ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2009, p. 4533–4536 Vol. 53, No. 10
0066-4804/09/$08.00+0 doi:10.1128/AAC.00519-09

Improved Murine Model of Malaria Using *Plasmodium falciparum* Competent Strains and Non-Myelodepleted NOD-*scid IL2Rγ*^{null} Mice Engrafted with Human Erythrocytes$^{\nabla}$

María Belén Jiménez-Díaz,[1] Teresa Mulet,[1] Sara Viera,[1] Vanessa Gómez,[1] Helen Garuti,[1] Javier Ibáñez,[1] Angela Alvarez-Doval,[1] Leonard D. Shultz,[2] Antonio Martínez,[1] Domingo Gargallo-Viola,[1]† and Iñigo Angulo-Barturen[1]*

ID CEDD Diseases of the Developing World, GlaxoSmithKline, c/Severo Ochoa 2, 28760, Tres Cantos, Spain,[1] *and The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609*[2]

Received 20 April 2009/Returned for modification 10 June 2009/Accepted 8 July 2009

Murine models of *Plasmodium falciparum* malaria may become crucial tools in drug discovery. Here we show that non-myelodepleted NOD-*scid IL2Rγ*^{null} mice engrafted with human erythrocytes support an infectious burden up to tenfold higher than that supported by engrafted NOD-*scid* β2*microglobulin*^{null} mice. The new model was validated for drug discovery and was used to assess the therapeutic efficacy of 4-pyridones, selective inhibitors of *P. falciparum* cytochrome bc_1.

Malaria is caused by the erythrocytic stages of protozoan parasites of the genus *Plasmodium*. Among the species pathogenic for humans, *Plasmodium falciparum* is responsible for 300 to 500 million cases of malaria and over a million deaths annually, particularly in developing countries. The development of new antimalarial medicines and vaccines is a key part of the global strategy for malaria eradication (6).

P. falciparum almost exclusively infects human erythrocytes (hE). As a result, candidate drugs and vaccines in early stages of preclinical development are usually tested in vivo by measuring their therapeutic efficacy against rodent-adapted plasmodial species and by assessing the antiparasitic response of non-human immune systems, respectively (11). To overcome the host specificity issue, two conceptually different murine models of erythrocytic stages of *P. falciparum* malaria have been developed. The first one requires chemical in vivo depletion of phagocytic cells from immunodeficient mice engrafted with hE in order to allow the growth of *P. falciparum* after intraperitoneal (i.p.) infection (2, 8). However, its variable kinetics of parasitemia and, particularly, the use of toxic reagents, which might affect the efficacy of antimalarials or effector cells, have limited its use in drug discovery (5). Recently, a new *P. falciparum* murine model that does not require in vivo myeloablative treatment of mice and is suitable for drug discovery was described (1). In this new model, NOD-*scid* mice genetically deficient in beta-2 microglobulin (β2 m^{tm1Unc}, abbreviated as β2 m^{null}) engrafted with hE (HM-β2 m^{null}) are infected intravenously with *P. falciparum* strains selected in vivo for their competence to grow reproducibly in hE-engrafted immunodeficient mice (1).

The NOD-*scid* β2 m^{null} mouse strain retains residual NK cell activity as well as other innate immune functions and shows a high incidence of early thymic lymphomas, which dramatically diminish their life span (4). These characteristics may be a serious problem for addressing long-term pharmacokinetic/pharmacodynamic (PK/PD) studies because of the relatively low total parasite burden per mouse achievable (1) and the short life span of NOD-*scid* β2 m^{null} mice (4). Interestingly, NOD-*scid* strains carrying a null mutation of the interleukin 2 (IL-2) receptor γ chain (*IL2Rγ*^{tm1Wjl}, abbreviated as *IL2Rγ*^{null}) have been developed (10). These murine strains lack fully mature NK cells and show additional defects in their innate immune system that explain their greater ability to support the engraftment of human leukocytes and their lower rate of lymphomas (13). Hence, NOD-*scid IL2Rγ*^{null} mice were tested as a model for a non-myelodepleted *P. falciparum* murine model.

The murine *P. falciparum* models require the engraftment of mice with hE. In the non-myelodepleted model, the engraftment of mice is achieved and maintained by daily i.p. injections throughout the experiments. Therefore, the ability of NOD-*scid IL2Rγ*^{null} mice to be engrafted with hE was tested. To assess this point, 8-week-old immunodeficient female NOD-*scid IL2Rγ*^{null} mice (The Jackson Laboratory, Bar Harbor, ME) were each injected i.p. daily with 1 ml of hE (AB Rh−; generously donated by the Spanish Red Cross Blood Bank, Madrid, Spain) suspended in isotonic solution (RPMI 1640, 25% decomplemented human serum, 3.1 mM hypoxanthine) at 50% hematocrit, as described previously (1). As shown in Fig. 1, the kinetics of engraftment in NOD-*scid IL2Rγ*^{null} fitted a one-phase exponential association function ($R^2 = 0.92$; half-life = 6.8 ± 0.1 days; 80 mice) as found previously for NOD-*scid* β2 m^{null} ($R^2 = 0.75$; half-life = 7.5 ± 0.6 days; 162 mice) (1). Hence; non-myelodepleted NOD-*scid IL2Rγ*^{null} mice could be efficiently and stably engrafted with hE by i.p. injection.

Next, the susceptibility of NOD-*scid IL2Rγ*^{null} mice engrafted with hE (HM-*IL2Rγ*^{null}) to infection by competent *P.*

* Corresponding author. Mailing address: GlaxoSmithKline, ID CEDD Diseases of the Developing World, Drug Discovery Biology, Therapeutic Efficacy, c/Severo Ochoa, 2, Tres Cantos 28760 Madrid, Spain. Phone: 34 91 807 57 40. Fax: 34 91 807 05 95. E-mail: inigo.x.angulo@gsk.com.

† Present address: Department of Toxicology and Safety Pharmacology, Ferrer Grupo, Barcelona, Spain.

$^{\nabla}$ Published ahead of print on 13 July 2009.

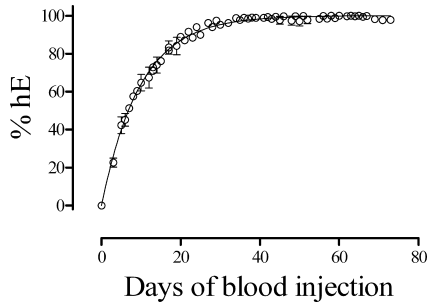

FIG. 1. Kinetics of engraftment of hE in NOD-*scid IL2Rγ*^{null} mice. Data are means ± standard errors of the means for TER-119$^-$ erythrocytes pooled from 80 mice in two separate experiments. Individual data fitted a one-phase exponential association equation. Only error bars that extend beyond the symbols are shown.

falciparum strains was assessed. The area under the curve for the concentration of murine erythrocytes (mE), hE, and infected hE (ihE) in peripheral blood versus time was evaluated in order to account for the temporal dimension of data (1). Those AUC variables were found to be normally distributed (1) and were analyzed using a multivariate general linear model (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL). For each blood sample, the degrees of engraftment (concentration of mE and hE) and parasitemia (concentration of ihE) were measured simultaneously by flow cytometry using the nucleic acid dye SYTO-16 (Molecular Probes, Leiden, The Netherlands) and anti-mouse erythrocyte monoclonal antibody TER-119 (Pharmingen, San Diego, CA), as described previously (7). Measuring engraftment and parasitemia simultaneously is essential in therapeutic efficacy assays in order to ascertain that a drug's effectiveness is not due to drug-induced destruction of hE. Accordingly, mE were gated as TER-119$^+$, hE as TER-119$^-$, and ihE as TER-119$^-$ SYTO-16$^+$. Once the methodology was established, two cohorts of HM-β2 *m*^{null} or HM-*IL2Rγ*^{null} mice (10 mice/cohort) were infected intravenously with 20×10^6 parasitized erythrocytes at day 10 after the start of blood injections. Half the mice from each murine strain were infected with Pf3D7$^{0087/N9}$ (generated from clone 3D7 of the NF54 strain; sensitive to all antimalarials) and the other half with PfV1/S$^{0176/N10}$ (generated from clone V1/S of the V1 strain; pyrimethamine and chloroquine resistant) (1). As shown in Fig. 2A, B, D, and E, the overall erythrocyte dynamics in peripheral blood of infected HM-*IL2Rγ*^{null} and HM-β2 *m*^{null} were similar. Interestingly, all parasite-mouse strain combinations showed a selective elimination of hE that was dependent on parasite growth, because it could be inhibited by treatment with standard antimalarials (data not shown) (1). Concomitant with the clearance of hE, a compensatory increase in mE was observed in all experimental groups. In contrast, the Pf3D7$^{0087/N9}$ infectious burden in HM-*IL2Rγ*^{null} mice (peak parasitemias around 12 to 14%) was 10-fold higher than that in HM-β2 *m*^{null} mice (Fig. 2C; Table 1). Similarly, the PfV1/S$^{0176/N10}$ infectious burden in HM-*IL2Rγ*^{null} mice (peak parasitemias around 30 to 40%) was 5-fold higher than that in HM-β2 *m*^{null} mice (Fig. 2F; Table 1). Interestingly, the infectious burden in PfV1/S$^{0176/N10}$-infected mice was always higher than that in Pf3D7$^{0087/N9}$-infected mice (Table 1). These results were confirmed in two additional observational experiments. It is worth

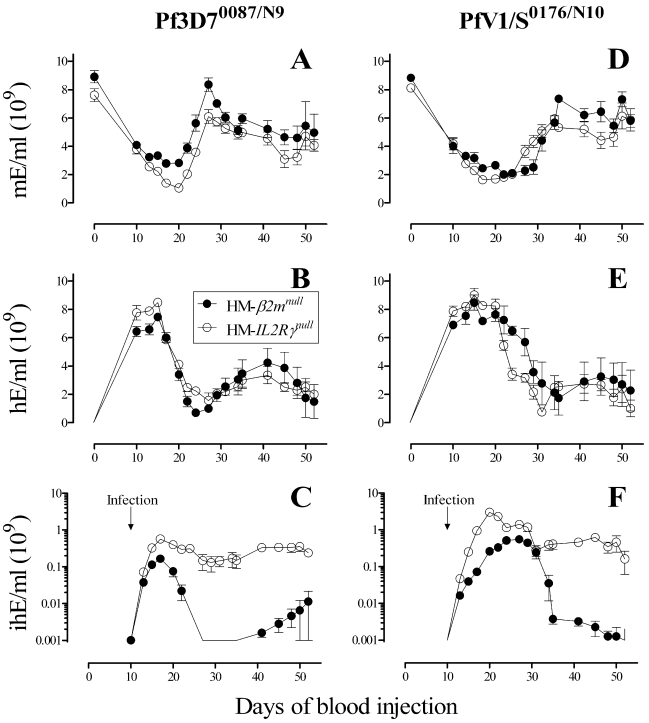

FIG. 2. Chimerism and infection by *P. falciparum* in HM-β2 *m*^{null} and HM-*IL2Rγ*^{null} mice. All mice were injected i.p. with hE daily from day 0 to day 52 after the start of injections. Data are means ± standard errors of the means for five mice per data point except for HM-β2 *m*^{null} mice infected with PfV1/S$^{0176/N10}$, for which only four mice were sampled. Note that the scale in panels C and F is logarithmic. Only error bars that extend beyond the symbols are shown.

mentioning that the parasite strain and not the murine background was the main factor governing the time required to achieve the maximum parasitemia (Cox regression analysis, $P < 0.001$), which was longer in PfV1/S$^{0176/N10}$-infected mice (Table 1). As a whole, our results indicate that HM-*IL2Rγ*^{null} mice are more susceptible to infection by competent *P. falciparum* strains than HM-β2 *m*^{null} mice. Moreover, the results suggest that the mechanism of hE destruction elicited by infection and the innate immune response against *P. falciparum* depend on different signaling pathways. One possibility is that signaling through cytokine

TABLE 1. Dynamics of infection in HM-β2 *m*^{null} and HM-*IL2Rγ*^{null} mice[a]

Parasite	Mouse	AUC$_{10–48}$[b]	C_{max}[c]	t_{max}[d]
Pf3D7$^{0087/N9}$	HM-β2 *m*^{null}	1,043 ± 286	164 ± 49	17
	HM-*IL2Rγ*^{null}	11,065 ± 2,100	581 ± 74	17
PfV1/S$^{0176/N10}$	HM-β2 *m*^{null}	5,863 ± 1,216	625 ± 158	27
	HM-*IL2Rγ*^{null}	33,108 ± 6,429	3,035 ± 393	20

[a] Data from five mice/group, except for HM-β2 *m*^{null} infected with PfV1/S$^{0176/N10}$, for which there were four mice/group.

[b] AUC$_{10–48}$, area under the curve for concentration of erythrocytes (10^6 ihE/ml) versus time (days) between days 10 and 48 after the start of blood injections. Data are means ± standard deviations.

[c] C_{max}, highest concentration of erythrocytes, expressed as 10^6 ihE/ml. Data are means ± standard deviations.

[d] t_{max}, day after the start of blood injections at which maximum concentration of ihE was achieved. All mice were infected at day 10 after the start of blood injections. Data are medians.

receptors sharing the IL2Rγ chain (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) may be responsible for the innate immune response against *P. falciparum* in engrafted mice. This signaling pathway is required for development of NK cells in NOD-*scid IL2Rγ*^{null} mice (13). Hence, our data point to defective interaction between NK cells and phagocytic accessory cells, which is important for triggering antimalarial immune responses (9), as a plausible explanation for the inability of NOD-*scid IL2Rγ*^{null} mice to control replication of *P. falciparum*.

The HM-*IL2Rγ*^{null} murine *P. falciparum* model was validated as a tool for drug discovery. In order to address this point, chloroquine, artesunate, and pyrimethamine were tested against Pf3D7$^{0087/N9}$ in a 4-day test as described previously (1). This assay was designed to measure the antimalarial efficacy of new compounds over two cycles of exponential *P. falciparum* growth in vivo. Briefly, 3 days after intravenous infection with 20×10^6 Pf3D7$^{0087/N9}$-infected hE, HM-*IL2Rγ*^{null} mice were randomly distributed into groups (three mice/group) and treated orally once a day for four consecutive days with different doses of each drug. In order to measure their potency, the effective doses that reduced parasitemias by 90% (ED_{90}) at day 7 after infection with respect to vehicle-treated mice were calculated by fitting data to a sigmoid dose-response curve (GraphPad 5.0; GraphPad Software, San Diego, CA). Data from observational studies indicated that the level of parasitemia at day 7 was a normally distributed variable (parasitemia, 8.2% ± 2.7%, 88 mice; $P = 0.986$ [Kolmogorov-Smirnov normality test with the Lilliefors correction of significance]). The sample size per experimental group ($n = 3$) was calculated as the minimum required to detect 50% reduction in parasitemia with respect to a vehicle-treated control group, assuming a value for α (confidence level) of 0.05 and a value for β (power of the assay) of 0.9. As shown in Fig. 3, all compounds showed a clear dose-response effect, and only parasites from mice treated with effective doses showed the characteristic microscopic alterations found previously (1). The following parameters of efficacy were estimated: $ED_{90(chloroquine)} = 4.9 \pm 0.5$, $ED_{90(artesunate)} = 12.9 \pm 1.0$, and $ED_{90(pyrimethamine)} = 0.5 \pm 0.2$ mg/kg. Therefore, their effectiveness was similar to that reported for the HM-β2 *m*^{null} model ($ED_{90(chloroquine)} = 4.4 \pm 1$, $ED_{90(artesunate)} = 12.7 \pm 1.3$, and $ED_{90(pyrimethamine)} = 0.9 \pm 0.2$ mg/kg) (1). To further quantitatively and qualitatively validate the *P. falciparum* HM-*IL2Rγ*^{null} model, we tested the aforementioned antimalarials against PfV1/S$^{0176/N10}$ in the 4-day test. As expected, the no-effect controls chloroquine and pyrimethamine were ineffective against this multiresistant strain at 50 mg/kg ($ED_{90(chloroquine,pyrimethamine)} > 50$ mg/kg, which is at least 10 and 100 times less efficacious, respectively, than against the susceptible strain Pf3D7$^{0087/N9}$), whereas the $ED_{90(artesunate)}$ was <20 mg/kg (no less efficacious than against the susceptible strain Pf3D7$^{0087/N9}$).

The HM-*IL2Rγ*^{null} model has been used in the preclinical evaluation of the therapeutic efficacy of 4(1H)-pyridones, which are selective inhibitors of cytochrome bc_1 from the electron transport chain present in *P. falciparum* mitochondrion (14). The candidate for clinical development GSK932121 was found to be as potent against *P. falciparum* Pf3D7$^{0087/N9}$ as pyrimethamine in both HM-β2 *m*^{null} mice ($ED_{90} = 0.6 \pm 0.2$ mg/kg) and HM-*IL2Rγ*^{null} mice ($ED_{90} = 0.6 \pm 0.1$ mg/kg) (Fig. 3) when administered orally in water–1% methylcellulose (Sigma). Interestingly, the $ED_{90(GSK932121)}$ against PfV1/S$^{0176/N10}$ in the 4-day test was found to be <1 mg/kg. These results suggest that GSK932121 might be useful for patient treatment in areas of high prevalence of chloroquine- and pyrimethamine-resistant isolates (e.g., Southeast Asia).

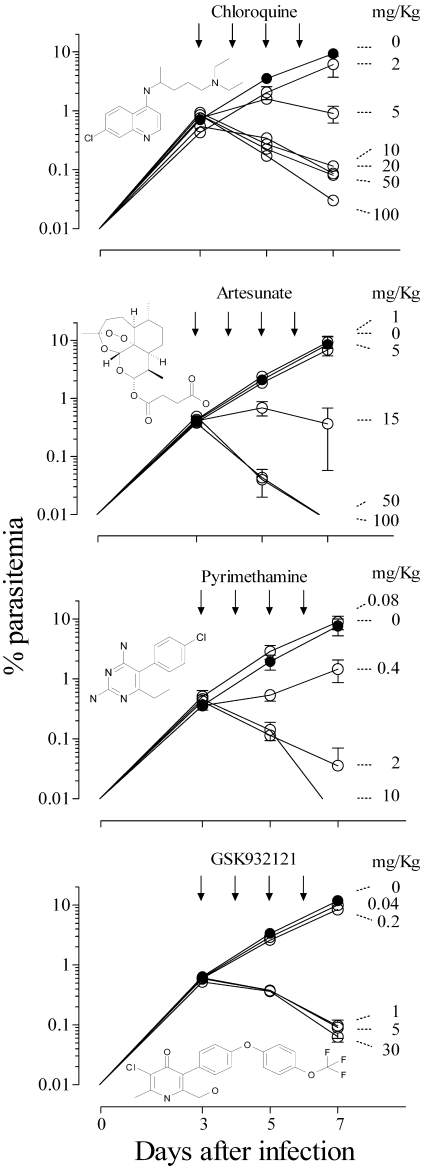

FIG. 3. Therapeutic efficacy of chloroquine, artesunate, pyrimethamine, and GSK932121 against *P. falciparum* Pf3D7$^{0087/N9}$ in the HM-IL2Rγ^{null} model. The arrows indicate the days of treatment in the standard *P. falciparum* 4-day test, and values are the level of parasitemia in peripheral blood of three mice/group. Data are means ± standard errors of the means. Only error bars that extend beyond the symbols are shown.

P. falciparum murine models are expensive and logistically demanding. However, with the exception of humans, they are the only in vivo models available for studying the parasite's biology inside hE. Here we show that the *P. falciparum* model in HM-*IL2Rγ*^{null} is as reliable for drug evaluation in vivo as the former HM-β2 *m*^{null} model and has two important advantages. First, the longer life span of HM-*IL2Rγ*^{null} mice has enabled us to perform long-term PK/PD experiments without mouse

deaths due to thymic lymphomas. In preliminary experiments, we found that HM-*IL2Rγ*^{null} mice harboring ~2 × 10^8 Pf3D7$^{0087/N9}$-infected hE each and treated orally with chloroquine at 50 mg/kg once a day for four consecutive days are free of parasites in peripheral blood at least 28 days after end of treatment (data not shown). A detailed PK/PD analysis of the therapeutic efficacy of GSK932121 is under way. Second, the higher susceptibility of HM-*IL2Rγ*^{null} mice to *P. falciparum* strains adapted to grow in hE-engrafted mice markedly enhances the range of infectious burdens achievable in HM-*IL2Rγ*^{null} mice for chemotherapy studies. For instance, the maximum infectious burden in HM-*IL2Rγ*^{null} mice infected with PfV1/S$^{0176/N10}$ is between 6 × 10^9 and 9 × 10^9 ihE/mouse (above 30% parasitemia) (Table 1), which is close to the lowest circulating parasite burden in patients included in clinical efficacy tests according to World Health Organization guidelines (3). In addition, the enhanced susceptibility of HM-*IL2Rγ*^{null} mice to *P. falciparum* infection may enable the development of reliable in vivo models to test the efficacy of vaccines against *P. falciparum* erythrocytic stages. This seems a feasible objective because NOD-*scid IL-2γ*^{null} mice are able to sustain high levels of engraftment with functional human hematolymphoid cells (12). Therefore, as the mouse innate immune system is unable to control infection, it should be possible to test the effect of candidate malaria vaccines on HM-*IL2Rγ*^{null} mice which have received adoptively transferred human immune cells. In conclusion, the use of NOD-*scid IL2Rγ*^{null} mice (13) engrafted with hE (HM-*IL2Rγ*^{null}) and appropriate competent *P. falciparum* strains opens up new possibilities for the preclinical evaluation of antimalarials and vaccines against erythrocytic stages of *P. falciparum*.

This work was supported in part by Medicines for Malaria Venture (Geneva, Switzerland) through GSK-Diseases of the Developing World/MMV agreement, by NIH grant HL077632, and by a grant from the Juvenile Diabetes Research Foundation.

We are indebted to Joaquín Rullas, Jose García-Bustos, and Geo Derimanov (GlaxoSmithKline, DDW) for critical review of the manuscript. We are deeply grateful to Emma Castro, Jose Luis Bueno, and Angel Pajares (Red Cross Blood Bank, Madrid, Spain) for the supply of blood to perform the experiments described in this paper. We also acknowledge the support of all the staff at Laboratory Animal Science Department in GlaxoSmithKline—Diseases of the Developing World in Tres Cantos (Spain) for providing and maintaining the mice used in this study.

All the experiments were approved by the DDW Ethical Committee on Animal Research, performed at DDW Laboratory Animal Science facilities accredited by AAALAC, and conducted according to European Union legislation and GlaxoSmithKline policy on the care and use of animals.

REFERENCES

1. **Angulo-Barturen, I., M. B. Jiménez-Díaz, T. Mulet, J. Rullas, E. Herreros, S. Ferrer, E. Jiménez, A. Mendoza, J. Regadera, P. J. Rosenthal, I. Bathurst, D. L. Pompliano, F. Gómez de las Heras, and D. Gargallo-Viola.** 2008. A murine model of *falciparum*-malaria by *in vivo* selection of competent strains in non-myelodepleted mice engrafted with human erythrocytes. PLoS One **3:**e2252.
2. **Badell, E., V. Pasquetto, P. Druilhe, and N. Van Rooijen.** 1995. A mouse model for human malaria erythrocytic stages. Parasitol. Today **11:**235–237.
3. **Bloland, P., P. Ringwald, and R. W. Snow.** 2003. Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated *falciparum* malaria. World Health Organization, Geneva, Switzerland. http://www.who.int/malaria/docs/ProtocolWHO.pdf.
4. **Christianson, S. W., D. L. Greiner, R. A. Hesselton, J. H. Leif, E. J. Wagar, I. B. Schweitzer, T. V. Rajan, B. Gott, D. C. Roopenian, and L. D. Shultz.** 1997. Enhanced human CD4+ T cell engraftment in β2-microglobulin-deficient NOD-*scid* mice. J. Immunol. **158:**3578–3586.
5. **Coslédan, F., L. Fraisse, A. Pellet, F. O. Guillou, B. Mordmüller, P. G. Kremsner, A. Moreno, D. Mazier, J.-P. Maffrand, and B. Meunier.** 2008. Selection of a trioxaquine as an antimalarial drug candidate. Proc. Natl. Acad. Sci. USA **105:**17579–17584.
6. **Greenwood, B. M., D. A. Fidock, D. E. Kyle, S. H. Kappe, P. L. Alonso, F. H. Collins, and P. E. Duffy.** 2008. Malaria: progress, perils, and prospects for eradication. J. Clin. Investig. **118:**1266–1276.
7. **Jiménez-Díaz, M. B., T. Mulet, V. Gómez, S. Viera, A. Alvarez, H. Garuti, Y. Vázquez, A. Fernández, J. Ibáñez, M. Jiménez, D. Gargallo-Viola, and I. Angulo-Barturen.** 2009. Quantitative measurement of *Plasmodium*-infected erythrocytes in murine models of malaria by flow cytometry using bidimensional assessment of SYTO-16 fluorescence. Cytometry A **75:**225–235.
8. **Moreno, A., E. Ferrer, S. Arahuetes, C. Eguiluz, N. V. Rooijen, and A. Benito.** 2006. The course of infections and pathology in immunomodulated NOD/LtSz-SCID mice inoculated with *Plasmodium falciparum* laboratory lines and clinical isolates. Int. J. Parasitol. **36:**361–369.
9. **Newman, K. C., D. S. Korbel, J. C. Hafalla, and E. M. Riley.** 2006. Cross-talk with myeloid accessory cells regulates human natural killer cell interferon-gamma responses to malaria. PLoS Pathog. **2:**e118.
10. **Pearson, T., D. L. Greiner, and L. D. Shultz.** 2008. Humanized SCID mouse models for biomedical research. Curr. Top. Microbiol. Immunol. **324:**25–51.
11. **Peters, W., and B. L. Robinson.** 1999. Malaria, p. 757–773. *In* O. Zak and M. A. Sande (ed.), Handbook of animal models of infection. Academic Press, New York, NY.
12. **Shultz, L. D., F. Ishikawa, and D. L. Greiner.** 2007. Humanized mice in translational biomedical research. Nat. Rev. Immunol. **7:**118–130.
13. **Shultz, L. D., B. L. Lyons, L. M. Burzenski, B. Gott, X. Chen, S. Chaleff, M. Kotb, S. D. Gillies, M. King, J. Mangada, D. L. Greiner, and R. Handgretinger.** 2005. Human lymphoid and myeloid cell development in NOD/LtSz-*scid* IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J. Immunol. **174:**6477–6489.
14. **Yeates, C. L., J. F. Batchelor, E. C. Capon, N. J. Cheesman, M. Fry, A. T. Hudson, M. Pudney, H. Trimming, J. Woolven, J. M. Bueno, J. Chicharro, E. Fernandez, J. M. Fiandor, D. Gargallo-Viola, F. Gomez de las Heras, E. Herreros, and M. L. Leon.** 2008. Synthesis and structure-activity relationships of 4-pyridones as potential antimalarials. J. Med. Chem. **51:**2845–2852.