-infected and sham-infected samples was also performed as these two cytokines are essential for RORγt induction and hence Th17 differentiation.[34,35] Expression of the *il6* gene was significantly up-regulated only in *S. pyogenes*-infected samples relative to naive controls in the draining lymph node and spleen (Fig. 3b). This was also mirrored by *tgfb* expression in GAS-infected spleen (Fig. 3c). As a result, up-regulation of *il6* and *tgfb* expression in the spleen seems to correlate with induction of *rorc* expression, whereas up-regulation of *il6* in the draining lymph nodes and in the absence of *tgfb* expression was not sufficient to induce *rorc* expression and Th17 cell differentiation.

Identification of innate and adaptive sources of IL-17A post-inoculation

The cellular source of IL-17A was determined by *ex vivo* intracellular cytokine flow cytometry, allowing identification of IL-17A producing cell types following either GAS or sham infection. IL-17A$^+$ cellular populations were determined in the draining lymph node of naive, sham-infected or GAS-infected mice. In the last 4 hr of i.m. immunization, BFA was given i.p. as part of the *ex vivo* staining protocol and PBS was used as control for this injection. The percentage of IL-17A$^+$ cells from the total population was not significantly different in the naive controls given either BFA or PBS. This suggests that the administration of chemicals into the peritoneal cavity did not affect the percentage of cells producing IL-17A (Fig. 4). Interestingly, mice treated with i.m. immunization (either sham-infected or GAS-infected) and BFA i.p. exhibited a slight (but not significant) decrease in the

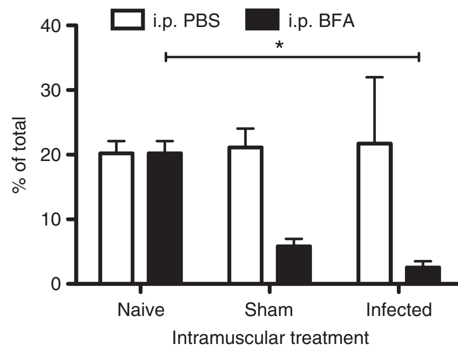

Figure 4. Flow cytometric staining of interleukin-17A-positive (IL-17A$^+$) cells after infection or immunization. Inguinal draining lymph nodes were harvested from untreated naive HLA-DQ8.Aβ^0 mice (n = 3) or *Streptococcus pyogenes*-infected (n = 4) or PBS-immunized (n = 3) mice at t = 24 hr. In the last 4 hr of immunization, PBS or brefeldin A (BFA) was given intraperitoneally before mice were culled and cells were stained for IL-17A production and analysed by flow cytometry. Fluorescence Minus One (FMO) controls were used to determine IL-17A$^+$ populations. The percentage of IL-17A$^+$ cells from total cells are shown for naive, sham and infected mice. Any significant differences are indicated (*P < 0·05) using the Kruskal–Wallis statistical test.

percentage of IL-17A$^+$-expressing cells compared with mice given PBS i.p. (Fig. 4). Infected mice given BFA contained a significantly lower percentage of IL-17A$^+$ cells compared with naive mice also treated with BFA (Fig. 4).

To determine the precise cells capable of producing IL-17A, a further *ex vivo* flow cytometric analysis was conducted (Fig. 5a). In naive animals, the predominant cell types in the draining lymph node to produce IL-17A upon treatment with PBS i.p. appeared to be NK and CD4$^+$ T cells (Fig. 5b). This was also true for sham-infected and GAS-infected mice given PBS i.p. (Fig. 5b). Mice treated with BFA i.p. had a reduced percentage of IL-17A$^+$ expression cells compared with mice given PBS (Fig. 5b). Significantly fewer NK cells and $\gamma\delta$ T cells expressed IL-17A in the sham-infected mice treated with BFA compared with the PBS control group (Fig. 5b). Naive mice treated with BFA had significantly fewer $\gamma\delta$ T cells compared with infected mice given BFA (Fig. 5b). This pattern of reduced cell types expressing IL-17A appears to agree with the decrease in the percentage of IL-17A$^+$ cells of the total population (Fig. 4). Overall, IL-17A synthesis in either naive, sham-infected or GAS-infected mice treated with PBS was primarily from NK and CD4$^+$ T cells.

Discussion

Interleukin-17A is the most widely studied from a family of five related cytokines from the IL-17 family (A–F).[36] Interleukin-17A has 50% homology with IL-17F and shares signalling via the heterodimeric IL-17RA and IL-17RC subunit receptor complex ubiquitously expressed on many different cells.[37] Hence, IL-17A can bring about immunological effects on various cell types (including non-lymphoid cells such as epithelial and mesenchymal cells) via a diverse array of target genes mediated by signal transduction downstream of its receptor.[38] Key examples of target genes include pro-inflammatory chemokines (CXCL1, CXCL8), cytokines (TNF-α, IL-6 and IL-1), anti-microbial peptides and tissue remodelling factors.[39]

Different roles in immunity have been postulated for IL-17A since its discovery in the early 1990s. One potent source of this pro-inflammatory cytokine was found to be adaptive CD4$^+$ T cells, termed Th17 cells in line with the Th1 and Th2 subsets postulated by Mosmann *et al.* in 1986.[40] Since then, most research has been dedicated to the role of Th17 cells in host defence in infectious diseases or contribution in the development of autoimmunity (e.g. experimental allergic encephalomyelitis, rheumatoid arthritis, inflammatory bowel disease).[5,41] However, in the past few years, interest has been generated as innate cell types such as $\gamma\delta$ T, NK and NKT cells were found to also secrete IL-17A in response to microbial stimuli.[4] Despite our current understanding, the precise interplay between innate and adaptive sources of, or the role of, IL-17A and downstream consequences during the early stages of acute bacterial infection have not been fully elucidated. In this study we sought to characterize both adaptive and innate sources of IL-17A during a time–course of acute *S. pyogenes* infection using a humanized murine model.

In this study, up-regulated *il17a* gene expression and protein production were detected following *S. pyogenes* infection in the inguinal draining lymph node, spleen and serum, respectively. However, using a stringent negative control not always included in infection protocols, it was noted that sham infection with sterile saline could induce changes of similar magnitude in *il17a* expression (Fig. 1) and protein production (Fig. 2) despite the absence of pathogen or antigen. Sham infection induced a marginally larger and longer lasting effect in *il17a* gene expression compared with infected samples.

The main sources of IL-17A in naive, sham or infected mice treated with PBS i.p. were NK and CD4$^+$ T cells, implying that both innate and unexpectedly early adaptive immune cells are involved in the production of IL-17A (Fig. 5). Interestingly, *rorc* transcription actually decreased following *S. pyogenes* infection or sham infection in the local draining lymph node when normalized to unimmunized naive controls, indicative of fewer Th17 cells migrating to the site of trauma or infection or even of Th17 differentiation. This was supported by down-regulation of expression of one of the key cytokines; *tgfb* required for Th17 differentiation in both sham-treated and GAS-treated samples compared with naive tissue. The CD4$^+$ T cells detected by *ex vivo* IL-17A staining in the draining lymph nodes of infected mice may be pre-existing

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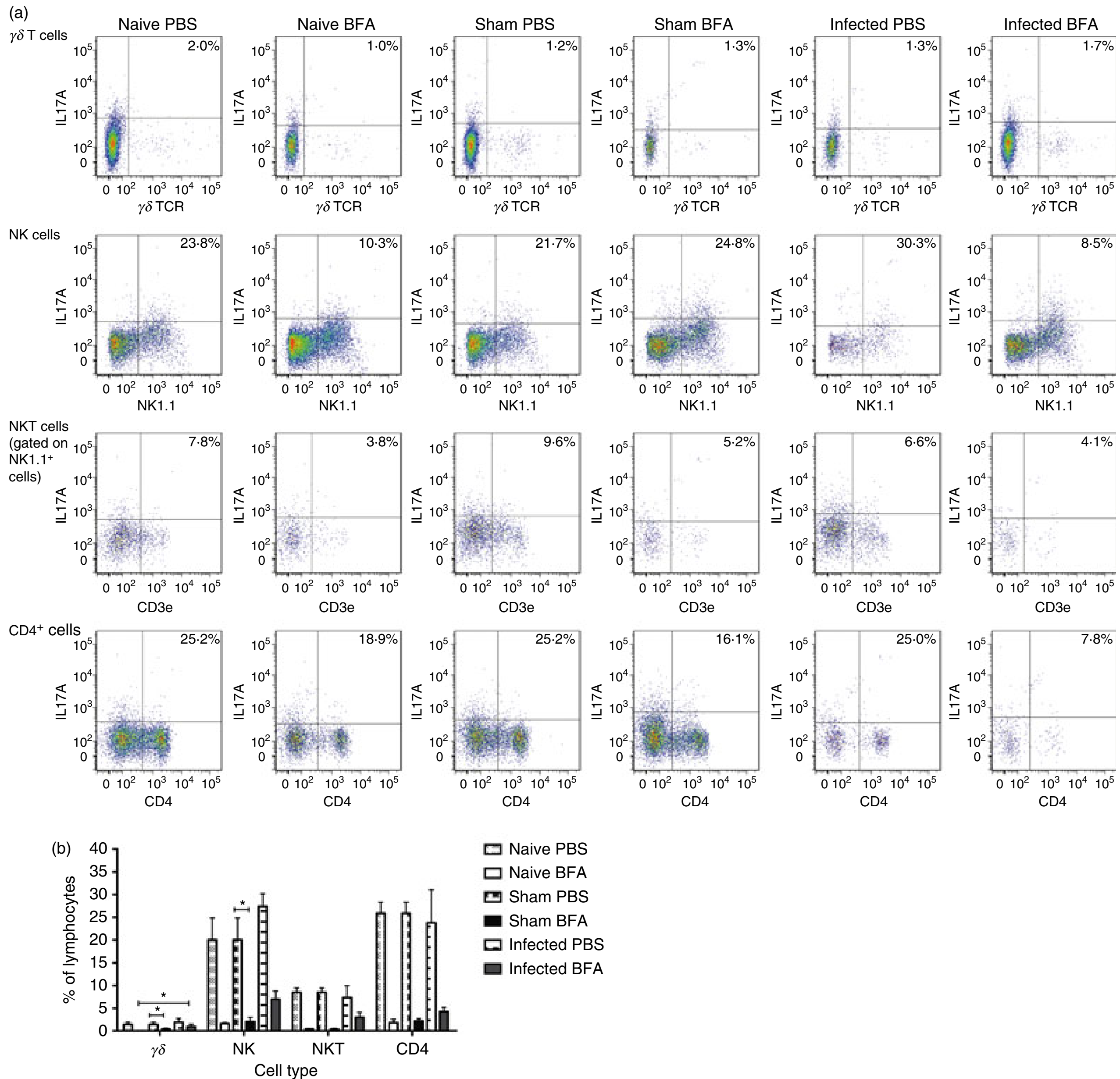

Figure 5. Immune cell populations producing interleukin-17A (IL-17A) during *Streptococcus pyogenes* infection or sham infection. Inguinal draining lymph nodes were taken from naive (n = 3) HLA-DQ8.Aβ^0 mice or from mice inoculated intramuscularly with $6{\cdot}5 \times 10^8$ CFU *S. pyogenes* (n = 4) or PBS (n = 3) for 24 hr. In the last 4 hr, PBS or brefeldin A (BFA) was given intraperitoneally before mice were culled and cells were stained for IL-17A production in various cell types and analysed by flow cytometry. Representative dot-plots are shown above (a) with the percentage of IL-17A$^+$ cells in the lymphocyte gate show in the top-right corner of the plot. Collective data are shown in (b) and any significant differences are indicated (*P < 0·05) using the Kruskal–Wallis statistical test.

memory cells, reminiscent of an IL-17A-producing memory Th17 population identified using superantigen-secreting *Staphylococcus aureus* isolates.[42]

The decreased percentage of IL-17A$^+$ cells of the total population in the sham-infected and GAS-infected samples treated with BFA i.p. compared with naive controls as shown by *ex vivo* IL-17A staining may be caused by increased cell death caused by the trauma of inoculation. This is particularly the case for *S. pyogenes*-infected mice, whereby this pathogen has been demonstrated to induce dramatic apoptosis in immune cell types.[43] The increased detection of IL-17A$^+$ cells in naive controls without treatment with BFA may represent a store of this pro-inflammatory cytokine and suggests that these cells are poised for quick release in response to trauma or infection.

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As no increase in *rorc* or *tgfb* gene expression was detected in the inguinal draining lymph node, it cannot be ruled out that Th17 cells may differentiate elsewhere upon sham or GAS infection. It has been shown that skin-migratory Langerhans cells can induce Th17 differentiation from naive $CD4^+$ T cells in the presence of IL-6 and IL-15.[44] Differentiated $CD4^+$ T cells were able to home to the skin and produce IL-17 and also interferon-γ to exert tissue damage. Therefore, it is not implausible to suggest that the trauma caused at the site of injection in our model may activate skin-migratory Langerhans cells to induce the differentiation of Th17 cells in a similar fashion and the production of IL-17A may contribute to the observed tissue damage and cell death. However, this would need to be confirmed.

The small difference between sham-infected and *S. pyogenes*-infected samples in terms of serum IL-17A levels is indicative of the very strong and volatile innate IL-17A response to local trauma. It has been long established that tissue damage and trauma can lead to the production of pro-inflammatory cytokines, such as IL-1, IL-2, TNF-α, IL-6, IL-12 and interferon-γ.[45] These cytokines and also chemokines act as danger signals, recruiting cells, such as NKT cells, to the site of injury to create a microenvironment suitable for tissue repair and wound healing[46] and also to guard against risk of infection.[47] Interleukin-17A is also associated with trauma and tissue damage. Systemic increases have been described in post-trauma patients. However, this was associated with Th17 cells as IL-23 induced by local macrophages and dendritic cells maintained the survival of this T-cell subset.[48] Elevated IL-17A has also been detected after severe burn injuries in both clinical patients and murine models.[49,50] $\gamma\delta$ T cells have been demonstrated to sense stress signals from dying cells and secrete IL-17A in response[51] as a form of immune surveillance.[52] In our acute sepsis model we found a significantly increased percentage of $IL\text{-}17A^+$ $\gamma\delta$ T cells in infected BFA-treated mice relative to naive BFA controls, indicating that this innate immune cell type may be playing a role in immune surveillance.

The major sources of IL-17A in this model were NK cells, $CD4^+$ cells, and to a lesser extent NKT cells, following *S. pyogenes* infection in terms of percentage of lymphocytes. Both pathogenic and protective roles for NK cells during bacterial sepsis have been demonstrated by depletion experiments using the caecal ligation puncture animal model of polymicrobial sepsis and other models of trauma.[53–56] Clearly, a potential mechanism for the interaction between *S. pyogenes* and immune cells leading to IL-17A production may be via secreted superantigens. For example, hepatic NKT cells can be activated by Kupffer cells in the presence of superantigens to produce interferon-γ and IL-12 and show enhanced cytotoxic activity.[57] We are currently defining the relative contributions of antigenicity and superantigenicity using superantigen-knockout GAS isolates.

Our experiments have shown potent and rapid release of IL-17A by both innate and adaptive cell types in response to trauma and infection. This appears to be an exquisitely reactive mechanism, poised to prime the host against potential infection and inflammation as perceived through tissue damage at the site of injury. Hence, extreme care is required in choosing appropriate controls for infection models.

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Disclosure

The authors have no financial disclosures.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of an isotype control and Fluorescence Minus One (FMO) control in the analysis of flow cytometry data of IL-17A positive cells.

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