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Role of gamma/delta T cell receptor-expressing lymphocytes in cutaneous infection caused by *Staphylococcus aureus*

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SUMMARY

The high number of γδ-expressing T cells found in the epithelial lining layer suggests that they form a first line of defence against invading pathogens. To evaluate the role of γδ T cell-receptor (TCR)-expressing cells in cutaneous infection caused by *Staphylococcus aureus*, mice lacking γδ-expressing T cells (TCRγδ−) were inoculated intradermally with *S. aureus*, and compared with *S. aureus*-infected congenic control mice. The number of bacteria recovered from the skin of TCRγδ− mice was significantly higher (*P = 0.0071*) at early time-points after inoculation compared to the number of bacteria isolated from infected TCRγδ+ congenic controls. Nevertheless, inflammatory responses measured as serum IL-6 levels, were significantly lower in TCRγδ− mice than in the control group. A possible explanation for this discrepancy was the observation of significantly decreased overall numbers of infiltrating cutaneous T lymphocytes, which are important producers of IL-6. These results support the notion that the γδ-expressing T cells that reside at the epithelial lining layer of the skin is of importance for early containment of the bacteria, thereby limiting their replication and spread.

Keywords dermatitis gamma/ delta TCR mice *Staphylococcus aureus*

INTRODUCTION

Recent experiments have demonstrated that gamma/delta (γδ) T cell-receptor (TCR)-expressing cells are involved in the regulation and resolution of inflammatory processes that are associated with infectious diseases and autoimmune. Nevertheless, the physiological role of γδT cells is not fully understood. γδT cells have been shown to accumulate at sites of inflammation that are associated with certain viral and parasitic infections, such as malaria and *Toxoplasma gondii* [1]. γδT lymphocytes also accumulate in certain human infectious diseases that are associated with granulomatous responses, such as the reactive lesions of leprosy and cutaneous leishmaniasis [2]. Experimental infection with intracellular bacteria, such as *Listeria monocytogenes*, has indicated that γδT lymphocytes may be involved in the regulation of the infection by suppressing the formation of liver lesions [3]. In addition, experimental infection with *Nocardia asteroides* showed an essential role for intraepithelial γδ lymphocytes in the survival of the host [4].

The majority of T cells bear the T-cell-receptor (TCR) alpha/beta complex (αβ) that recognizes antigen peptides only in the context of self major histocompatibility complex (MHC) molecules [5]. In man, γδTCR lymphocytes constitute on average 5% of all the T cells in normal peripheral blood [6], in organized lymphoid organs and in the skin- and gut-associated lymphoid tissues [2]. Most human γδ T cells display the Vγ9Vδ2 rearrangement and lack the expression of both CD4 and CD8 accessory molecules. These T cells constitute a small subset both in the neonatal and adult thymus, but the population expands with age in the peripheral blood, which suggests that positive selection occurs in the periphery following sustained antigenic stimulation [7,8].

In the mouse, γδ T cell populations with distinct clonal compositions have been found in the epidermis, intestine, lung, female reproductive tract, tongue and lactating mammary gland. In the murine epidermis, the vast majority of T lymphocytes express TCR γδ rather than TCR αβ. In the mouse epidermis, the γδ T cells which are also known as Thy-1+ dendritic epidermal T cells (DETCs) [9,10], expresses essentially a single type of γδ heterodimer [11], which is known as the junctionally monomorphic Vγ9/Vδ2+ TCR [12]. The γδ T cells vary in density between different skin sites and different strains [9], with values as high as 580 cells/mm² in ear epidermis, measured in whole mounts of epidermis (horizontal sections). Precursors of DETC appear primarily
in the fetal liver and traverse the fetal thymus as a single wave of cells on their way to the epidermis. At later time-points in thymic ontogeny, cells that express other subsets of γδ TCR receptors appear in similar waves but migrate preferentially to other tissues [13–15]. The DETCs that appear in the skin cannot be replaced in the adult animal by new precursors that express the same subset of γδ TCR receptors, because the generation of DETC precursors is restricted to specific stages of fetal development. In animals that congenitally lack these γδ DETCs, the epidermis is populated by bone marrow-derived TCR αβ-, CD8+ DETCs [16].

To investigate the role of γδ TCR-expressing lymphocytes in cutaneous staphylococcal infection, we compared bacterial clearance in γδ TCR knock-out (TCRδ−) mice and heterozygous littermate control mice (TCRδ+) using a recently described model of cutaneous S. aureus infection [17,18]. Our results indicate that resident cutaneous γδT cells act as an early antibacterial defence.

MATERIALS AND METHODS

Mice

Male mice of mixed (129 × C57BL/6) background, which lacked γδ T cells as a result of targeted germline mutation in the TCRD gene [19], were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mutated TCRD locus was back-crossed onto the DBA/1 (originally from the Jackson Laboratory) background for six generations. By subsequent intercrossing of mice that were either heterozygous or homozygous for the mutated TCRD, we obtained both homozygous (γδ T cell-deficient) or heterozygous (normal T cell phenotype) offspring litters. The different T cell phenotypes were determined by flow cytometry analysis of blood cells, using a monoclonal antibody (GL3) that was specific for TCRγδ.

Female, 12–26-week-old DBA/1 mice, were housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg, under standard conditions of light and temperature and fed standard laboratory chow and water ad libitum.

Bacterial strain

The DBA/1 mice were inoculated intracutaneously with S. aureus LS-1 strain, which is harboured naturally on the skin of many mouse strains [20]. LS-1 strain has been shown to produce large amounts of toxic shock syndrome toxin 1 (TSST-1) and to be coagulase and catalase positive. The bacteria were kept frozen at −20°C in 5% bovine serum albumin and 10% dimethylsulphoxide (C2,H10,OS) in phosphate buffered saline (PBS) (pH 7.4) until use. Just prior to use, the bacteria were thawed and washed in PBS. Viable counts were used to check the number of live bacteria in each bacterial solution.

Experimental protocol

Two experiments were performed using DBA/1 mice. The mice were inoculated with bacteria intracutaneously on the shaved back during neurolept analgesia (Dormicum®, Hoffmann-La Roche Ltd., Basel, Switzerland; Hypnorm®, Janssen Pharmaceuticals, Beerse, Belgium). In experiment I, 31 γδ T cell receptor knock-out mice (TCRδ−) and 31 congeneric control mice (TCRδ+) were inoculated at two sites with 0·1 ml of saline that contained either 2 × 107 or 2 × 108 colony-forming units (CFU) of S. aureus (a total of 4 × 107 or 4 × 108 CFU per mouse). In experiment II, 19 γδ+ T cell receptor knock-out mice (TCRδ−) and 19 control mice (TCRδ+) were inoculated at a single site with 1 × 107 CFU of S. aureus (a total of 1 × 108 CFU per mouse). The mice were monitored individually and sacrificed by cervical dislocation 2 days or 7 days after bacterial inoculation. The mice were evaluated clinically for local inflammatory reactions and weight development. In experiment I, skin samples corresponding to single injection sites on each mouse were dissected for histopathological and immunohistochemical evaluation at the time intervals indicated. Skin samples of the second injection site were resected for bacterial analysis. Blood samples were taken for bacterial counts, granulocyte counts, total white blood cell counts, interleukin-6 (IL-6)-levels, immunoglobulins (IgG1, IgG2a, IgG3 and IgM), and for specific antibodies to staphylococcal cell walls and TSST-1 (see below).

In experiment II, skin samples from the injection site as well as blood samples were obtained for bacterial analysis at the time intervals indicated. Blood samples were also analysed for granulocyte counts and total white blood cell counts.

Histopathological examination

Skin samples from 62 mice (experiment I) were examined histopathologically after routine fixation and staining with haematoxylin–eosin. Blinded microscopical evaluation was carried out to characterize the size and density of the inflammatory infiltrates.

Immunohistochemical examination

Skin samples from the mice in experiment I were also analysed regarding the occurrence of CD11b+ cells (i.e. macrophages) and CD3+-expressing T cells. Briefly, skin samples were frozen in isopentane prechilled with liquid nitrogen, and kept at −70°C until cryosectioned. All the sections were fixed in cold acetone for 5 min and washed in PBS. The sections were incubated overnight in a humid atmosphere at +4°C with unlabelled rat anti-CD11b (Mac-1; M1/70) [21] or hamster anti-CD3 (clone 145–2C11, PharMingen, San Diego, CA, USA) monoclonal antibodies [22], which were diluted in PBS containing 1% bovine serum albumin (BSA). After several washes, endogenous peroxidase was depleted by treatment with 0·3% H2O2 for 5 min. Biotin-labelled rabbit antirat Ig (Vector Laboratories, Burlingame, CA, USA) and mouse antihamster IgG (were a cocktail of clones G70-204 and G94-56; PharMingen) diluted in PBS–BSA were used as secondary antibodies. The binding of biotin-labelled secondary antibodies was detected by stepwise incubation with streptavidin–biotin-complex/HRP (Dako, Denmark) and 3-amino-9-ethyl-carbazole containing H2O2. All sections were counterstained with Meyer’s haematoxylin.

Bacterial culture

Skin and blood samples were obtained for bacterial analysis. After surface disinfection with 70% alcohol, skin samples that corresponded to the injection site were deposited in sterile plastic bags, homogenized and suspended in 10 ml PBS. Skin suspensions were plated in appropriate dilutions on agar plates containing 5% horse blood and incubated at 37°C for 24 h. The number of CFUs per skin sample and per 100 μl of blood were counted and the bacterial colonies were tested for coagulase and catalase activity.

Serological analyses

IL-6 assay. The cell line B13·29, which is dependent on interleukin-6 (IL-6) for growth, has been described previously [23–25]. For IL-6 determinations, the more sensitive subclone B9 was used. B9 cells were harvested from tissue culture flasks, seeded

were estimated by an ELISA in which poly L-lysine (25
antibodies that were specific for
logit-log models [27,28].

were constructed with a computer program that employed weighted
dilutions of a pool of reference sera. The calibration curves were
that were based on the optical density values obtained from serial

at 414 nm (A414) was measured in a SpectraMax Plus spectropho-
tometer (Molecular Devices). All optical density values were con-

Microscopic evaluation
Microscopic evaluation of infected skin specimens revealed a
dense infiltrate of granulocytes and macrophages at days 2 and 7
in mice that received 2 × 10^8 CFU of S. aureus. The bacteria were
clearly visible in some skin specimens. In contrast, mice that received
2 × 10^7 CFU of S. aureus showed only mild inflammatory
infiltrates and the bacteria were not easily detectable. This result
is in accordance with previously reported findings [17]. There
were no clear-cut differences between the KO and control mice
with respect to inflammatory infiltrate size.

Immunohistochemical analysis
Immunohistochemical analysis was performed to determine the
occurrence of CD11b^+ cells (phagocytic cells) and CD3^+ cells (T
lymphocytes). The number of CD3^+ T cells in the epidermis was
significantly higher in the control mice compared to the γδ T cell-
deficient animals (Fig. 2), both in mice that received 2 × 10^8 CFU
of S. aureus and in mice that were inoculated with 2 × 10^7 CFU of S. aureus at days 2 and 7 (Table 1). No significant differences were
noted between the groups regarding the number of CD3^+ cells in
the dermis, where a number of approximately 10 CD3^+ T cells/mm^2
were noted. The number of CD11b^+ (Mac-1) cells was high
(>50/mm^2) in all the mouse groups, with no significant differences
noted at days 2 or 7 (Fig. 2).

RESULTS

Bacterial cultures
Skin samples from γδ T cell-deficient mice that received 2 × 10^8
CFU of bacteria had significantly higher numbers of bacteria
than littermate controls 2 days after inoculation (P = 0.0071) (Fig. 1). There were no significant differences in bacterial num-
bbers between mice that received 2 × 10^7 CFU of S. aureus 7
days after bacterial inoculation, and no significant differences
between groups of mice that were inoculated with 2 × 10^7 CFU
of S. aureus (data not shown). Bacterial cultures from blood
samples 2 days after bacterial inoculation were positive in six
of eight mice (75%) in the γδ T cell-deficient group that received
a total of 4 × 10^7 CFU, and in eight of nine mice (89%) in the control group (n.s.) that received the same dose of bacteria. Seven days after staphylococcal inoculation, 2/7
mice (29%) in both groups showed bacteraemia. Bacteraemia
was absent in mice that received a total of 4 × 10^6 CFU of S. aureus.

Haematological analysis
Total white blood cell counts (WBC) were determined using a haemacytometer (Toa Medical Electronics, Kobe, Japan). Blood
smears were prepared and stained by the May–Grunwald–
Giemsa method for differential counts.

Statistical evaluation
The differences between mean values were tested for significance
using the non-parametric Mann–Whitney U-test. P-values ≤ 0.05
were considered to be statistically significant.

Fig. 1. Numbers of S. aureus (± s.d.) in skin samples (n = 7–9 for each time
time period and group) recovered at defined time intervals after initial intrac-

taneous inoculation with 2 × 10^7 CFU of S. aureus. □, γδ TCR^+; □, γδ

TCR^-.
Serological analyses

IL-6. The serum levels of IL-6 in mice that received a total of $4 \times 10^8$ CFU *S. aureus* were significantly lower in γδ knock-out mice than in control mice ($P < 0.02$), with peak values around 600 pg/ml in γδ T cell-deficient mice compared to 1200 pg/ml in the controls (Fig. 3). No significant differences were noted at day 7 in groups of mice that received a total of $4 \times 10^8$ CFU or $4 \times 10^7$ CFU of *S. aureus*.

*B lymphocyte responses.* Because IL-6 is an efficient B lymphocyte-stimulating factor, we decided to analyse the serum levels of immunoglobulins and specific antibodies to staphylococcal components. Serum levels of IgG1 and IgG3 were not increased significantly 7 days after bacterial inoculation in any mouse group, and there were no significant differences between the γδ T cell-

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**Table 1.** Number of epidermal CD3+ T cells (mean ± s.e.m./skin section) is significantly decreased in TCR δ− mice. Two to five sections were counted per mouse.

<table>
<thead>
<tr>
<th>No. of <em>S. aureus</em> inoculated</th>
<th>TCR status</th>
<th>Day 2</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 5–8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^8$</td>
<td>TCR δ−</td>
<td>48 ± 11</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>$10^8$</td>
<td>TCR δ−</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$10^7$</td>
<td>TCR δ−</td>
<td>40 ± 7</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>$10^7$</td>
<td>TCR δ−</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

that received 4 day 7 in control mice (Table 2), with a twofold increase also seen in γδ T cell-deficient mice (n.s.) that were inoculated with a total of 10^8 CFU of bacteria. The serum levels of TSST-1-specific antibodies of IgM class were also significantly increased (P = 0.006) between day 2 and day 7 in control mice that received 4 × 10^8 CFU of bacteria, while the serum levels of TSST-1 specific IgM antibodies in γδ T cell-deficient mice showed only a slight increase (n.s.). No increase in serum levels of TSST-1 specific antibodies of IgG class were detected (data not shown).

Clinical and haematological analysis
No significant changes in weight development were noted after inoculation with S. aureus between γδ T cell-deficient mice and their congenic controls. Analysis of blood smears showed an increase in the percentage and number of granulocytes after bacterial inoculation, but no significant differences were noted between the groups (Table 3).

DISCUSSION
The presence of large numbers of γδ TCR-expressing lymphocytes at epithelial surfaces suggests that they have a role in the first line of defence against invading pathogens. Nevertheless, the timing of influx and regulatory responses of γδ TCR-expressing T cells is debated and controversial. Some investigators have shown that γδ T cells are more prominent during subacute infections, or in the recovery phase following certain viral infections. The predominance of early or late γδ T cell responses following infection may be related to the type and dose of bacteria as well as to the site of infection and sampling time-points [1]. Indeed, our study suggests that the cutaneous deposition of 2 × 10^8 CFU of S. aureus but not of lower bacterial doses leads to differential immune responses in mice that have intact T cell repertoires and those being knocked out for the γδ T cell receptor. The reasons for this threshold effect...
are presently unknown. In the present study, significantly higher numbers of \textit{S. aureus} were recovered from skin samples 2 days after intracutaneous inoculation of bacteria in TCR\(\gamma\delta\)− mice compared to heterozygous TCR\(\gamma\delta\)− littermate control mice. Similar findings have been reported in experimental \textit{Listeria monocytogenes} infection, in which mice that were depleted of \(\gamma\delta T\) cells succumbed to infection induced by high doses of this intracellular bacterium \[1\]. In that report, the authors proposed that one function of \(\gamma\delta T\) cells is to down-regulate the responses of infection-activated macrophages by inducing their apoptosis, thereby restoring macrophage homeostasis and preventing the development of chronic inflammation. The mechanisms by which \(\gamma\delta T\) cells kill activated macrophages have not been determined, but examples of cytotoxicity induced by \(\gamma\delta\) T cells have been reported, which include the perforin pathway and Fas ligation on target cells \[29,30\].

Although it has been demonstrated clearly that \(\gamma\delta\) TCRs do not recognize processed peptide Ags that are complexed to self-MHC molecules \[31\], there is evidence that they can recognize highly conserved non-protein Ags. In this respect, peptidoglycans and/or teichoic acid, both of which are constituents of the staphylococcal cell wall may provide activating signals to \(\gamma\delta T\) cells that, directly or indirectly, enhance bacterial elimination. In addition, it has been recently shown that \(\gamma\delta T\) cells may be activated directly by staphylococcal TSST-1 \[32\], a superantigen that is produced by the staphylococcal strain used in the present study. In the present study, skin infection with TSST-1-producing staphylococci induced predominantly IgG2a production in mice that lacked \(\gamma\delta\) TCR-expressing cells. Furthermore, decreased levels of IL-6, which is a Th2 cytokine, were found in the same mice. This finding indicate either decreased ability to produce IL-6 or decreased inflammatory stimulus (i.e. number of bacteria). Since the \(\gamma\delta\) knockout mouse displayed higher rather than lower bacterial load, the first hypothesis is more valid. Interestingly, it has been suggested previously that IL-6 stimulates antibacterial macrophage activities \[33\], thereby decreasing the risk of infection.

Our observations of early and transient deficiencies in bacterial elimination in \(\gamma\delta\) T cell-deficient mice need further clarification. One possibility, put forward recently by Ottones et al. \[34\], is that activated \(\gamma\delta T\) cells act directly as antibacterial effector cells in the early stages of infection. A paucity of \(\gamma\delta T\) cells would thus hamper the efficient early elimination of staphylococci, whereas in the later stages of infection compensatory mechanisms would have emerged. Altogether, our results strongly support the notion that cutaneous \(\gamma\delta\)-expressing T cells act as an early barrier to prevent staphylococcal infections of the skin.

### Table 3. Number of leucocytes in peripheral blood 7 days after intracutaneous inoculation with \textit{S. aureus} LS-1. The values shown represent mean ± s.d., \(n = \) number of mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>((n))</th>
<th>Size of total inoculum (CFU)</th>
<th>WBC (10(^6)/ml)</th>
<th>Total granulocytes (10(^6)/ml)</th>
<th>Granulocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\gamma\delta^+)</td>
<td>9</td>
<td>(4 \times 10^7)</td>
<td>(48 \pm 3.3)</td>
<td>(0.9 \pm 0.7)</td>
<td>18.8 ± 6.0</td>
</tr>
<tr>
<td>(\gamma\delta^-)</td>
<td>8</td>
<td>(4 \times 10^7)</td>
<td>(41 \pm 2.2)</td>
<td>(0.8 \pm 0.5)</td>
<td>18.8 ± 4.5</td>
</tr>
<tr>
<td>(\gamma\delta^+)</td>
<td>10</td>
<td>(1 \times 10^6)</td>
<td>(7.6 \pm 2.1)</td>
<td>(1.7 \pm 0.5)</td>
<td>22.9 ± 3.3</td>
</tr>
<tr>
<td>(\gamma\delta^-)</td>
<td>10</td>
<td>(1 \times 10^6)</td>
<td>(5.8 \pm 2.0)</td>
<td>(1.5 \pm 0.6)</td>
<td>24.8 ± 3.8</td>
</tr>
<tr>
<td>(\gamma\delta^+)</td>
<td>7</td>
<td>(4 \times 10^6)</td>
<td>(9.0 \pm 2.7)</td>
<td>(2.8 \pm 0.9)</td>
<td>281 ± 3.1</td>
</tr>
<tr>
<td>(\gamma\delta^-)</td>
<td>7</td>
<td>(4 \times 10^6)</td>
<td>(9.8 \pm 2.9)</td>
<td>(2.7 \pm 1.2)</td>
<td>290 ± 6.1</td>
</tr>
</tbody>
</table>

### ACKNOWLEDGEMENTS

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