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Essential Role of Extrathymic T Cells in Protection Against Malaria[1]

M. Kaiissar Mannoor, Ramesh C. Halder, Sufi Reza M. Morshed, Anoja Ariyasinghe, Hanaa Y. Bakir, Hiroki Kawamura, Hisami Watanabe, Hiroho Sekikawa, and Toru Abo[2]

Athymic nude mice carry neither conventional T cells nor NKT cells of thymic origin. However, NK1.1$^-$TCR^{int} cells are present in the liver and other immune organs of athymic mice, because these lymphocyte subsets are truly of extrathymic origin. In this study, we examined whether extrathymic T cells had the capability to protect mice from malarial infection. Although B6-*nu/nu* mice were more sensitive to malaria than control B6 mice, these athymic mice were able to survive malaria when a reduced number of parasitized erythrocytes (5×10^3 per mouse) were injected. At the fulminant stage, lymphocytosis occurred in the liver and the major expanding lymphocytes were NK1.1$^-$TCR^{int} cells (IL-2Rβ^+TCR$\alpha\beta^+$). Unconventional CD8$^+$ NKT cells (Vα14$^-$) also appeared. Similar to the case of B6 mice, autoantibodies (IgM type) against denatured DNA appeared during malarial infection. Immune lymphocytes isolated from the liver of athymic mice which had recovered from malaria were capable of protecting irradiated euthymic and athymic mice from malaria when cell transfer experiments were conducted. In conjunction with the previous results in euthymic mice, the present results in athymic mice suggest that the major lymphocyte subsets associated with protection against malaria might be extrathymic T cells. *The Journal of Immunology,* **2002, 169: 301–306.**

Many investigators still believe that conventional T and B cells are associated with protection against malaria. Therefore, several Ags that are expressed by *Plasmodium* are used for immunization against malaria (1–6). These trials examined the concept of the induction of anti-malaria Abs produced by conventional B cells and the induction of cellular immunity mediated by conventional T cells. This concept arises from earlier reports that CD4$^+$ (7–13) or CD8$^+$ (14) T cells are associated with protection against malaria. However, all these studies were performed before introduction of the concept of extrathymic T cells.

In a series of recent studies, we and other investigators have reported that extrathymic T cells are present in the digestive tract in such organs as the liver (15–18) and intestine (19–23) and that these T cells comprise an innate immune system in conjunction with autoantibody-producing B-1 cells. When we observed immunoparameters in mice infected with *Plasmodium*, there was no evidence of the activation of conventional T cells (i.e., TCR^{high} cells) (24, 25). Rather, these mice showed thymic atrophy during malarial infection, suggesting the arrest of conventional T cell differentiation. Inversely, primordial T cells (i.e., TCR^{int} cells) were highly activated in number and function, especially in the liver and spleen, and the sera always contained autoantibodies.

Primordial T cells include the NK1.1$^+$TCR^{int} subset (i.e., NKT cells) and the NK1.1$^-$TCR^{int} subset (17). NKT cells are primarily derived from the thymus (through an alternative intrathymic pathway but not the mainstream of the intrathymic pathway) and home to the liver (26–28), whereas NK1.1$^-$TCR^{int} cells are truly of extrathymic origin (29). Almost all T cells that are identified in athymic nude mice are NK1.1$^-$TCR^{int} cells.

In light of these findings, we further characterized the properties of T cells in athymic nude mice with malarial infection. If protection against malaria is achieved as the result of innate immunity as we propose, athymic mice should survive malarial infection due to the action of extrathymic T cells and B-1 cells. This possibility was investigated in the present study.

Materials and Methods

Mice and parasites

C57BL/6 (B6) and B6-*nu/nu* mice at the age of 8–15 wk were used. The mice were maintained at the animal facility of Niigata University (Niigata, Japan) under specific pathogen-free conditions. *Plasmodium yoelii* 17XNL (nonlethal strain), a generous gift of Dr. S. Waki (Gunma Prefectural College of Health Science, Maebashi, Japan), was used (24). Parasites were maintained by routine in vivo passages in mice. Mice were infected by an i.p. injection of 10^4 or 5×10^3 parasitized erythrocytes per mouse. Parasitemia in the blood was observed by Giemsa staining every 2 or 3 days and the mice were sacrificed at the indicated days after infection. Lymphocytes were obtained from the liver, spleen, and thymus (in the case of euthymic mice) in control and infected mice.

Cell preparation

Hepatic mononuclear cells (MNC)[3] were isolated by a previously described method (25). Briefly, the liver was removed, pressed through 200-gauge stainless steel mesh, and suspended in Eagle's MEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5 mM HEPES and 2% heat-inactivated newborn calf serum. After being washed once with medium, the cells were fractionated by centrifugation in 15 ml of 35% Percoll solution (Amersham Pharmacia Biotech, Piscataway, NJ) for 15 min at 2000 rpm. The pellet was resuspended in erythrocyte lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA-Na, and 170 mM Tris (pH 7.3)). Splenocytes and thymocytes were obtained by forcing the spleen and thymus through stainless steel mesh. Splenocytes were used after erythrocyte lysing.

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[3] Abbreviation used in this paper: MNC, mononuclear cell.

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Immunofluorescence test

FITC-, PE-, or biotin-conjugated reagents of mAbs were used and biotin-conjugated reagents were developed with tricolor-conjugated streptavidin (Caltag Laboratories, San Francisco, CA) (30). The mAbs used here were anti-CD3 (145-2C11), anti-IL-2Rβ (TM-β1), anti-NK1.1 (PK136), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-TCR$\alpha\beta$ (H57-597), anti-TCR$\gamma\delta$ (GL3), and anti-erythrocyte (TER119) mAbs (BD PharMingen, San Diego, CA). Cells were analyzed by FACScan (BD Biosciences, Mountain View, CA). To prevent nonspecific binding of mAbs, CD16/32 (2.4G2; BD PharMingen) was added before staining with labeled mAb. Dead cells were excluded by forward scatter, side scatter, and propidium iodide gating.

RT-PCR for Vα14 mRNA

Total RNA was extracted from MNC by the acid guanidium thiocyanate-phenol-chloroform method. cDNA was synthesized using Moloney leukemia virus transcriptase (Takara, Tokyo, Japan) and random hexamer primer (Takara). PCR amplification of synthesized cDNA was conducted as previously described (24). PCR products as well as markers were estimated by staining with ethidium bromide. A control experiment was done by using G3PDH mRNA.

Histology

Tissues were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections 4 μm in thickness were stained with H&E.

Serum levels of anti-DNA Ab

Measurements of IgG and IgM Abs reacting with ssDNA by the ELISA method were modified as previously described (31). Standard sera were obtained from MRL-*lpr*/*lpr* (*lpr*) mice (after the onset of disease) and arbitrarily determined to contain 100 U of anti-DNA Ab. In each test, the titer was expressed as the percentage in comparison with the standard sera.

Cell transfer experiments

Liver MNC that were isolated from mice with or without malarial infection were used for cell transfer experiments (32). A total of 5×10^6 liver MNC were i.v. injected into 4-Gy-irradiated B6 or B6-*nu*/*nu* mice. These mice were infected with malaria within 1 day after cell transfer.

Results

Survival conditions for athymic nude mice infected with malaria

Control B6 mice were able to recover from malarial infection when 10^4 *P. yoelii*-infected erythrocytes (per mouse) were injected (Fig. 1*A*). However, the same injection resulted in death in athymic nude mice, which showed severe parasitemia in the blood (up to 60% parasitemia). Therefore, we reduced the number of infected erythrocytes to 5×10^3 per mouse. Although the parasitemia continued longer in athymic mice than in B6 mice, athymic mice finally recovered from malarial infection. Under the above conditions of application, immunologic responses seen in athymic nude mice were investigated thereafter.

Time kinetics of the number of lymphocytes in the liver and spleen during malarial infection

As was the case in a previous study (24), the major organs where lymphocytes expanded during malarial infection were the liver and spleen. We then compared the pattern of variation in the number of lymphocytes in the liver and spleen between control B6 mice and athymic mice (Fig. 1*B*). Severe lymphocytosis was seen in the liver and spleen of both B6 mice and athymic mice after malarial infection (5×10^3 *P. yoelii*-infected erythrocytes per mouse). The onset of lymphocytosis tended to be retarded in athymic mice, but the maximum number on day 21 was greater in athymic mice than in B6 mice.

Because the major lymphocyte subsets that expanded during malarial infection were NK1.1$^+$CD3^{int} and NK1.1$^-$CD3^{int} cells in B6 mice, the time kinetics of these subsets were also identified (Fig. 1*C*). Two-color staining of lymphocytes for CD3 and NK1.1 was conducted in the liver of both B6 and athymic mice in this experiment.

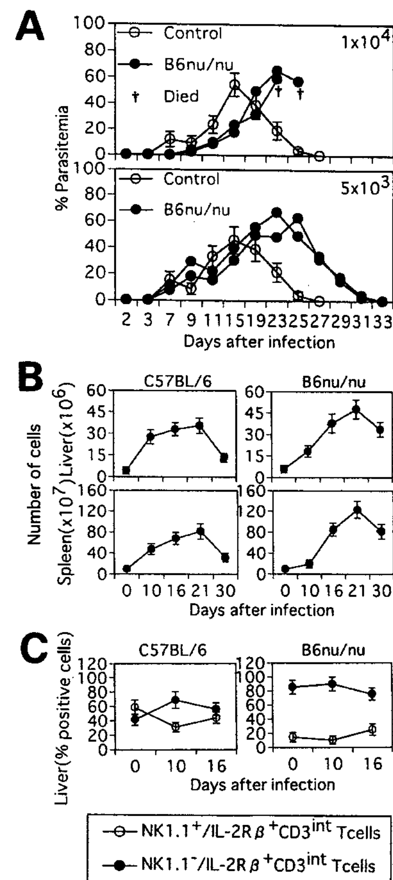

FIGURE 1. Parasitemia and immunoparameters in control B6 mice and athymic nude mice after malarial infection. *A*, Parasitemia and death. *B*, Time kinetics of the number of lymphocytes and lymphocyte subsets in the liver (or the spleen). *C*, Proportional changes of NK1.1$^+$TCR^{int} and NK1.1$^-$TCR^{int} cells among total TCR^{int} cells in the liver during malarial infection. Malaria-infected erythrocytes (10^4 or 5×10^3) were injected into B6 mice and athymic nude mice. In the case of B6 mice, the mean and 1 SD were produced from four mice. NK1.1$^+$CD3^{int} (IL-2Rβ^+) and NK1.1$^-$CD3^{int} (IL-2Rβ^+) subsets were identified by two-color staining for NK1.1 and CD3. All these subsets were IL-2Rβ^+ (see the subsequent phenotypic study). The mean and 1 SD were produced from four mice.

In both types of mice, the major expanding subset was NK1.1$^-$CD3^{int} cells. This was more striking in the athymic nude mice.

Details of the phenotypic characterization of lymphocytes that expanded in the liver and spleen during malarial infection

Two-color staining for CD3 and IL-2Rβ is represented first (Fig. 2*A*). This staining simultaneously identified IL-2Rβ^+CD3$^-$ NK cells, IL-2Rβ^+CD3^{int} cells, and IL-2Rβ^-CD3^{high} conventional T cells, especially in the liver and spleen of normal B6 mice (see Fig. 2*A*, *left column*). However, this general staining pattern was prominently changed in B6 mice after malarial infection. IL-2Rβ^+CD3^{int} cells became a major lymphocyte subset in both the liver and spleen (Fig. 2*A*, arrowheads in the liver). Because the intensity of IL-2Rβ expression decreased slightly in CD3^{int} cells, the area of CD3^{int} cells spread to that of IL-2Rβ^-CD3^{high} cells. In the case of athymic nude mice, IL-2Rβ^+CD3$^-$ NK cells were abundant in the liver, and T cells that existed in the liver and spleen were only IL-2Rβ^+CD3^{int} cells. During malarial infection, the proportion of NK cells decreased while that of CD3^{int} cells increased in the liver (Fig. 2*A*, arrowhead on day 21) and in the spleen.

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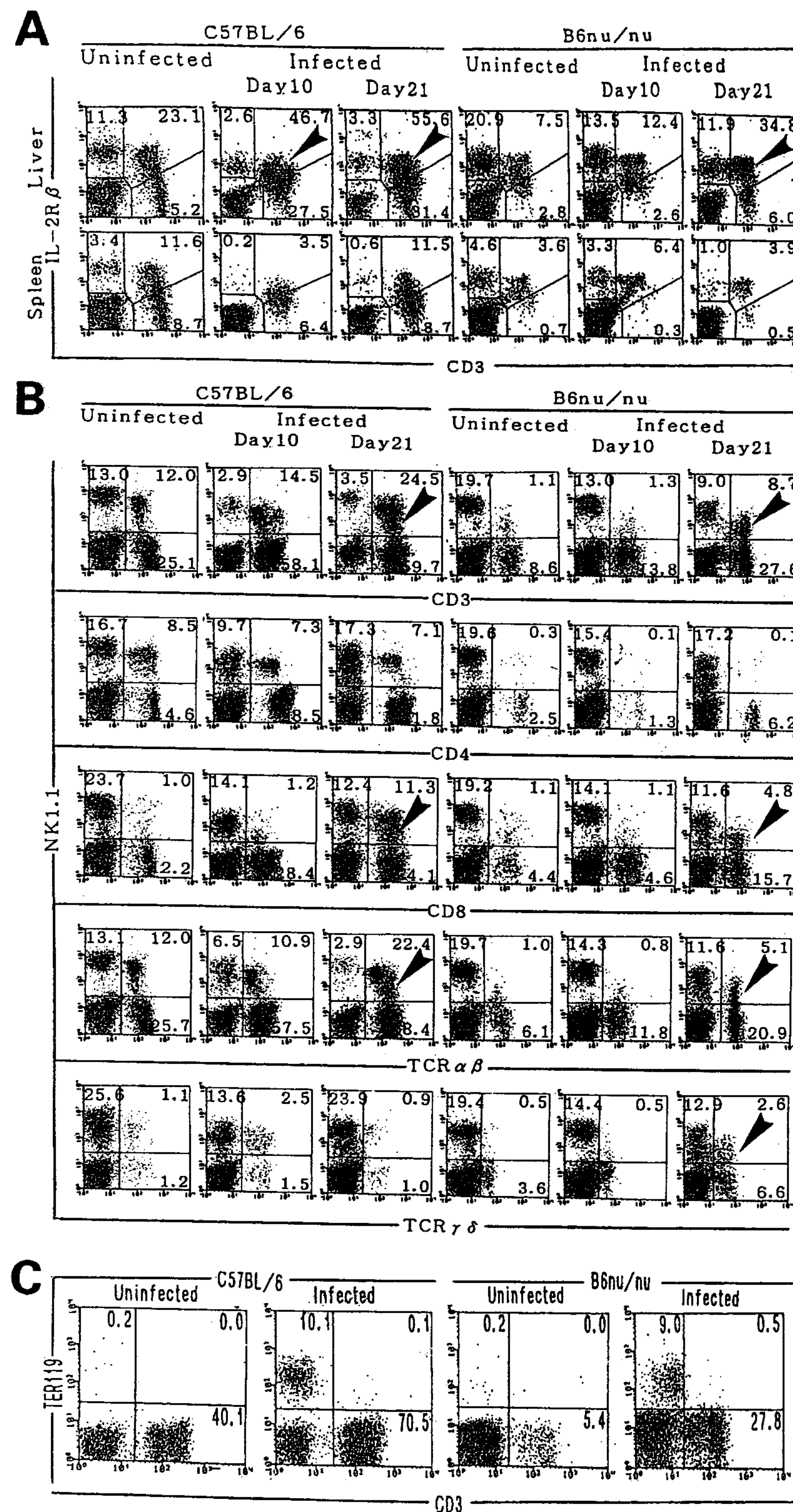

FIGURE 2. Phenotypic characterization of lymphocytes in the liver and spleen of B6 mice and athymic nude mice. *A*, Two-color staining for CD3 and IL-2Rβ. *B*, Two-color staining for NK1.1 and other markers. *C*, Two-color staining for TER119 and CD3. Lymphocytes were isolated at days 10 and 21 after malarial infection. Numbers represent the percentage of fluorescence-positive cells in the corresponding areas. The data shown are representative of three experiments. The expansion of IL-2Rβ^+CD3^{int} cells and NK1.1$^+$ T cells is indicated by arrowheads.

We have previously reported that both NKT cells and NK1.1$^-$CD3^{int} cells were stimulated by malarial infection, although the expansion of NK1.1$^-$CD3^{int} cells was much more dominant (25). This result was confirmed by two-color staining for CD3 and NK1.1 in the liver of B6 mice (Fig. 2*B*, arrowhead on day 21). In the case of B6 mice, such NKT cells were estimated to be CD4$^+$ or CD8$^+$TCR$\alpha\beta^{int}$ cells (Fig. 2*B*, rows 2–5).

In the case of athymic mice, NKT cells, which are primarily of thymic origin, were extremely few before malarial infection. However, NKT cells expanded in the liver of athymic mice (Fig. 2*B*,

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arrowhead on day 21). Two-color stainings for other combinations revealed that these NKT cells were $CD8^+TCR\alpha\beta^{int}$ or $CD8^+$ $TCR\gamma\delta^+$. $CD4^+$ NKT cells never appeared in conjunction with malarial infection in nude mice.

During malarial studies in humans and mice, we have often observed clusters of cells in the parenchymal space of the liver; such clusters contain not only lymphoid cells but also erythroid cells. In this regard, we conducted erythrocyte ($TER119^+$) staining by using liver MNC (Fig. 2*C*). These liver MNC were used after RBC lysis; therefore, denucleated RBC were not present in these preparations. Irrespective of whether the mice were euthymic or athymic, nucleated $TER119^+$ erythrocytes newly appeared after malarial infection (day 21). It was speculated that extramedullary erythropoiesis began in the liver as the result of malarial infection.

No appearance of $V\alpha14J\alpha281^+$ NKT cells before and after malarial infection in athymic mice

Phenotypic study showed that $CD4^+$ NKT cells did not appear in athymic nude mice even after malarial infection. This result was confirmed by RT-PCR method using $V\alpha14J\alpha281$ mRNA (Fig. 3). Thus, conventional $CD4^+$ NKT cells preferentially use an invariant chain of $V\alpha14J\alpha281$ gene for $TCR\alpha$ (17). The sign of $V\alpha14$ mRNA was detected in liver lymphocytes of euthymic B6 mice and this sign was almost unchanged in these mice after malarial infection. However, irrespective of malarial infection, the sign of $V\alpha14$ mRNA was not detected at all in liver lymphocytes of athymic nude mice.

Appearance of cell clusters in the liver after malarial infection

The expansion of $NK1.1^-TCR^{int}$ cells and nucleated erythrocytes in the liver after malarial infection suggested a new generation of these cells in the liver. This possibility was examined by histology (Fig. 4). There were no clusters in the liver of athymic mice without malarial infection (Fig. 4*A*). In sharp contrast, large cell clusters appeared in the parenchymal space of the liver in both euthymic mice (Fig. 4*B*) and athymic mice (Fig. 4*C*) after malarial infection (day 21).

Autoantibody production during malarial infection

There was intimate cooperation between extrathymic T cells and B-1 cells in certain autoimmune diseases and during malarial infection (25). This possibility was examined in athymic nude mice infected with malaria (Fig. 5). B6 mice infected with malaria were examined in parallel. In the case of B6 mice, both IgG and IgM types of autoantibodies against denatured DNA were detected. However, only the IgM type of autoantibodies was detected in athymic mice after malarial infection. In these experiments, sera of *lpr* mice were used as a positive control (adjusted to 100 U/ml).

Capability of protection against malaria by extrathymic T cells

To directly prove the capability of protection against malaria by extrathymic T cells, cell transfer experiments of liver MNC isolated from athymic nude mice with or without malarial infection were conducted (Fig. 6). B6 mice and athymic mice were used as recipients after 4-Gy irradiation. Transferred cells were prepared from normal athymic nude mice or from athymic nude mice that had recovered from malaria (within 4 mo after recovery). When 4-Gy-irradiated B6 mice and 4-Gy-irradiated athymic mice (no cell transfer) were infected with malaria, all of these mice died as a consequence of parasitemia. This was also the case when naive MNC (5×10^6 per mouse) were transferred into these mice (Fig. 6, *lower panels*). In sharp contrast, when immune MNC (5×10^6 per mouse) were transferred into these mice, both B6 and athymic mice were able to survive and showed no parasitemia. In other words, only liver lymphocytes isolated from athymic mice that had recovered from malaria were able to protect mice from malaria.

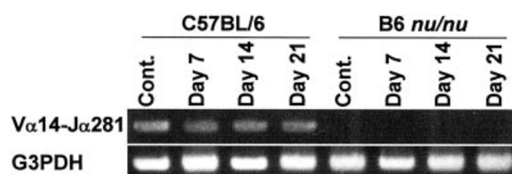

FIGURE 3. No expansion of $V\alpha14J\alpha281^+$ NKT cells during malarial infection. To confirm that $CD4^+$ NKT cells did not expand after malarial infection, RT-PCR method was applied to detect $V\alpha14J\alpha281$ mRNA. Total RNA was extracted from liver lymphocytes of B6 and B6-*nu/nu* mice before (Cont.) and after the infection (days 7, 14, and 21).

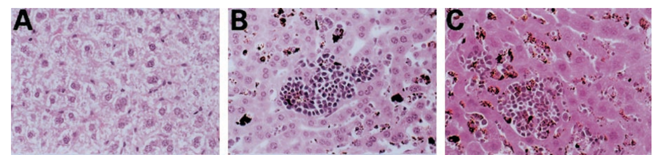

FIGURE 4. Histology of the liver in light microscopy (×400). *A*, The liver of normal B6 athymic mice. *B*, The liver of B6 mice after malarial infection (day 21). *C*, The liver of B6 athymic mice after malarial infection (day 21). H&E staining was conducted.

Discussion

In the present study, we demonstrated that the major expanding T cells were $NK1.1^-IL\text{-}2R\beta^+CD3^{int}$ cells when athymic nude mice were infected with malaria and subsequently recovered from it. Moreover, by cell transfer experiments, these $NK1.1^-IL\text{-}2R\beta^+CD3^{int}$ cells were found to have the ability to protect mice from malaria if such lymphocytes were isolated from the liver of mice that had recovered from malaria. These results suggest that the protection from malarial infection might be the result of immunological events achieved by extrathymic T cells. This notion is also supported by the phenomenon of autoantibody production in athymic mice during malarial infection. Namely, the activation of extrathymic T cells is always accompanied by the autoantibody production by B-1 cells.

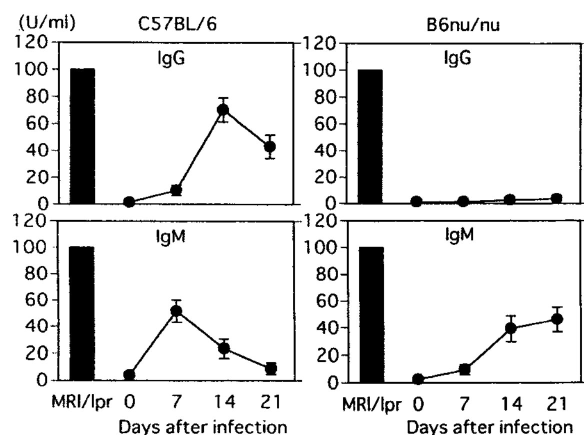

FIGURE 5. Autoantibody production in B6 mice and athymic nude mice after malarial infection. Sera were obtained at the indicated points of time. Both IgG and IgM types of autoantibodies against denatured DNA were detected by ELISA. Sera of *lpr* mice (after the onset of autoimmune disease) were used as a positive control (adjusted as 100 U/ml). The mean and 1 SD were produced by four experiments.

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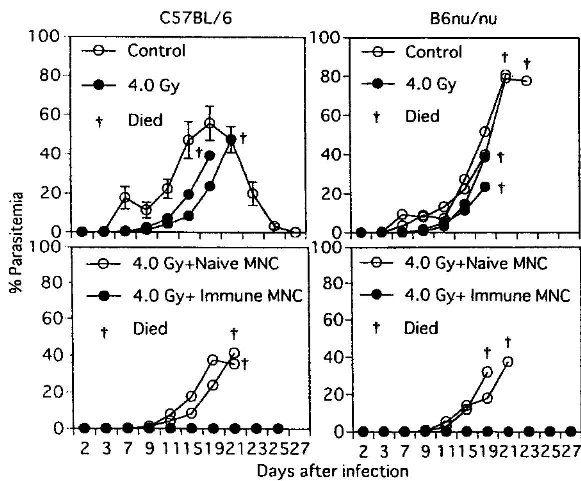

FIGURE 6. Protection against malaria as shown by cell transfer experiments using liver MNC isolated from athymic mice recovered from malaria. Normal B6 mice and athymic nude mice were used as controls. Irradiated (4 Gy) B6 mice and athymic nude mice were also used. These irradiated mice were also used as recipients for cell transfer experiments. In the case of control B6 mice, the mean and 1 SD were produced from four mice. Liver MNC were isolated from normal athymic mice and athymic mice that had recovered from malaria (within 4 mo of the recovery). A total of 5×10^6 liver MNC per mouse were injected into recipient mice.

For a long time, protection against malaria has been considered to be due to immunological events mediated by conventional T cells, including $CD4^+$ and $CD8^+$ T lymphocytes (7–14). However, this conception raises several questions: 1) why does immunological memory that humans or animals once acquired by malarial infection disappear 1 year or more after the infection (24, 33, 34), 2) why do autoantibodies often appear during malarial infection (35–39), and 3) why is thymic atrophy always accompanied by malarial infection (25)? The third question is serious. If conventional T cells are important for protection against malaria, the arrest of intrathymic T cell differentiation by severe thymic atrophy during malarial infection is not easily explained.

According to the above-mentioned reasons, we conducted experiments of malarial infection in athymic nude mice that carry only extrathymic T cells (29). Under athymic conditions, these mice primarily lack two important T lymphocyte subsets of thymic origin, namely, conventional T cells (i.e., $NK1.1^-IL\text{-}2R\beta^-TCR^{high}$ cells) and NKT cells (i.e., $NK1.1^+IL\text{-}2R\beta^+TCR^{int}$ cells). Although NKT cells are abundant in the liver of euthymic mice, these primordial T cells or their precursors originate in the thymus through an alternative intrathymic pathway (27, 28). Indeed, as shown in the present study, athymic nude mice carry only extrathymic T cells (i.e., $NK1.1^-IL\text{-}2R\beta^+TCR^{int}$ cells). In this situation, we found that extrathymic T cells had the ability to protect mice from malarial infection.

In this study, we used 5×10^3 *P. yoelii*-infected erythrocytes to induce the blood stage malarial infection. This was due to the fact that athymic nude mice were more sensitive to malarial death than euthymic mice with the same genetic background (B6). We have to consider the possibility that some other lymphocyte subsets may cooperatively act with extrathymic T cells for malarial protection. In this case, one of the candidates is NKT cells. Under euthymic conditions, we have reported that NKT cells were also activated during malarial infection and that NKT-deficient mice (e.g., CD1d knockout mice) were more susceptible to malarial death than normal mice (25).

Extrathymic T cells carry many properties as primordial T cells (15–18); one such property is that the activation of extrathymic T cells is often accompanied by autoantibody production by primordial B-1 cells (25). When syngeneic denatured liver tissue was injected into mice, mice fell victim to autoimmune hepatitis. At this time, thymic atrophy, the activation of extrathymic T cells, and autoantibodies against denatured DNA were simultaneously induced (our unpublished observation). Chronic graft-vs-host disease is also known as an autoimmune-like state that accompanies thymic atrophy, the activation of extrathymic T cells, and autoantibody production (40, 41). Under both euthymic conditions and athymic conditions (the present study), a similar phenomenon (activation of extrathymic T cells and autoantibody production) was evoked during malarial infection.

Primarily, $CD4^+$ NKT cells that use $V\alpha14J\alpha281$ are absent in athymic nude mice (42–45). Even after malarial infection, such $CD4^+$ NKT cells did not appear in these mice. However, it was found that a significant proportion of NKT cells did newly appear in the liver of athymic mice after malarial infection. All of these NKT cells were $CD8^+$ or DN cells that use $TCR\alpha\beta$ or $TCR\gamma\delta$. None of them used an invariant chain of $V\alpha14J\alpha281$ as shown by the RT-RCR method. Another interesting finding was the new appearance of $TER119^+$ nucleated erythrocytes in the liver after malarial infection. This was seen in both euthymic and athymic mice. It is conceivable that the large cell clusters in the parenchymal space of the liver (see Fig. 4) may comprise not only newly generated extrathymic T cells but also newly generated erythrocytes (i.e., extramedullary erythropoiesis).

In the case of athymic mice, only the IgM type (but not IgG type) of autoantibodies against denatured DNA appeared after malarial infection. In contrast, autoimmune MRL-*lpr*/*lpr* mice and euthymic mice with malaria produced both types of the autoantibodies. It is speculated that some help (e.g., by conventional T cells) may be required for the complete production of autoantibodies.

In a final cell transfer experiment, we demonstrated that extrathymic T cells had the ability to protect mice from malarial death. Namely, when extrathymic T cells were isolated from the liver of athymic nude mice that had recovered from malarial infection, these T cells rescued both 4-Gy-irradiated euthymic and athymic mice from death. In conjunction with the expansion of limited lymphocyte subsets (i.e., extrathymic T cells and possible B-1 cells) during malarial infection, it is presumed that the protection against malaria may be the result of immunological events mediated by the innate immune system rather than by the developed immune system (mediated by conventional T and B cells).

Acknowledgments

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