A Profound Alteration of Blood TCRβ Repertoire Allows Prediction of Cerebral Malaria

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A Profound Alteration of Blood TCRB Repertoire Allows Prediction of Cerebral Malaria

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Cerebral malaria (CM) is one of the severe complications of Plasmodium infection. In murine models of CM, Tαβ cells have been implicated in the neuropathogenesis. To obtain insights into the TCRB repertoire during CM, we used high throughput CDR3 spectratyping and set up new methods and software tools to analyze data. We compared PBL and spleen repertoires of mice infected with Plasmodium berghei ANKA that developed CM (CM⁺) or not (CM⁻) to evidence modifications of the TCRB repertoire associated with neuropathology. Using distinct statistical multivariate methods, the PBL repertoires of CM⁺ mice were found to be specifically altered. This alteration is partly due to recurrently expanded T cell clones. Strikingly, alteration of the PBL repertoire can be used to distinguish between CM⁺ and CM⁻. This study provides the first ex vivo demonstration of modifications of Tαβ cell compartment during CM. Finally, our original approach for deciphering lymphocyte repertoires can be transposed to various pathological conditions. The Journal of Immunology, 2004, 173: 4568–4575.

Malaria is more than ever a health problem. A total of 300–500 million people are infected each year. Approximately two million, mostly African children, die of complications of infection by Plasmodium falciparum, particularly cerebral malaria (CM). This situation could soon worsen as resistance to chemotherapies are spreading and mosquito resistance to insecticides has hampered programs to diminish vector prevalence (1).

Pathogenesis of CM is a complex process involving both host and parasite factors. Several cytokines and mediators have also been associated with CM (2). However, no mechanism has been clearly established. Several lines of evidence implicate T lymphocytes in the development of CM. In humans, a class I MHC allele as well as a class II MHC haplotype have been associated with protection from severe forms of malaria (3). In mouse CM models that mimic only partially the neuropathology in humans (4), the immune system plays an active deleterious role, as evidenced by the protective effect of immunosuppressive treatments (5–7).

The Tαβ cells, both CD4⁺ and CD8⁺ subpopulations, are necessary for the development of experimental CM as shown in nude, knockout, and T cell-depleted mice (6, 8–12). Tγδ cells could also play a role in the neuropathogenesis because depletion by anti-CD8 Ab prevents CM (11). However, they are not required because CD8 knockout mice still develop CM (11, 12).

Balance between deleterious and protective roles of immune responses is a common feature in many infectious diseases in which pathogens manipulate the host immune system (13–16). In such complex situations, deciphering lymphocyte subpopulation implication in pathology vs protection requires the exhaustive description of lymphocyte populations in clinical trials involving numerous individuals. Lymphocyte responses can be described by their Ag-specific receptor diversity, which is produced by somatic DNA rearrangements of V, D, and J segments later spliced to C segments (17). The product of the V(D)J joining, called the CDR3, is in contact with the Ag. This region is imprecise in the number and the nature of nucleotides that are removed or added, and is therefore variable in amino acid length and composition. The CDR3 spectratyping method precisely describes diversity of a lymphocyte population repertoire by the analysis of the CDR3 length use (18, 19).

In this study, we set up a new strategy associating large scale CDR3 spectratyping, original software tools, and multifactorial statistical analyses that enabled us to decipher the TCRB repertoire during infection by Plasmodium berghei ANKeras/Kasapa (ANKA) and distinguish between pathogenic and protective repertoires. We show that the PBL repertoire of CM⁺ mice is altered and identify potentially pathogenic recurrent clones expanded in CM⁺ mice, but not in infected mice that do not develop CM (CM⁻).

Materials and Methods

Mice and parasites

Eight-week-old B10.D2 mice were purchased from Harlan (Oxon, U.K.). The clone 1.49L of P. berghei ANKA (20) was kindly given by D. Walliker (Institute of Genetics, Edinburgh, U.K.) and is maintained in our laboratory on C57BL/6J female mice. This clone induces in mice a neurological syndrome partly mimicking the one of human CM. Erythrocytic stages of the parasite are cryopreserved in liquid nitrogen as stabiles in Alservé’s solution containing 10% glycerol. Infection was induced by i.p. injection of 10⁴ parasitized RBC. Between days 7 and 10 after infection, 90% of the mice develop CM characterized by ataxia, paralysis, deviation of the head, and convulsions, followed by deep coma and death. These mice
constituted the CM⁺ group. CM⁻ mice were sacrificed between days 11 and 16 after infection, not having shown signs of CM during the critical period and exhibiting a parasitemia above 20%.

**Cell preparation**

Blood was obtained on heparin by retroorbital puncture. Mononuclear cells were isolated on Ficol-Hypaque gradient (Pharmacia, Uppsala, Sweden). Spleens were removed, and cells were suspended by surging in 3% FBS-PBS. RBCs were lysed with ammonium chloride buffer for 5 min at room temperature. The brain lymphoid cells were obtained after tissue homogenization in RPMI 1640 medium and centrifugation at 500 × g for 20 min in 35% (v/v) Percoll (Pharmacia). The brain lymphoid cells of four CM⁻ mice were pooled, stained with PE-conjugated anti-mouse CD3 mAb (145-2C11), and sorted by use of a MoFlo cell sorter (DakoCytomation, Fort Collins, CO, and BD Pharmingen, San Diego, CA). Dead cells were excluded using propidium iodide at a final concentration of 1 μg/ml (Sigma-Aldrich, Saint Quentin Fallavier, France).

Cell preparations were then washed twice with PBS. Lymphoid cells were counted using Malassez cell in presence of eosin to exclude dead cells.

**TCRB repertoire**

Total RNA was extracted from >90,000 mononuclear cells for each sample using the TRI REAGENT kit (Molecular Research Center, Cincinnati, OH). A total of 20 μg of glycogen (Roche, Meylan, France) was used to ensure optimal precipitation of RNA and pellet visualization. Protocols for TCR BV-BC and BV-BJ CDR3 spectratyping have already been explained elsewhere in detail (18, 21). BV, BV, and BJ primer sequences were as described earlier (18), except BV5.3 (TGCTGCAACTCTAAATAGGA) and BV13 (AGGCCCTAAAGGAACTAACTCCAC). As BV5.3 – BV8.2 were not amplified. BV-BJ repertoires were analyzed for BV2, BV3, BV4, BV5.1, BV6, BV7, BV8.1 – 3, BV9, BV14, and BV16 genes. PCR products were loaded on a 36-well ABI373 automated sequencer (Applied Biosystems, Foster City, CA) and separated according to their nucleotide length, forming a profile of peaks for each primer combination, spaced by 3 nt as expected for in-frame transcripts. Each peak corresponds to a CDR3 length.

The Immunoscope software (18) was used to obtain peak area and nucleotide length and CDR3 profile displays from sequencer raw data.

**BV-BJ direct sequencing**

Direct sequencing was performed with BV and BJ primers for peaks representing between 65 and 100% of the BV-BJ profile, following the recommendations of the ABI Prism Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems). BV-BJ PCR products were first reamplified. PCR products were then incubated with 0.5 U of shrimp alkaline phosphatase (United States Biochemistry, Cleveland, OH) and 0.25 U of exonuclease I (United States Biochemistry) at 37°C for 40 min, followed by 20 min at 80°C. DNA alignments were performed using the GCG package (Genetics Computer Group, Accelrys, Cambridge, U.K.)

**Methods and software tools for CDR3 spectratype analyses**

We developed the ISEApiks software package (©2000–2002; Institut Pasteur, France, Paris) to extract, smooth, manage, and analyze the large amount of data obtained in this study (25, 26). As the intensity of CDR3 peaks is not comparable between different amplifications, we considered the percentage of use of each CDR3 length by dividing the area of CDR3 peaks by the total area of all peaks within the profile. Perturbation of BV-BC repertoire was compared with a control group, as explained elsewhere (27, 28). Sample perturbation (μDBV-BC) is the mean perturbation of each BV segment (DBV-BC). We extended this perturbation index to BV-BJ repertoires by computing μμDBV-BJ, the mean of the DBV-BJ for all BV and BJ segments. BV-BC and BV-BJ perturbations range from 0, identical profiles, to 100, completely different profiles.

Recurrence of CDR3 expansions was assessed quantitatively with OligoScore (25), which scores each peak in each group of samples. The maximum score of the control group is used as a threshold for other groups. Peaks with a score above this threshold are considered recurrently expanded.

**Results**

**Analysis of TCRB repertoire during CM by the Immunoscope method**

The TCRB repertoire during CM induced in B10.D2 mice by P. berghei ANKA was analyzed using the exhaustive CDR3 length spectratyping approach (18).

BV-BC repertoires were studied with total cDNA, prepared from noninfected control (CTR) mice and infected mice that did (CM⁺) or did not (CM⁻) develop CM. In PBL and spleen of naive mice, CDR3 profile for each BV-BC combination is bell shaped, indicative of a diverse polyclonal repertoire (Fig. 1, and data not shown). The TCRB repertoires of the CM⁺ and CM⁻ mice are profoundly altered: almost all CDR3 profiles are different from a bell-shaped profile, and multiple expansions are evidenced (Fig. 1). Among these complex repertoire modifications, some could contribute to the protective response against P. berghei ANKA infection, while others are involved in pathology. The comparison between CDR3 profiles from CM⁺ and CM⁻ mice enables the identification of potentially pathogenic clones associated with CM that would be present in CM⁺ mice, but absent in CM⁻ mice.

**Specific alterations of TCRB repertoire during CM**

To identify CM-associated alterations, we studied ex vivo the PBL and splenocyte TCRB repertoires of several individuals for CTR, CM⁻, and CM⁺ groups. A total of 1150 BV-BC CDR3 profiles was obtained for 55 samples. The analysis of this set of BV-BC profiles, each including 6–8 peaks, required the use of an original approach that combines bioinformatic tools and multivariate statistics.

All peak data of TCRB repertoire were extracted, formatted, and edited with the ISEApiks software to construct the peak database. PCA was used to reduce the initial number of variables. The new data set retains 97% of the original information. We used linear DA to evidence global modifications due to infection in CM⁺ and CM⁻ mice. DA determines whether multivariate observations of different groups are samples of the same statistical population. As shown on Fig. 2, all members of a given group are closely clustered together. Moreover, sample groups can be separated in four statistically significant clusters, as only the first three discriminant
functions are significant: CM\(^+\) PBL, CM\(^-\) PBL, CM\(^-\) spleen, and a group containing CM\(^+\) spleen, CTR PBL, and CTR spleen.

To further characterize the repertoires, we used another method that computes CDR3 spectratype perturbation index for each sample (27). All repertoires were compared with the spleen CTR repertoire to obtain a perturbation index for each BV-BC combination (DBV-BC). The average of BV-BC perturbations, \(DBV-BC\), were compared by two-way ANOVA between compartments (PBL and spleen) and the different groups of mice (CTR, CM\(^+\), and CM\(^-\)) (Fig. 3A). ANOVA compares the effect of qualitative factors on a quantitative dependent variable. Infection by \(P.\) berghei ANKA leads to significant alteration of the TCRB perturbation both due to the groups of mice (\(p < 0.0001\)) and to the lymphoid compartment (\(p = 0.0002\)). PBL repertoires of CM\(^+\) mice (mean = 20.2) were more perturbed than in CM\(^-\) mice (mean = 15.2; \(t\) test, df = 21, \(p < 0.0001\)). To strengthen this observation, we used \(k\)-mean clustering to see whether groups could be separated on the basis of BV-BC perturbations without knowledge of group composition, which was the case of DA and ANOVA. For \(k = 3\) (or \(k = 4\), all 13 CM\(^+\) PBL samples clustered together in a group containing 17 (or 16) samples that had the probability \(p = 1.64E-9\) (\(p = 3.86E-10\)) to occur by chance (Fig. 3B). Because PBL BV-BC repertoires appear to be specifically altered in CM\(^+\) as compared with CM\(^-\) mice, further analyses were performed on PBL only.

**BV-BC perturbation of PBL repertoires allows prediction of CM**

We investigated whether the alteration of PBL BV-BC repertoire could enable us to classify samples. Because the sample number is not large enough to divide it in training and testing sets, we used the jackknife method: each sample is left out in turn and DA is performed on the new set of variables obtained with PCA. Bivariate graphics represent the value of two discriminant functions. Discriminant functions are linear combination of the variables that try to separate groups. F1 to F5 stands for each discriminant function, sorted decreasingly by the associated eigenvalue. Samples (8 CTR PBL, 6 CTR spleen, 10 CM\(^+\) PBL, 8 CM\(^+\) spleen, 13 CM\(^-\) PBL, and 10 CM\(^-\) spleen) are represented on the bivariate plots, but are scarcely visible because DA groups samples very well. Confidence (0.95) ellipses, centered on group centroid, are overlaid. The density normal curve of each group is shown on the diagonal. Vertical and horizontal axes represent canonical scores for each function. The density normal curves of F1 and F5 show that groups are not well separated, consistent with the nonsignificance of these functions. All CDR3 profiles of this study can be obtained on the supplement web page (www.pasteur.fr/recherche/unites/fmschan/addit2.html).
performed on the remaining samples to obtain classification functions. These functions are then used to determine to which group the left-out sample belongs. Table I shows that 85% of CM+ PBL samples were correctly classified, indicating that perturbation of PBL repertoire can be used to discriminate between CM+ and CM− mice.

**BV-BJ PBL repertoire analysis during infection**

To describe more precisely the TCRB repertoire during CM, we performed BV-BJ repertoire analysis for 9 BV genes of 21, which represent more than two-thirds of the total TCRB repertoire. A total number of 2300 BV-BJ profiles was generated.

To compare perturbation results obtained from BV-BC and BV-BJ repertoire analysis, we first computed the average DBV-BJ for all BV and BJ genes for each sample (μDBV-BJ). The three PBL groups (CTR, CM−, and CM+) were significantly different (ANOVA, p < 0.0001). Again, CM+ PBL repertoires (mean = 32.3) were more altered than those of CM− PBL (mean = 24.5; p = 0.0035). By using k-mean clustering with DBV-BJ data, all three groups could also be reconstituted without prior knowledge of group composition (Fig. 3C). Five of six CM+ PBL samples (p = 0.0001) clustered together apart from CTR and CM− samples.

To determine which BV gene(s) contributed to this difference, we computed the perturbation of BV genes on the basis of BV-BJ repertoires (μDBV-BJ perturbation). MANOVA analysis showed that the three groups are statistically different (p = 0.01). However, only μBV8.1-BJ (ANOVA, p = 0.01) was significantly more altered in CM+ mice compared with CM− mice. Finally, analysis of the contribution of BJ segments in BV8.1-positive T cells by MANOVA, followed by ANOVA, implicated only DBV8.1-BJ1.1, DBV8.1-BJ1.6, DBV8.1-BJ2.1, and DBV8.1-BJ2.2.

ISEApeaks was used to compare BJ percentages for each BV gene in PBL samples. These percentages were compared by MANOVA. BJ use was not significantly different among the CTR, CM−, and CM+ groups (data not shown). Thus, perturbations of BV-BJ CDR3 spectratypes are not correlated with modifications of the BJ use.

**Identification of pathogenic recurrent clones**

We searched the PBL BV-BC and BV-BJ repertoire data for pathogenic T cell clones associated with CM. We concentrated on the identification of pathogenic clones that are recurrently present in CM+, but not in CM− mice. We used OligoScore, which scores peaks for their recurrence in each group (25). For BV-BC repertoires, no peak in CM− or CM+ groups has a score higher than the threshold of CTR groups (data not shown). Hence, no BV-BC peak is found recurrently expanded.

We used the same scoring approach to identify recurrent clones in BV-BJ repertoires. A total of 122 peaks in the PBL CM+ mice has a score above the corresponding threshold defined with the CTR PBL group. They were compared with those of the CM− group by subtracting the corresponding peak scores. Peaks were sorted decreasingly to identify the most recurrent in CM+, but absent in CM−. The three most differentially expressed recurrent peaks belong to BV8.1-BJ1.5 and BV8.1-BJ2.2 profiles (Table II and Fig. 4). BV2-BJ1.3 peak is given as an example of a peak that is recurrently expanded both in CM+ and CM− mice and thus low ranked in Table II (this point will be discussed later). BV8.1 peaks were directly sequenced. For each of the two BV8.1-BJ combinations, similar amino acid sequences were found in several CM+ individuals (Table III). On the contrary, direct sequencing for the PCR products in CM− samples yielded no readable CDR3 sequence (data not shown).

Finally, we have explored the representation of the identified BV-BJ combination in the brain of CM+ mice. Due to cell number limitation and to obtain a global view of BV-BJ repertoire in the brain, we have pooled PBL, spleen, and brain samples from four CM+ mice. BV08.1-BJ1.5, BV08.1-BJ2.2, and BV02-BJ1.3 were analyzed together with BV08.1-BJ2.3 and BV02-BJ2.3 as controls.
are also found in the brain of CM/H11001 neuropathology of CM. in the brain, the site of pathogenesis, and could thus mediate the explained by recurrent clones that are present in CM spleen. This perturbation of the TCRB repertoire is partly ex-
ed in the brain (65.0%), to a similar extent to the PBL identified in all three organs (70.0%). The BV08.1-BJ2.2 was again identi-
ed in all three organs (70.0%). The BV08.1-BJ1.5 combination was also noted in the brain compartment (65.7%), (65.7%) and spleen (62.9%) compartments. The BV08.1-BJ2.2 and BV08.1-BJ1.5 clones, identified as differentially expanded in CM+ mice by comparison with CM− mice, are thus also present in the brain, the site of pathogenesis, and could thus mediate the neuropathology of CM.

Discussion

The aim of the present study was to exhaustively characterize the TCRB repertoire during CM in B10.D2 mice infected with P. berghei ANKA clone 1.49L. To this end, we devised new global methods and tools, based on a large-scale use of the CDR3 spectratyping approach. Our results show that the TCRB repertoire is specifically altered in the PBL of CM+ mice as compared with PBL of CM− mice, whereas no difference was evidenced in the spleen. This perturbation of the TCRB repertoire is partly explained by recurrent clones that are present in CM+ and absent in CM− mice. Preliminary data indicate that these recurrent clones are also found in the brain of CM− mice.

Analysis of the repertoire using the CDR3 spectratyping describes the entire ex vivo TCRB repertoire of a sample by up to 2400 measurements. As a high number of parameters are measured, it must be ensured that the cell quantity used is sufficient. In particular, paucity of material tends to favor stochastic PCR amplifications. We therefore carefully determined by repertoire analysis of a set of cell dilutions that the cell numbers we used were sufficient to guarantee the quality of our repertoire data (A. Six and O. Gorgette, unpublished data). Furthermore, the identification of recurrent BV-BJ CDR3 expansions shows that the repertoire modifications documented in this study are not artifacts (Table III).

The original bioinformatic tools we devised enabled us to analyze the 3450 CDR3 profiles generated in this study. All three independent multivariate statistics consistently gather in a similar manner the six experimental groups into three to four clusters. Moreover, as expected, the CTR PBL and CTR spleen groups are not separated for DA, ANOVA, and k-mean clustering (with k = 3). We demonstrated for the first time that T cell repertoire data can give diagnostic/prognostic information when analyzed by class prediction. The alteration of the BV-BC repertoire enabled the group classification of 85% of the PBL samples of CM+ mice. PBL samples of CM− mice are less correctly classified (50%). However, only 1 of 10 CM− PBL samples is erroneously classified as a CM+ PBL, indicating that the risk to predict falsely that an infected individual is developing CM is small.

An original quantitative scoring method, OligoScore (25), was used to identify recurrent expansion of T cell clones among the 1040 BV-BJ CDR3 peaks. It should be noted that existence of perturbation in a particular BV-BC or BV-BJ profile does not imply existence of recurrent expansion within this profile because private expansions can also distort it. Surprisingly, no recurrent peaks were found at the level of BV-BC. Two explanations can be given. Recurrent peaks in the BV-BC repertoires might be below the detection limit of our scoring method. This is unlikely because OligoScore enabled detection of recurrence that were not visible by eye, even with a small number of samples (25). More likely, it might be the consequence of a buffer effect between BV-BC and BV-BJ data (32). Variation at the more precise level of BV-BJ repertoires can be averaged in the corresponding BV-BC repertoires because these repertoires are the addition of all BV-BJ repertoires. An example of this buffer effect can be visualized on Fig. 4 for BV8.1+ cells, in which modifications seen at the BV8.1–BJ1.5 and BV8.1–BJ2.2 levels are smoothed at the BV8.1-BC level. This effect is also seen on PBL sample perturbation of CM− mice, which is 20.2 when estimated on BV-BC repertoires and 32.3 on BV-BJ repertoires. It is because BV-BJ repertoires are 12 times more precise than BV-BC and therefore give a more accurate description of the repertoire. Finally, as BV-BJ repertoires are quantitative inside a given BV gene (33, 34), it is possible to test

| Table I. Perturbation of BV-BC repertoires allows prediction of CM+ |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | CM+ PBL | CM+ Spleen | CTR PBL | CTR Spleen | % Correct Prediction |
| CM+                        |         |            |         |            |                   |
| PBL                        | 13      | 11         | 0       | 0          | 85                |
| Spleen                     | 10      | 2          | 1       | 1          | 40                |
| CM−                        | 10      | 1          | 5       | 3          | 50                |
| PBL                        | 8       | 1          | 0       | 5          | 63                |
| Spleen                     | 6       | 0          | 1       | 3          | 50                |
| Total                      | 16      | 10         | 7       | 10         | 60                |

* BV-BC perturbation data were analyzed by DA, with the jackknife method. Each sample has been left out in turn, while classification functions were computed on the remaining samples. These functions were used to predict the class membership for this sample. Left-out cases are in row and attributed categories in columns.

* The percentage of correct prediction is obtained by dividing the correctly classified samples by the total number of samples.

An original quantitative scoring method, OligoScore (25), was used to identify recurrent expansion of T cell clones among the 1040 BV-BJ CDR3 peaks. It should be noted that existence of perturbation in a particular BV-BC or BV-BJ profile does not imply existence of recurrent expansion within this profile because private expansions can also distort it. Surprisingly, no recurrent peaks were found at the level of BV-BC. Two explanations can be given. Recurrent peaks in the BV-BC repertoires might be below the detection limit of our scoring method. This is unlikely because OligoScore enabled detection of recurrence that were not visible by eye, even with a small number of samples (25). More likely, it might be the consequence of a buffer effect between BV-BC and BV-BJ data (32). Variation at the more precise level of BV-BJ repertoires can be averaged in the corresponding BV-BC repertoires because these repertoires are the addition of all BV-BJ repertoires. An example of this buffer effect can be visualized on Fig. 4 for BV8.1+ cells, in which modifications seen at the BV8.1–BJ1.5 and BV8.1–BJ2.2 levels are smoothed at the BV8.1-BC level. This effect is also seen on PBL sample perturbation of CM− mice, which is 20.2 when estimated on BV-BC repertoires and 32.3 on BV-BJ repertoires. It is because BV-BJ repertoires are 12 times more precise than BV-BC and therefore give a more accurate description of the repertoire. Finally, as BV-BJ repertoires are quantitative inside a given BV gene (33, 34), it is possible to test

### Table II. Identification of recurrent expansions in CM+ mice in BV-BJ CDR3 profiles by OligoScore

<table>
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<tr>
<th>Rank</th>
<th>BV</th>
<th>BJ</th>
<th>CDR3 (aa)</th>
<th>OS CM+</th>
<th>OS CM−</th>
<th>ΔCM+−</th>
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<td>1</td>
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</table>

* Differences (ΔCM+−) between the OligoScore of the CM+ PBL (OS CM+) and the CM− (OS CM−) groups were sorted decreasingly to identify recurrent CDR3 peaks differently expressed in CM+ PBL but not in CM− PBL. All OligoScores can be obtained on the supplemental data web page.

* Only one or two CM− PBL samples were analyzable for these peaks, whereas four or more were for the other peaks and groups.
PBL repertoires during CM, despite the numerous *P. berghei* molecules that stimulate the immune system during infection. By contrast with recurrent responses against single Ags (35, 36), the recurrent response against *P. berghei* is modest because it was not found at the BV-BC level. This could be related to a general activation of T cells, possibly due to *Plasmodium* mitogens (37–39) that prevent the expansion of Ag-specific clones. The stability of BJ use observed between groups is consistent with this observation (data not shown).

Modification of the TCRB repertoire is evidenced only in the PBL of CM* mice in contrast with the usually well-accepted idea that PBL reflects spleen. This is also in contrast with spleen being necessary for the occurrence of CM (6, 40, 41). Absence of specific alteration in the spleen of CM* mice could be explained by the dilution of stimulated cells in the bulk of T cells present in this organ and the fact that they leave to recirculate upon activation (42).

T cell clones associated with neuropathogenesis can be of different types. First, they can be either private, specific to one individual, or recurrent, present in different individuals and sometimes designated as public clones. Second, their function might be pathogenic, protective, or regulatory. Based on our BV-BJ repertoire analysis of PBL, we have identified in this study recurrent clones associated to neuropathogenesis by assessing their presence in PBL reactivity against single Ags (35, 36), the recurrent response against *P. berghei* is modest because it was not found at the BV-BC level. This could be related to a general activation of T cells, possibly due to *Plasmodium* mitogens (37–39) that prevent the expansion of Ag-specific clones. The stability of BJ use observed between groups is consistent with this observation (data not shown).

In our preliminary experiment on brain lymphocytes pooled from four mice presented in Table III, we show that these expanded BV-BJ combinations are also found in the brain of CM* mice. This relates to the absence of CM in mice in contrast with the usually well-accepted idea that PBL reflects spleen. This is also in contrast with spleen being necessary for the occurrence of CM (6, 40, 41). Absence of specific alteration in the spleen of CM* mice could be explained by the dilution of stimulated cells in the bulk of T cells present in this organ and the fact that they leave to recirculate upon activation (42).

We did not identify any CM*–specific recurrent peak (data not shown), which could be involved in protection, but a BV2-BJ1.3

<table>
<thead>
<tr>
<th>BV BJ</th>
<th>CDR3 Length (aa)</th>
<th>Mice</th>
<th>Amino Acid Sequences</th>
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<td>8.1 2.2</td>
<td>9/10</td>
<td>CM*</td>
<td>CAS SGDDXGQL YFG</td>
</tr>
<tr>
<td>8.1 1.5</td>
<td>9</td>
<td>CM*</td>
<td>CAS SGDDQGQAP LFG</td>
</tr>
<tr>
<td>2 1.3</td>
<td>9</td>
<td>CM*</td>
<td>CTC SETGSGNTL YFG</td>
</tr>
<tr>
<td>CM*</td>
<td>1</td>
<td>CTC SXTGSGNTL YFG</td>
<td></td>
</tr>
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</table>

* BV-BJ PCR products were directly sequenced for indicated PBL samples using both a BV- and a BJ-specific primer.

<table>
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<tr>
<th>BV BJ</th>
<th>CDR3 Length (aa)</th>
<th>Mice</th>
<th>Amino Acid Sequences</th>
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| BS/BC profiles with our BV-BJ data (data not shown). We were indeed able to reconstruct BV-BC profiles with our BV-BJ data (data not shown).

The implication of T cell clones associated with neuropathogenesis in malaria has been demonstrated with depletion by Abs (6, 9–11) and use of nude (8) or knockout mice (11, 12). Our present results add to these previous studies by demonstrating, ex vivo, a profound perturbation and the existence of recurrent CDR3 peaks in TCRB

FIGURE 4. BV-BJ CDR3 profiles of recurrent expansions identified by OligoScore and perturbation analysis. PBL cDNA were amplified with BV8.1- or BV2-specific primers with a BC-specific primer. PCR products were then subjected to runoff with appropriate BJ-specific primers. Products were separated on an automated sequencer and analyzed with ImmunoScope and ISEApeaks. All PBL samples for which those combinations were analyzed are represented. Horizontal axis represents the nucleotide size, and vertical axis the fluorescence intensity, in arbitrary units.

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PBL repertoires during CM, despite the numerous *P. berghei* molecules that stimulate the immune system during infection. By contrast with recurrent responses against single Ags (35, 36), the recurrent response against *P. berghei* is modest because it was not found at the BV-BC level. This could be related to a general activation of T cells, possibly due to *Plasmodium* mitogens (37–39) that prevent the expansion of Ag-specific clones. The stability of BJ use observed between groups is consistent with this observation (data not shown).

Modification of the TCRB repertoire is evidenced only in the PBL of CM* mice in contrast with the usually well-accepted idea that PBL reflects spleen. This is also in contrast with spleen being necessary for the occurrence of CM (6, 40, 41). Absence of specific alteration in the spleen of CM* mice could be explained by the dilution of stimulated cells in the bulk of T cells present in this organ and the fact that they leave to recirculate upon activation (42).

T cell clones associated with neuropathogenesis can be of different types. First, they can be either private, specific to one individual, or recurrent, present in different individuals and sometimes designated as public clones. Second, their function might be pathogenic, protective, or regulatory. Based on our BV-BJ repertoire analysis of PBL, we have identified in this study recurrent clones associated to neuropathogenesis by assessing their presence in CM* mice and absence in CM* mice. It is of interest to investigate the relation of these expansions with neuropathology, in particular to determine whether these expansions are also found in the brain. We and others have recently observed the accumulation of CD8* Tcβ lymphocytes in the brain of CM* mice (43, 44). However, due to the small number of infiltrating lymphocytes in the brain, even when pathology occurs (43, 44), it was difficult to investigate this point at the same time in individual mice. These experiments are underway in the laboratory. In our preliminary experiment on brain lymphocytes pooled from four mice presented in this study, we show that these expanded BV-BJ combinations are also found in the brain of CM* mice. Five of the 10 most recurrent and differentially expressed peaks use the BV8.1 segment. Observations that support their having a pathogenic role is that deletion of BV8.1/2* cells diminishes the incidence of CM from 90 to 40% (12). Others peaks, including the ones using BV6, BV7, and BV9 segments, have been identified in CM* mice (Table II). This relates to the absence of CM in mice treated with a superantigen that deletes BV6, 7, 8.1, and 9 using T cells (12, 45).

We did not identify any CM*–specific recurrent peak (data not shown), which could be involved in protection, but a BV2-BJ1.3
FIGURE 5. BV-BJ CDR3 profiles of recurrent expansions identified in brain, PBL, and spleen. Brain, PBL, and spleen cDNA were prepared from pooled lymphocytes isolated from four CM+ mice. cDNA were amplified with BV8.1- or BV2-specific primers with a BC-specific primer. PCR products were then subjected to run off with appropriate BJ-specific primers. Products were separated on an automated sequencer and analyzed with ImmunoScope and ISEApeaks. BV02-BJ1.3, BV08.1-BJ1.5, and BV08.1-BJ2.2 are BV-BJ combinations that were found recurrently expanded in PBL of individual CM+ mice. BV02-BJ2.3 and BV08.1-BJ2.3 combinations serve as controls. Horizontal axis represents the nucleotide size, and vertical axis the fluorescence intensity, in arbitrary units.

peak is recurrently present both in CM+ and CM− mice, as judged by the high score obtained for the two groups (Table II and Fig. 4). This peak is by far the most expanded peak in CM− mice (data not shown). Mechanisms contributing to neuropathogenesis could thus be the result of a regulatory pathway between BV2- and BV8.1-expanded clones, leading to alteration of their cytokine profiles (46).

Altogether, these results suggest that few T cell clones are implicated in the development of CM. The immunological history for each individual shapes the emergent repertoire differentially between inbred individuals (47). These variations could explain why, among a group of genetically identical mice infected with the same stable parasite, only some develop CM.

In a previous study, we had observed by flow cytometry an increase of BV8.1/2− T cells in the PBL of CM+ mice, while no expansion was seen in the spleen of CM+ mice nor in the PBL and spleen of CM− mice (12) (data not shown). This increase, if caused by the expansion of few T cell clones, should distort the bell-shaped CDR3 length distribution of BV8.1/2-BC profiles. However, the PBL BV8.1/2-BC repertoire of CM+ mice is not perturbed by comparison with CTR mice (ANOVA, p = 0.71) or CM− mice (ANOVA, p = 0.29). In addition, no modification of the BJ segment use was observed in the CM+ mice. The increase of the representation of BV8.1/2 cells in the PBL of CM+ mice thus cannot be attributed to a mono- or oligoclonal increase, and is therefore polyclonal. BV8.1/2 could be stimulated by a superantigen-like molecule, as observed in Plasmodium yoelii infection (48). Implications of such molecules in pathogenesis have been reported for infections by Toxoplasma gondii (49) and Leishmania infantum (A. Sassi, B. Largueche-Darwaz, A. Collette, A. Six, D. Laouini, P. A. Cazenave, and K. Dellagi, unpublished results).

Plasmodium parasites being constituted of a very complex pool of molecules, it can comprise different types of activities, such as mitogens, superantigens, and conventional Ags that together shape the immune response qualitatively and quantitatively. In our P. berghei ANKA-infected B10.D2 mice, one can hypothesize that among expanded BV8.1/2 T lymphocytes, Ag-specific T cell clones could have a pathogenic function. This is under investigation in our laboratory by determining their function and fine specificity.

The original method presented in this work allows the exhaustive analysis of immune repertoires. Applied to a mouse model of malaria, it demonstrates that the neuropathology induced by P. berghei ANKA is associated with a global perturbation of TCRB repertoires specifically found in PBL together with the recurrent expansion of few T cell clones.

It will be of interest to determine whether the CM+ perturbed repertoire is responsible for neuropathology. We have recently described two CM resistance loci in a wild-derived inbred strain mice (50), and are currently in the process of obtaining a double congenic B6 strain resistant to CM, but still susceptible to infection. This model will be best suited to test whether an adoptive transfer of CM+ T lymphocytes from B6 mice can alter the course of infection and shift pathology to CM in genetically CM-resistant mice.

The repertoire analysis method reported in this work can easily be transposed to human malaria because PBL are easily accessible to experiment. It is intriguing to know whether the same association between PBL perturbation and neuropathology can be found in P. falciparum malaria. Furthermore, classification experiments allowed separation of the CM+ and CM− mice, and thus provide new tools for a better understanding of the immune response during malaria in humans. We are currently testing our hypotheses by studying cohorts of malaria patients. Finally, our original approach for deciphering lymphocyte repertoires can be transposed to various pathological conditions. For instance, this methodology is used in clinical follow-ups of patients after bone marrow transplantation or vaccination. The results and approach we present provide a promising basis for the development of innovative investigation strategies in the field of immunology.

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References


