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Vγ9Vδ2 T-Cell Anergy and Complementarity-Determining Region 3-Specific Depletion during Paroxysm of Nonendemic Malaria Infection

Federico Martini,[1]* Maria Grazia Paglia,[1] Carla Montesano,[1] Patrick J. Enders,[2] Marco Gentile,[3] C. David Pauza,[2] Cristiana Gioia,[1] Vittorio Colizzi,[4] Pasquale Narciso,[3] Leopoldo Paolo Pucillo,[1] and Fabrizio Poccia[1]

Laboratory of Clinical Pathology,[1] 4th Clinical Division,[3] and ICAERI,[4] National Institute for Infectious Diseases "Lazzaro Spallanzani" I.R.C.C.S., Rome, Italy, and Institute of Human Virology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21021[2]

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Vγ9Vδ2 T lymphocytes strongly respond to phosphoantigens from *Plasmodium* parasites. Thus, we analyzed the changes in Vγ9Vδ2 T-cell function and repertoire during the paroxysm phase of nonendemic malaria infection. During malaria paroxysm, Vγ9Vδ2 T cells were early activated but rapidly became anergic and finally loose Jγ1.2 Vγ9 complementarity-determining region 3 transcripts.

Malaria affects hundreds of millions of persons worldwide, and *Plasmodium falciparum*, the most common species responsible, causes a severe disease resulting in millions of deaths. The degree of malaria pathology depends on both parasite and host factors (27). T cells bearing the γδ T-cell receptor (TCR) represent a variable fraction (1 to 10%) of peripheral blood lymphocytes. The main subset expresses a skewed Vγ9Vδ2 TCR rearrangement. These cells are activated during malaria infection and recognize nonpeptidic phosphoantigens present in *P. falciparum* (1, 2) and in red blood cells that could be released by erythrocyte rupture (4). After stimulation, Vγ9Vδ2 T lymphocytes can produce significant amounts of gamma interferon and tumor necrosis factor alpha (TNF-α) (8, 14, 22) that can mediate killing of blood stage malaria parasites (11). However, the role of γδ T cells in malaria infection is still unclear.

In this study, we analyzed the longitudinal pattern of γδ T-cell activation during the early events of nonendemic malaria infection, both during the paroxysm stage and during recovery after successful therapy. Four patients with nonendemic *P. falciparum* malaria from the "L. Spallanzani" Institute were enrolled (mean age, 44 years; age range, 32 to 53 years; 3 males, 1 female). Informed consent was obtained from all persons prior to enrollment, and only residual blood samples from the diagnostic routine were used. Blood samples were collected before antimalarial treatment, when patients showed high fever, chills, and rigors (paroxysm), and after recovery from clinical symptoms (after 24 to 36 h of antimalarial treatment). Malaria diagnosis was made by microscopic examination of thick blood film and confirmed by species-specific PCR (26). The clinical state was evaluated by using a clinical score as previously described (12). Table 1 shows clinical data obtained during paroxysm and after successful antimalarial treatment. Six healthy donors were used as controls. Surface staining was performed by using anti-CD3 (immunoglobulin G1 [IgG1], clone UCHT1), anti-HLA-DR (IgG2a, clone I2), and anti-TCR Vδ2 (IgG1, clone IMMU389) monoclonal antibodies directly coupled to fluorochromes (fluorescein isothiocyanate, phycoerythrin, and Cy5; ImmunoTech-Coulter, Miami, Fla.). In vitro Vγ9Vδ2 expansion after stimulation with 120 μM isopentenyl pyrophosphate (IPP) was determined as previously described (20, 21). Supernatants from cultures stimulated for 24 h with IPP were analyzed for TNF-α production by enzyme-linked immunosorbent assay kits used in accordance with the manufacturer's (Endogen, Woburn, Mass.) instructions. Vγ9 TCR complementarity-determining region 3 (CDR3) length heterogeneity was determined as previously described (6, 7). Group comparison was performed with the Mann-Whitney U test, and values of P that were ≤ 0.05 were considered significant.

TABLE 1. Clinical parameters of patients with nonendemic *P. falciparum* malaria[a]

Period and patient	Thick blood film[b]	PCR[c]	Clinical score
Paroxysm			
200299	T	+	13
120299	T, G	+	11
230399	T	+	7
190399	T, G	+	6
Recovery			
200299		+	2
120299		−	0
230399	T	+	0
190399		−	0

[a] The diagnosis was made by microscopic examination of thick blood film and confirmed by species-specific PCR (26). Clinical status was evaluated by a clinical scoring (12). Patients were studied at paroxysm (before therapy) and after successful antimalarial treatment (24 to 36 h after paroxysm).
[b] T, trophozoites; G, gametocytes.
[c] +, positive; −, negative.

* Corresponding author. Mailing address: National Institute for Infectious Diseases "Lazzaro Spallanzani" I.R.C.C.S., Via Portuense 292, 00149 Rome, Italy. Phone: 39 06 55170 907. Fax: 39 06 55170 904. E-mail: martini@inmi.it.

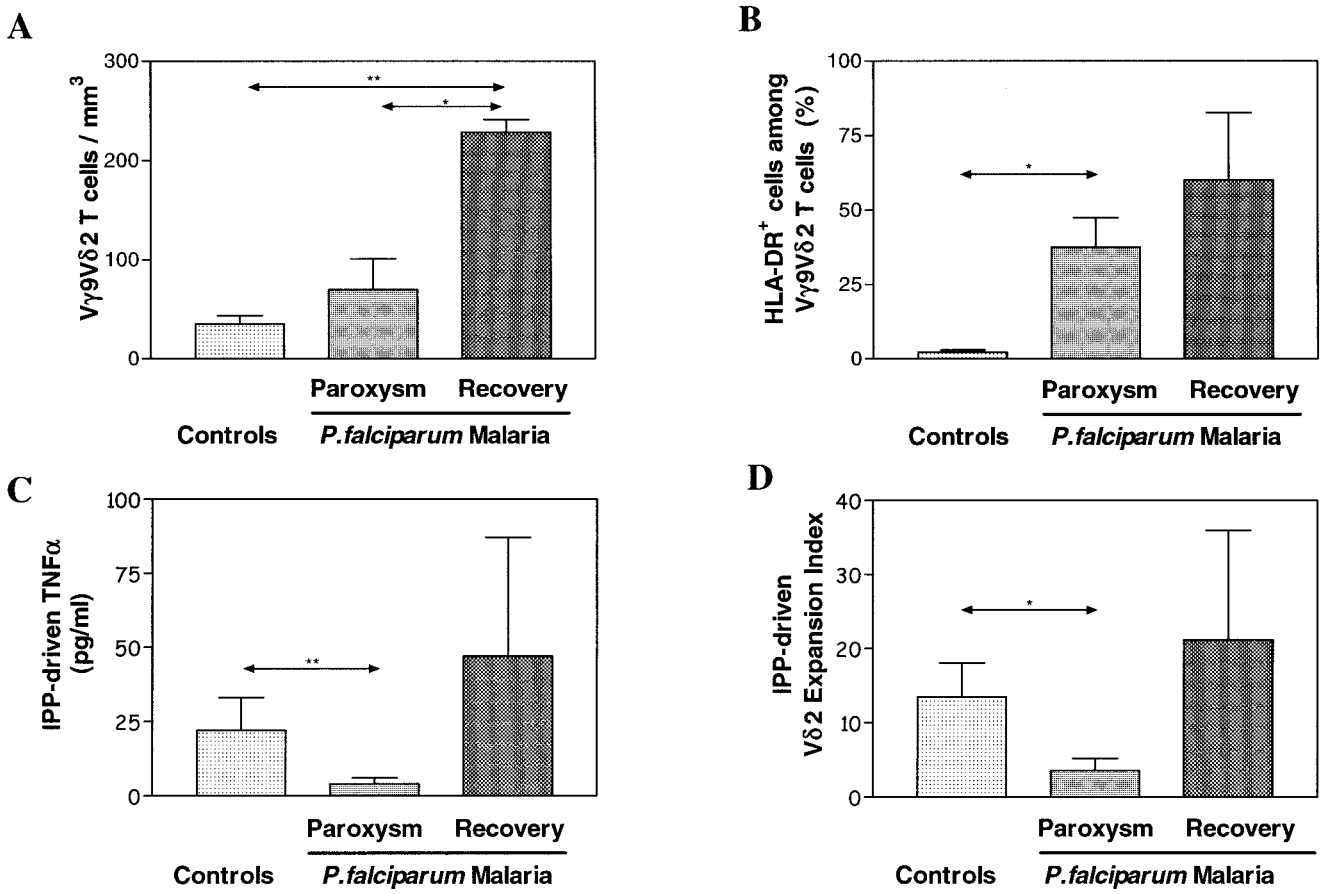

FIG. 1. Malaria-induced changes in ex vivo Vγ9Vδ2 T-cell subset absolute counts (A), ex vivo activation phenotype (HLA-DR$^+$ percentage in the Vγ9Vδ2 subset of T cells) (B), in vitro phosphoantigen-driven TNF-α synthesis (C), and Vδ2 expansion index (D). Results are expressed as medians plus the upper quartiles. Four *P. falciparum* patients were studied in the paroxysm phase and after successful antimalarial treatment (24 to 36 h after paroxysm). As controls, six uninfected persons were studied. Differences between groups were evaluated with the Mann-Whitney U test. *, $P \leq 0.05$; **, $P \leq 0.01$.

In Fig. 1A, a significant increase in the absolute Vδ2 T-cell subset number was found in malaria patients only after recovery. However, an increased percentage of activated HLA-DR$^+$ Vδ2 T cells (Fig. 1B) was observed during paroxysm (1). Peripheral Vγ9Vδ2 T cells were stimulated in vitro with IPP ligand (20, 21), and 24-h supernatants were collected and studied for TNF-α production by enzyme-linked immunosorbent assay (Fig. 1C). Interestingly, Vγ9Vδ2 T cells were unable to produce TNF-α upon phosphoantigen stimulation during paroxysm. After 10 days, the CD3/Vδ2/HLA-DR phenotype was assessed by flow cytometry and a relative expansion index was determined as previously described (20, 21) (Fig. 1D). During paroxysm, the IPP reactivity was significantly reduced but it reverted after successful chemotherapy. This anergy to IPP stimulation was specific for Vγ9Vδ2 T cells, since the αβ T-cell response to phytohemagglutinin was unaffected (data not shown). Figure 2 shows flow cytometry panels from a representative patient. An increase in the Vδ2 T-cell number and an increased frequency of Vδ2$^+$ HLA-DR$^+$ activated cells were observed after recovery (Fig. 2A and B). When stimulated in vitro for 10 days with IPP, more than half of the lymphocytes were Vδ2$^+$ HLA-DR$^+$ after recovery (Fig. 2F), compared with less than 1% in the same culture performed during paroxysm (Fig. 2E). Since it has recently been shown that Vγ9Vδ2 T cells stimulated with nonpeptidic antigens mostly exhibit Jγ1.2 in the Vγ9 TCRγ chains (17), we used spectratyping analysis to assess Vγ9 TCR heterogeneity (18). In Fig. 3, Vγ9 CDR3 length profiles from a representative patient with *P. falciparum* infection are shown both during paroxysm and after recovery (panel A). Two normal controls are shown in Fig. 3B. In healthy donors, the Vγ9 CDR3 lengths were biased to favor Vγ9 mRNA open reading frames 990 to 996 bases in length (Fig. 3B), corresponding to Jγ1.2 fragment usage (6, 7). In contrast, a shift from longer to shorter Vγ9 lengths was shown during paroxysm in malaria patients (Fig. 3A). Indeed, Vγ9 transcripts of 990 to 996 bases (Jγ1.2) accounted for 75% ± 13% (standard deviation) in healthy controls, compared to only 11% ± 16% ($P < 0.005$) and 42% ± 14% ($P < 0.05$) in malaria patients during paroxysm and recovery, respectively. Interestingly, there were increases in the proportions of normally minor 972-, 975-, and 978-base Vγ9 transcripts for both the paroxysm samples and the recovery samples compared to uninfected controls (Fig. 3A and B).

The possible role of the host immune response in determin-

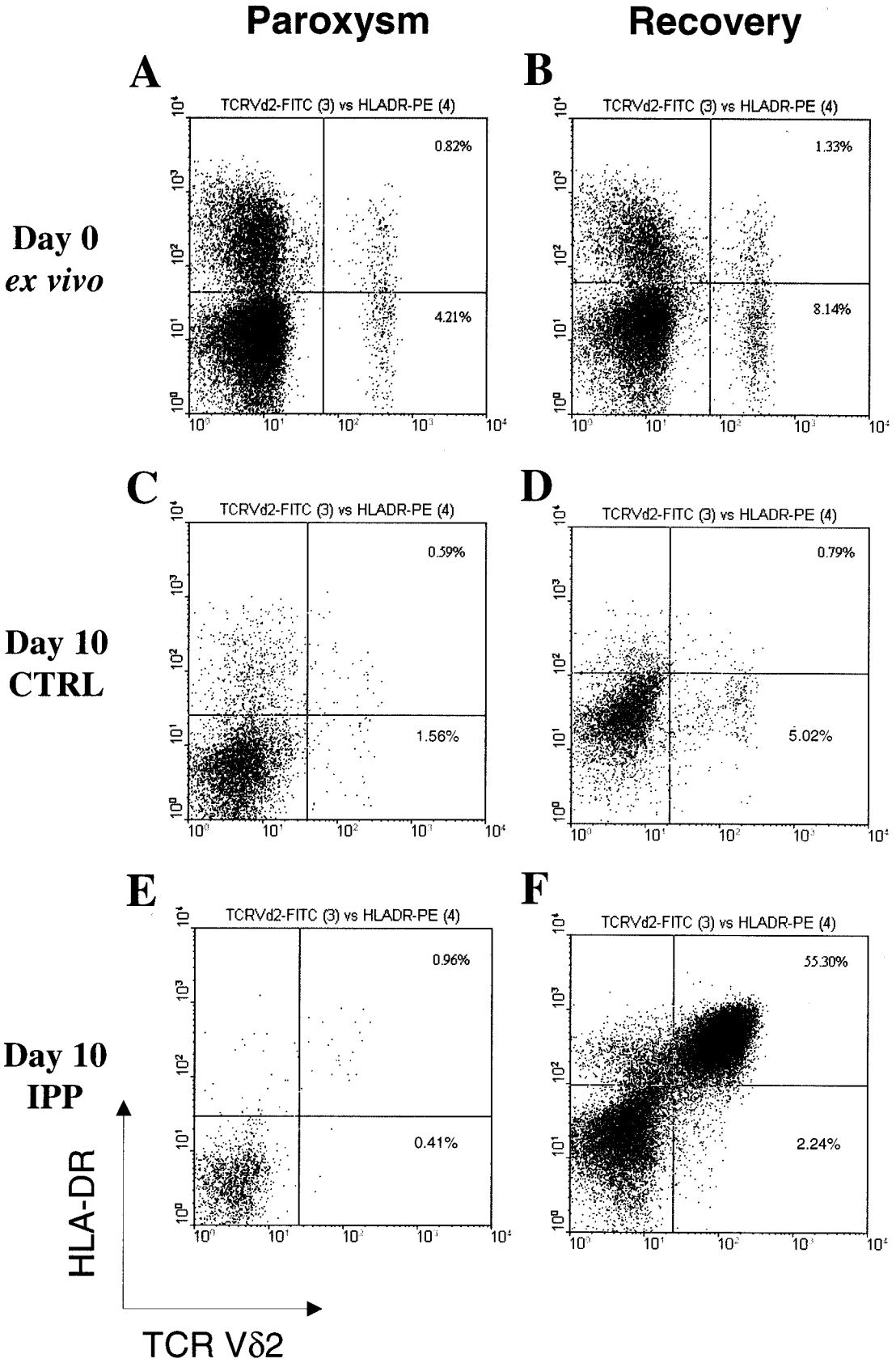

FIG. 2. Representative cytometric panel from a *P. falciparum* patient (120299) shown as Vδ2/HLA-DR plots during paroxysm and after successful antimalarial treatment (24 h after paroxysm). In panels A and B, ex vivo distributions are shown. In panels C and D, in vitro cultures stimulated with phosphoantigen (PPD) plus interleukin-2 for 10 days are shown. In panels E and F, in vitro cultures stimulated with phosphoantigen (interleukin-2 plus IPP) for 10 days are shown. Percentages refer to the indicated quadrant among total T cells. FITC, fluorescein isothiocyanate; PE, phycoerythrin; CTRL, control.

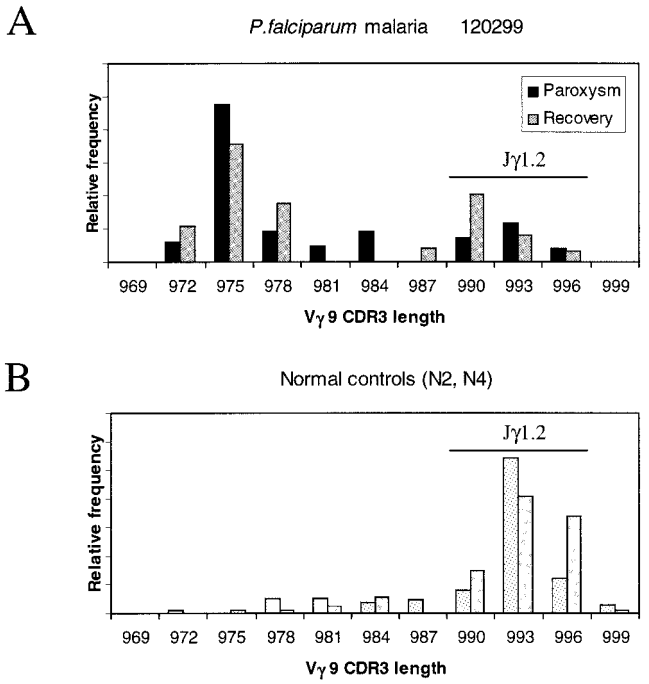

FIG. 3. Vγ9 CDR3 length profile frequencies of a representative *P. falciparum* patient (120299) studied during the paroxysm and recovery phases (A) and those of two normal controls (B). CDR3 lengths 990 to 996 refer to phosphoantigen-reactive Vγ9/Jγ1.2 clones.

ing the clinical outcome of malaria infection is controversial (27), with data supporting both a protective (9) and an immunopathogenetic (5, 13) role mediated by cytokines. In this context, Vγ9Vδ2 T lymphocytes have been shown to undergo deep changes during acute malaria infection: Vγ9Vδ2 expansion at onset was described by some authors (19, 23), while others found a delayed but persistent expansion occurring only after recovery (25). In this study, we observed a paroxysm-linked decrease in response to phosphoantigens, as both in vitro TNF-α synthesis and expansion, presumably as a consequence of specific hyperactivation. Moreover, γδ T-cell function recovered after successful antimalarial treatment, confirming that γδ T-cell anergy during infectious diseases is reversible (16). Interestingly, a study of experimental infection with *P. falciparum* (24) showed fast activation of Vγ9Vδ2 T cells, associated with a steep decline, linked to the erythrocyte cycle during which both *Plasmodium* phosphometabolites and red cell components are released. The overall outcome of Vγ9Vδ2 T-cell stimulation strongly depends on the activation level. At the early phases of infection, a massive antigenic exposure makes possible the induction of an abortive response (10, 15), as activated Vγ9Vδ2 T cells are highly susceptible to activation-induced cell death (4) triggered by Fas-FasL interaction (15). Moreover, the phosphoantigen 2,3-diphosphoglycerate, contained in red blood cells at millimolar concentrations, could be released by erythrocyte rupture and act as an antagonist (4), thus contributing to γδ T-cell anergy. Response to phosphoantigens is dependent on the Vγ9Vδ2 receptor (3). Vγ9Vδ2 T-cell populations maintain diversity in the CDR3s of Vγ9 mRNA after phosphoantigen stimulation, indicating that the response is polyclonal or oligoclonal, and are enriched for Vγ9 TCR chains containing the Jγ1.2 segment (7). During paroxysm, the changes in the Vγ9 repertoire indicated a preferential depletion of Jγ1.2 Vγ9 transcripts with higher affinity to phosphoantigens. Furthermore, human immunodeficiency virus-infected individuals were shown to have significant reductions in the frequency of Vγ9 containing the Jγ1.2 segment (6). Thus, the decrease in Vγ9Vδ2 response during active infectious diseases may reflect a specific depletion of Vγ9/Jγ1.2$^+$ phosphoantigen-responding clones by a mechanism of hyperactivation-apoptosis. The recovery phase associated with successful chemotherapy is followed by fast reconstitution of the Vγ9Vδ2 T-cell number and response capability (16). This pattern of rapid activation, anergy depletion, and recovery after resolution of the infection may represent a common behavior of Vγ9Vδ2 T cells in response to acute-phase infections.

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