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Signaling through the IL-2 receptor γ_c peptide (CD132) is essential for the expression of immunity to *Plasmodium chabaudi adami* blood-stage malaria

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SUMMARY

A genetic dissection approach was employed to determine whether the IL-2 receptor complex (IL-2R) comprised of α , β and γ chains is required for the suppression of *Plasmodium chabaudi adami* parasitemia. Blood-stage infections in IL-2R $\gamma_c^{-/y}$ mice failed to cure with parasitemia remaining elevated for >50 days indicating the IL-2R γ_c through which all members of the γ_c family of cytokines signal, has an essential role in protective immunity against blood-stage malaria parasites. In contrast, the curing of parasitemia in IL-2/15R $\beta^{-/-}$ mice, deficient in both IL-2 and IL-15 signaling was significantly delayed but did occur, indicating that neither cytokine plays an essential role in parasite clearance. Moreover, the observation that the time-course of parasitemia in IL-15 $^{-/-}$ mice was nearly identical to that seen in controls suggest that the parasitemia-suppressing role of stimulating through the IL-2/15R β chain is due to IL-2 signaling and not a redundant function of IL-15.

Keywords

IL-2 receptor complex; *Plasmodium chabaudi adami*; knockout mice; γ_c cytokine family

INTRODUCTION

With the aim of revealing potential vaccine targets, we have been searching for host genes that are crucial for the clearance of blood-stage malaria parasites. The common γ chain of the interleukin 2 receptor (IL-2R γ_c) gene appears to be closely linked to susceptibility to infectious agents. In humans, mutations in the IL-2R γ_c gene result in X-linked severe combined immunodeficiency disease (XSCID), making the host exceedingly vulnerable to opportunistic infections (1,2). IL-2R γ_c deficient mice, while displaying many of the immunodeficiencies seen in XSCID patients are B cell deficient as well (3,4). Surprisingly, XSCID mice survive acute phase infections caused by different intracellular pathogens, including *Toxoplasma gondii* (5) and *Listeria monocytogenes* (6). They accomplish this by activating IFN γ –dependent mechanisms of innate immunity.

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Cytokines signaling through the common γ chain of the IL-2 receptor (γ_c) (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) play important roles in the development, activation, proliferation, differentiation and regulation of lymphocytes and a variety of other cell types (7,8,9). Interleukin-2, IL-15 and IL-7 in particular have critical roles in regulating lymphoid homeostasis: IL-4 is required for the differentiation of T_H2 cells. Moreover, γ_c cytokines play essential roles in the adaptive immune responses to most infectious agents. The mechanisms by which these cytokines appear to function depend on the different signaling pathways that they activate in vivo, the differentiation status of the cells being stimulated and the environment in which the target cells reside (8,10).

Previous studies indicate that IL-2 does not play an essential role in immunity to experimental murine malaria, resulting from blood-stage infections with either *Plasmodium chabaudi adami* (11), hereafter referred to as *P. c. adami*, or the more virulent, *P. c. chabaudi* AS strain (12). Although the suppression of parasitemia is delayed in gene-targeted IL-2 KO mice infected with either subspecies of the parasite, their infections eventually cure. IL-15 functions redundantly with IL-2 in certain aspects of lymphocyte biology while having specific activities of its own (13). Ing et al, (14) report that the duration of *P. c. chabaudi* parasitemia is prolonged in IL-15 KO mice compared to intact control mice but they too eventually cure. $Th1$ cytokine production, dendritic cell and NK cell function are impaired in these mice, suggesting that IL-15 functions in both innate and adaptive immunity to the parasite. Although both IL-2 and IL-15 contribute to immunity against blood-stage *P. chabaudi* malaria, neither cytokine appears to have an essential role i.e., the absence of either cytokine merely delays the suppression of parasitemia but doesn't prevent it. Whether these observations can be explained by the redundant function of the 2 cytokines signaling through the interleukin 2/15 receptor β chain (IL-2/15R β) of the IL-2R (15) or other mechanisms remains to be elucidated.

In the present study, we have examined the roles played by components of the IL-2R complex, namely, the IL-2/15R β and the IL-2R γ_c chains, in immunity to *P. c. adami* by comparing the time-courses of parasitemia in KO mice deficient in these peptides with those seen in intact controls. Our findings indicate that the IL-2R γ_c chain is essential for parasite clearance. In contrast, the IL-2/15R β chain, through which only IL-2 and IL-15 signal (9,15), does not play a crucial role in the suppression of parasitemia.

MATERIALS & METHODS

Female and male IL-2/15R $\beta^{-/+}$ mice backcrossed to C57BL/6 mice for 5 generations (16) and C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME, [USA](#)). Breeding stocks of IL-15 $^{-/-}$ mice on a C57BL/6 background (17) and IL-2R $\gamma_c^{-/y}$ mice (4) backcrossed to C57BL/6 mice for more than 5 generations were kindly provided by Dr. Elaine Thomas (Immunex Corporation, Seattle, WA, [USA](#)) and Dr. Warren J. Leonard (NIH, Bethesda, MD, [USA](#)) respectively. Mice were bred in the AAALAC-accredited animal facility at the University of Wisconsin, Madison, WI, USA to produce male IL-2R $^{-/y}$ mice lacking functional IL-2R γ chains and male IL-2R $^{+/y}$ control mice which expressed functional IL-2 receptors. Mice homozygous for nonfunctional IL-2/15R β chains served as test mice whereas heterozygous mice were used as controls. Time courses of *P. c. adami* parasitemia in heterozygous IL-2/15R $\beta^{-/+}$ mice and C57BL/6 mice were identical (data not shown). Age and sex matched C57BL/6 mice served as controls for IL-15 $^{-/-}$ mice. All procedures were approved by the University of Wisconsin Institutional Animal Use and Care Committee.

The avirulent malarial parasite *P. c. adami* 556KA was maintained and used as described previously (18). Experimental mice were injected i.p. on day 0 with 1×10^6 erythrocytes

parasitized with *P. c. adami*. The resulting parasitemia was assessed by enumerating parasites in 200 to 1,000 erythrocytes on Giemsa-stained thin blood films prepared every other day, beginning day 5 post-infection (PI). Groups of 3 to 6 sex- and age-matched mice between 6 and 16 weeks of age were used in each experiment. Data are presented as the percent parasitemia, calculated as number of parasitized erythrocytes/total number of erythrocytes $\times 100$.

Two-color cytofluorimetric analysis was performed on splenocyte single-cell suspensions as described previously (19). The biotinylated antibodies were anti-CD3 and anti-NK1.1 mAbs (Boehringer Mannheim, Indianapolis, IN, USA) and anti-TCR β , anti-TCR γ , and a hamster immunoglobulin G (IgG) control (BD Biosciences PharMingen, San Diego, CA, USA). Streptavidin-phycoerythrin was obtained from Southern Biotechnology Associates (Birmingham, AL, USA). Fluorescein isothiocyanate-conjugated antibodies were as follows: anti-CD3 (Boehringer Mannheim) and anti-CD4, anti-CD8, anti-CD45R (B220), and rat IgG isotype controls (BD Biosciences PharMingen). Propidium iodide was added shortly before data acquisition to allow electronic exclusion of dead cells. Data were acquired on a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer and analyzed with the use of CellQuest and Attractors (Becton Dickinson) programs.

Sera were obtained from IL-2/15R $\beta^{-/-}$, IL-2/15R $\beta^{+/-}$, and C57BL/6 mice following the suppression of parasitemia 34 days after inoculation with 1×10^6 parasitized erythrocytes. *P. chabaudi*-specific antibodies in the sera of test and control mice were measured by a modification of the enzyme-linked immunoabsorbent assay (ELISA) described previously (20). ELISA plates (Easy-Wash; Corning Costar Corporation, Cambridge, MA, USA) were coated with 2.5 $\mu\text{g}/\text{well}$ of a crude preparation of *P. c. adami* blood-stage antigen. Following overnight incubation at 4°C, wells were washed, and blocked. Starting at 1/250, serial 2-fold dilutions of each serum sample were prepared and added to antigen-coated wells in duplicate (50 $\mu\text{l}/\text{well}$). Following a 2 hr incubation at room temperature, antigen-specific antibodies were detected with a horseradish peroxidase (HRP)-conjugated rabbit antibody (Zymed Laboratories, South San Francisco, CA, USA) specific for the heavy chains of mouse IgM, IgG, and IgA with ABTS, [2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid)] as the substrate. For each serum, A₄₀₅ values between 1.0 and 0.1 were plotted and titer was calculated as the reciprocal of the dilution of serum yielding an A₄₀₅ = 0.2.

Statistical analysis was performed with the unpaired, two-tailed, Student's t test and GraphPad Prism 3 software (GraphPad Software Inc., San Diego, CA, USA). A *P* value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

To determine whether the IL-2R is essential for the development of immunity against acute blood-stage infection, the time course of *P. c. adami* parasitemia was compared in IL-2R $\gamma_c^{-/y}$ mice and IL2R intact controls. This choice was based on the knowledge that all members of the γ_c cytokine family signal through the IL-2R γ_c (7).

Ascending parasitemia following the i.p. injection of 1×10^6 parasitized erythrocytes was similar in both groups of mice, reaching peak values of 20.7% $\pm$ 12.5% on day 9 post-inoculation (PI) in knockout (KO) mice lacking functional genes for the expression of the IL-2R γ_c peptide and 11% on day 7 in control mice. Whereas parasitemia in control mice was suppressed to $\sim$ 0.01% by day 13 PI, parasitemia in IL-2R $\gamma_c^{-/y}$ mice remained at high unremitting levels (8-29%) for >7wks PI when the experiment was terminated (Figure 1a). This finding that parasitemia was prolonged at high levels in IL-2R $\gamma_c^{-/y}$ mice indicates that signaling through the IL-2R complex is essential for the suppression of *P. c. adami* parasitemia. Acute blood-stage *P. c. adami* infections in mice are suppressed by antibody

mediated immunity (AMI) dependent on CD4⁺ T cells and B cells (21) and/or cell-mediated immunity (CMI) dependent on CD4⁺ T cells and $\gamma\delta$ T cells (22, 29). The observation that IL-2R $\gamma_c^{-/y}$ mice failed to clear *P. c. adami* parasites from their blood indicates that both AMI and CMI against the parasites were defective in these mice lacking a functional IL-2R due to a mutation of a single gene, the IL-2R γ_c gene. IL-2R $\gamma_c^{-/y}$ mice have been reported previously by others to be deficient in $\alpha\beta$ T cells, $\gamma\delta$ T cells and B cells (3, 4). As indicated in Table 1, we observed similar deficiencies in these cell populations.

Because IL-2 and IL-15 may have redundant roles in immunity to blood-stage malaria, we determined the time courses of *P. c. adami* parasitemia in IL-2/15R $\beta^{-/-}$ mice and intact controls following inoculation with 1×10^6 parasitized erythrocytes. Parasitemia was prolonged in IL-2/15R $\beta^{-/-}$ mice by ~3 weeks as compared to control mice (Figure 1b), but the mice eventually cured. Both $\gamma\delta$ T cells and B cells were deficient in the spleens of IL-2/15R $\beta^{-/-}$ mice compared to infected control mice (Table 1) with numbers similar to those seen in IL-2R $\gamma_c^{-/y}$ mice. In addition antibodies reactive with crude malaria antigen were detected in the sera of IL-2/15R $\beta^{-/-}$ mice, following the suppression of parasitemia albeit at approximately half the concentrations seen in control mice (Table 2). This difference was not statistically different.

Both IL-2 and IL-15 stimulate through the IL-2/15R β (9,13). Whereas IL-2 deficient mice exhibit *P. c. adami* parasitemia of prolonged duration before spontaneously clearing (11), the effects of IL-15 deficiency on the course of malaria caused by the *adami* subspecies of the parasite had not yet been determined. To assess whether IL-15 contributes to the suppression of acute parasitemia, we compared time-courses of *P. c. adami* parasitemia initiated with 1×10^6 parasitized erythrocytes in IL-15 KO mice versus C57BL/6 controls. Parasitemia in experimental and control groups followed a similar time-course, reaching a peak level on day 9 in the KO group and day 7 in the control group and declined to subpatent levels at 18 days PI (Figure 1c). At peak, the mean parasitemia percentages in IL-15 $^{-/-}$ and control mice were similar, $10.43 \pm 2.66\%$, and $9.81 \pm 5.44\%$ respectively. Differences in the results published by Ing et al. (14) and our findings may be attributed to the differences in virulence of the subspecies of *P. chabaudi* used in the different studies.

Our results indicate that the IL-2R complex has an essential protective role in immunity to blood-stage malaria. Protection is achieved by γ_c cytokine family members signaling through the IL-2R γ_c signifying the importance of a single gene in immunity to malaria but leaves unanswered 2 important questions. 1/ Which members of the γ_c cytokine family are responsible for stimulating protective immunity to blood-stage parasites and 2/ what are the protective mechanisms activated through IL-2R γ_c signaling?

IL-2R $\gamma_c^{-/y}$ mice are also deficient in NK cells, NKT cells and CD8⁺ T cells (24). However, our recent findings do not suggest a protective role for any of these cells in immunity to blood-stage malaria (25). Although the roles of IL-7, IL-21 and IL-9 are unknown in blood-stage infections caused by *P. c. adami*, no single member of the γ_c cytokine family has been identified as having such a protective role. Furthermore, our data indicate that neither IL-2 nor IL-15 signaling separately through the IL-2R has an essential role in protective immunity. Whether they or other γ_c cytokine family members can function together sequentially, additively or synergistically to activate protective immunity to blood-stage malaria parasites remains to be determined. As a model, the IL-2R $\gamma_c^{-/y}$ mouse provides a unique opportunity to analyze down-stream gene activation and its contribution to immunity.

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Figure 1.

Time-course of parasitemia in experimental and control mice following i.p. inoculation with 1×10^6 erythrocytes parasitized with *P. c. adami*, Panel A, IL-2R γ ^{-/-} mice and IL-2R γ ^{+/+} controls. Panel B, IL-2/15R β ^{-/-} mice and IL-2/15R β ^{+/+} controls. Panel C, IL-15^{-/-} mice and C57BL/6 controls. * $P \leq 0.05$. The experiments in Panels A, B, and C were repeated producing similar results.

Cytofluorimetric analysis of mononuclear splenocytes from IL-2R deficient mice and controls infected with *P. c. adami* parasitized erythrocytes.

Table 1

Mouse Type	Number	Day Post Inoculation	% Live Splenocytes				
			CD4 ⁺ /CD3 ⁺	CD8 ⁺ /CD3 ⁺	γδ ⁺ /CD3 ⁺	Ig ⁺ /B220 ⁺	
IL-2R ^{γ/γ}	4	53	23.95±/5.71	5.85±/1.06	0.26±/0.12	2.52±/0.71	
IL-2R ^{γ/γ}	3	53	18.14±/0.25	11.07±/0.95	2.03±/0.97	59.21±/0.33	
IL-2R ^{γ/γ}	3	0	19.42±/3.12	1.09±/0.40	0.24±/0.20	0.52±/0.28	
IL-2R ^{β/-}	3	34	44.70±/10.99	8.74±/5.66	0.29±/0.17	1.35±/0.23	
IL-2r ^{β/+}	6	34	22.11±/1.28	10.61±/1.12	2.20±/1.61	49.53±/2.17	
C57BL/6	4	34	18.63±/3.81	9.10±/1.37	5.92±/5.09	47.09±/7.61	

Mice were infected i.p. with 1×10⁶ *P. c. adami* parasitized erythrocytes on day 0. Splens were harvested and processed for cytofluorimetry on the days post-inoculation indicated.

Table 2

Antibody titers for IL-2/15Rb^{-/-} mice and controls infected with *P. c. adami*.

Mouse Type	Number	Antibody Titer (Mean +/- SD)
IL-2/15Rb ^{-/-}	8	597 +/- 394
IL-2/15Rb ^{+/+}	7	1137 +/- 1390
C57BL/6	8	1150 +/- 1052
Uninfected C57BL/6 controls	Pooled sera N=5	<250

Sera were harvested 34 days PI with 1×10^6 parasitized erythrocytes injected i.p. Antibody titers (Mean +/- SD) were determined by ELISA as described in the Material and Methods section and expressed as the reciprocal of the serum dilution which yielded an A₄₀₅ = 0.2. Differences between IL-2/15Rb^{-/-} mice and control mice were not significant at the 0.05 level.