Immunology 1994 **81** 216–221

Expression of the IL-1 receptor discriminates Th2 from Th1 cloned CD4$^+$ T cells specific for *Plasmodium chabaudi*

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SUMMARY

The expression of selected interleukin receptors by cloned CD4$^+$ T cells specific for the murine malaria parasite *Plasmodium chabaudi chabaudi* (*P. chabaudi*) representative of the T-helper (Th) 1 and Th2 subsets was examined. Both sets of clones expressed receptors for those interleukins for which they had a growth factor requirement *in vitro*. Each Th1 clone expressed receptors for, and was responsive to, interleukin (IL)-2 and IL-4, while each Th2 clone expressed receptors for, and was responsive to, IL-2, IL-4 and IL-1. IL-1 receptor (IL-1R) expression by the Th1 clones was either negligible or could not be detected. The disparity in expression of IL-1R by the Th1 and Th2 clones was more clear-cut than has been previously reported and IL-1R provided a definitive phenotypic marker for clones of the Th2 subset. Should IL-1R expression prove to be a feature of other Th2 cells cultured long-term *in vitro*, this will be invaluable for investigations involving the phenotyping, depletion or selection of CD4$^+$ T cells of either Th1 or Th2 subset.

INTRODUCTION

In recent years it has been appreciated that mature murine CD4$^+$ T cells can be separated into two distinct groups based upon their restricted cytokine secretion profiles.[1] Th1 cells secrete interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β) whereas Th2 cells secrete IL-4, IL-5, IL-6 and IL-10.[2] Much attention has since focused on whether cells with this functional dichotomy can be discriminated on the basis of cell-surface markers. Several laboratories have reported surface markers that appear to be expressed differentially on Th1 and Th2 cells, including CD45 isoforms[3-5] and immunoglobulin Fc receptors.[6] Although preferential expression of these molecules can be used to assign cells to either CD4$^+$ subset, this may be of only limited importance as the difference in levels of expression reported are quantitative rather than absolute. Another molecule which has been proposed as a potential discriminator between Th1 and Th2 cells is IL-1R.[7,8] IL-1α and IL-1β are two polypeptide cytokines which, although products of distinct genes,[9] have similar biological activities and bind to the same IL-1R.[10] This has been shown to be expressed at high affinity by Th2 clones but not by Th1 clones.[7,8] Differential expression of IL-1R appears to affect the proliferative responses of the two subsets. The proliferation of Th1 clones is independent of, and not influenced by, IL-1. For Th2 clones, however, in the absence of IL-1, exogenous IL-4 enhances only minimally responses to concanavilin A (Con A). The failure of Th1 cells to respond to IL-4 in the presence of IL-1 is considered likely to reflect their relative lack of IL-1R.

We have established a panel of eight antigen-specific CD4$^+$, major histocompatibility complex (MHC) class II-restricted T-cell clones derived from mice infected with *Plasmodium chabaudi*.[11] Four of the clones are characteristic of Th1, secreting IL-2 and IFN-γ, and four are typical of Th2 cells in that they secrete IL-4 and IL-10. All the clones used herein are stable *in vitro*, maintaining their respective cytokine secretion profiles during numerous rounds of culture and cryopreservation. Furthermore, clones of both types may confer protection by adoptive transfer to mice rendered CD4-depleted by treatment with appropriate monoclonal antibodies (mAb).[12] Unreconstituted CD4-depleted mice are severely immunologically depressed and either die from the infection or suffer high chronic parasitaemia. As these cloned CD4$^+$ cells are protective against malaria infection, they provide valuable tools for investigating immunity to blood stage malaria parasites. This would necessarily include an examination of the antigen-driven expansion of these cells and their trafficking patterns in the peripheral blood and lymphoid organs of malarious mice. To perform such novel studies would require the segregation of these Th1 and Th2 CD4$^+$ T-cell clones, not only on the basis of secretion of different cytokines but also on the basis of a phenotypic distinction. This led us to explore the possibility of the differential expression of one or more cell-surface markers on clones belonging to these different polarized Th subsets.

In the present study, we have examined both sets of CD4$^+$ clones for responsiveness to cytokine stimulation for extended periods *in vitro* and analysed the expression of the corresponding IL R under these conditions. The principal finding from this

Received 9 July 1993; revised 3 September 1993; accepted 20 October 1993.

Abbreviations: Con A, concanavilin-A; IL, interleukin; IL R, interleukin receptor; mAb, monoclonal antibody; MFI, mean fluorescence intensity; TcR, T-cell receptor; Th, T-helper.

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work was that each Th2 clone expressed IL-1R constitutively, whereas IL-1R was undetectable on the Th1 clones. The differential expression of this cytokine receptor provides a valuable marker for the dissection of the role of Th1 and Th2 cells in immunity to malaria, and clearly has implications for the study of the role of these subsets in other T-cell-mediated events, should this dichotomy in IL-1R prove to apply generally.

MATERIALS AND METHODS

Cells

Each of the clones used has been described in detail previously.[11,12] In brief, eight T-cell lines cloned by limiting dilution were raised to, and shown to be specific for, a lysate of *P. chabaudi*-parasitized erythrocytes. Each was phenotyped as a homogeneous population of $CD3^+$ $CD4^+$ $CD8^-$ $TcR\alpha\beta^+$ $TcR\gamma\delta^-$ cells. Four of the clones, WEP 996, 997, 998 and 999, are Th1-type cells, secreting IL-2 and IFN-γ and helping IgG2a production; four other clones, WEP 985, 986, 987 and 988, are Th2 cells, secreting IL-4, IL-10 and helping IgG1 synthesis. The surface phenotype, proliferation to *P. chabaudi* antigen and cytokine secretion of all the clones was reconfirmed by flow cytometry, tritium incorporation and IFN-γ and IL-4 ELISA, respectively at the start of this study (data not shown).

Cell culture

Cells were thawed from liquid N_2 storage, washed and resuspended in RPMI-1640 medium (Flow Laboratories, Irvine, U.K.) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 20 μg/ml gentamicin sulphate. Cells were incubated at 2×10^5/ml as 10-ml volumes in 25-ml tissue culture flasks in the presence of 1 μg/ml Con A. Cultures were maintained at 37°, 5% CO_2 and split every 2–3 days with medium replenished for up to 6 days, at which time sufficient cells were available for flow cytometric analysis or for performing a proliferation assay. For the time-course of expression of IL R in response to stimulation with IL-4 (Table 1), cells were incubated with Con A plus 100 U/ml recombinant murine IL-4 (Genzyme, Cambridge, MA) for up to 28 days before harvesting for analysis.

Proliferation assay

Cells were incubated at 1×10^6/ml with 1 μg/ml Con A in 96-well microtitre plates. Where appropriate, cytokines were added at 100 U/ml IL-1, IL-2 or IL-4 (all Genzyme) in a final volume of 200 μl/well. Cells were pulsed with 1 μCi/well [^{3}H]TdR (Amersham International, Amersham, U.K.) for the final 12 hr of a 48-hr incubation period and radioactive incorporation was measured by standard liquid scintillation counting. Results are given as counts per minute (c.p.m.).

Flow cytometry

Single-colour fluorescence was performed by incubating a sample of each $CD4^+$ clone with rat anti-mouse IL-2R (Pharmingen, San Diego, CA), IL-1R type I or IL-4R (both Genzyme), followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG polyclonal (Serotec, Oxford, U.K.). For two-colour fluorescence, cells were incubated first with rat anti-mouse IL-2R, then the FITC conjugate, followed by secondary staining with biotinylated rat anti-mouse IL-1R type I or IL-4R, revealed using phycoerythrin (PE)-conjugated streptavidin (Sera Lab, Crawley Down, U.K.). At each step, cells were washed twice and resuspended in binding buffer [phosphate-buffered saline (PBS) containing 5% FCS and 0·1% sodium azide] at 10^6/100 μl and incubated with an optimal dilution of the appropriate reagent for 45–60 min at 4°. Fc receptors were blocked by the inclusion of excess normal mouse immunoglobulin in the binding buffer at all stages. Control samples of unstained cells or of cells incubated with either FITC conjugate or PE–streptavidin only were included. After completion of labelling, cells were fixed with 1% paraformaldehyde in PBS and analysed on a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA). Dead cells were excluded on the basis of forward and right-angle light scatter. For each sample, 10,000 live cells were counted and the percentage of positive cells and their mean fluorescence intensity (MFI) (linear conversion of $\log_{10}$ fluorescence) were determined after correction for non-specific fluorescence of controls. The density of expression of surface IL receptors is given as the MFI ratio, using the following formula:

$$\text{MFI ratio} = \frac{\text{MFI of cells treated with test mAb}}{\text{MFI of cells treated with control mAb}}.$$

Statistical analysis

Results given for proliferation and fluorescence data are expressed as means ± SD. Student's *t*-test was applied to determine the significance of differences between paired values.

RESULTS

Cytokine requirements for proliferation of Th1 and Th2 cells

Each $CD4^+$ T-cell clone was examined for proliferation to Con A plus IL-2 or IL-4. All eight clones studied responded well to Con A *in vitro*, but in each case growth was enhanced significantly ($P < 0{\cdot}01$) by the addition of 100 U/ml IL-2 (Fig. 1). There was no noticeable difference in the degree of growth enhancement attained with IL-2 between the Th1 and Th2 clones. When cultured with IL-2, both Th1 and Th2 clones expressed IL-2R at a high density. After incubation with Con A plus IL-2 for 14 days, for all clones >98% of cells were $IL\text{-}2R^+$ and had an MFI ratio of >400 (data not shown). In contrast, the proliferative response of the Th1 and Th2 clones to Con A plus IL-4 was not significantly different from that to Con A alone (illustrated for representative clones of Th1 and Th2, WEP 999 and WEP 988, respectively in Fig. 2).

For the Th1 clones, proliferation to Con A could be enhanced significantly by the addition of IL-2 ($P < 0{\cdot}01$) but not of either IL-4 or IL-1 ($P > 0{\cdot}05$) (Fig. 2a). The level of proliferation to IL-2 plus IL-4 was significantly greater than that to IL-2 as a single supplement ($P < 0{\cdot}05$), indicating that IL-4 acts as a co-stimulator of IL-2 on these Th1 cells. In contrast, when IL-1 was added to cultures of the Th1 clones incubated with Con A plus IL-2, proliferation was not increased significantly ($P > 0{\cdot}05$) (Fig. 2a). Likewise, the Th1 cells responded no better to IL-4 and IL-1 together than to either alone. Thus, IL-1 had no apparent stimulatory effect on the proliferation of the Th1 clones tested, while IL-4 did so only in combination with IL-2.

Similarly to the Th1 clones, the proliferation of each Th2 clone to Con A plus IL-2 was significantly enhanced ($P < 0{\cdot}01$)

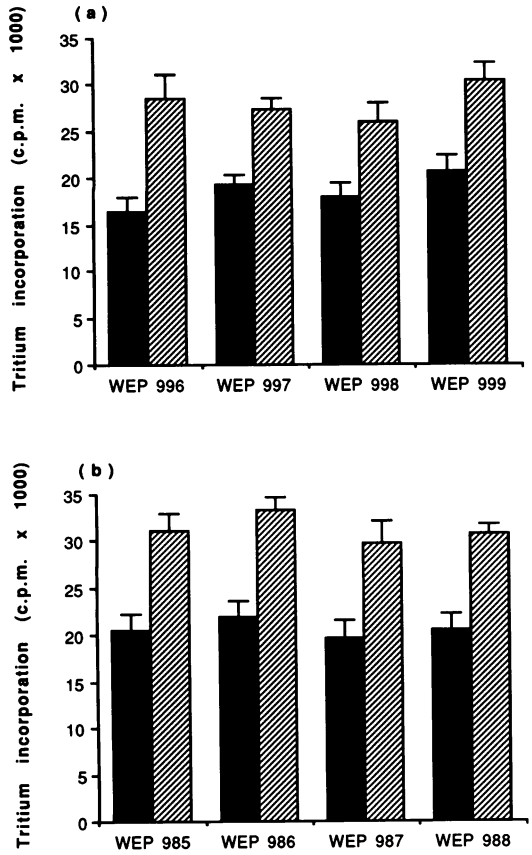

Figure 1. Proliferation of CD4+ clones when stimulated with Con A with or without IL-2. Th1 clones (a) and Th2 clones (b) were incubated with 1 μg/ml Con A (■) or 1 μg/ml Con A plus 100 U/ml IL-2 (▩). Values represent means ± SD of triplicates for one of three similar experiments. Responses to IL-2 alone (without Con A) were significantly greater than background ($P < 0.01$) but < 5000 c.p.m. (data not shown).

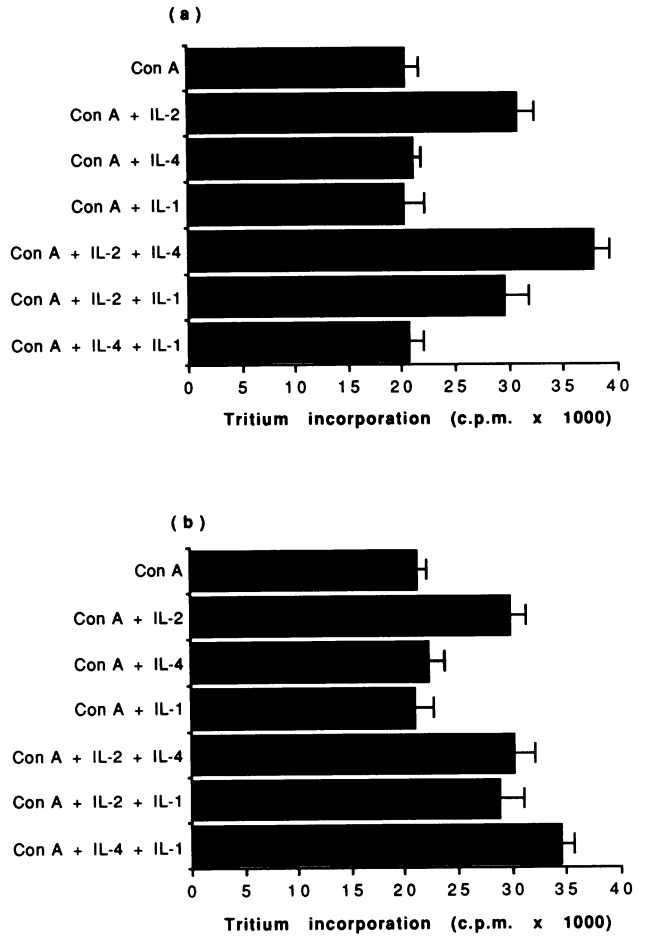

Figure 2. Effect of cytokines on the proliferation of two representative CD4+ clones, WEP 999 (Th1) (a) and WEP 988 (Th2) (b). Cells were incubated with 1 μg/ml Con A, either alone or with IL-2, IL-4 or IL-1 separately or in combination. All cytokines were used at a concentration of 100 U/ml. Values represent means ± SD of triplicates for one of three similar experiments. Responses to cytokines alone were < 4000 c.p.m. (data not shown).

compared to that to Con A alone or with added IL-4 or IL-1 (Fig. 2b). As for the Th1 clones, each Th2 clone also responded significantly better to IL-2 plus IL-4 than to IL-4 as growth factor supplements to Con A ($P < 0.01$). However, unlike the Th1 cells, the level of growth was the same as that attained with only IL-2 added to Con A. This suggested that the greater proliferation observed compared to that with Con A alone could be attributed solely to IL-2, and that for the Th2 cells IL-2 and IL-4 had no co-stimulatory activity. Proliferation of all the Th2 clones tested was enhanced significantly by adding IL-1 to cultures containing Con A plus IL-4 ($P < 0.01$) (Fig. 2b), indicating that IL-1 is a co-stimulator of IL-4 on these Th2 cells.

Cytokine receptor expression of Th1 and Th2 cells

The above findings indicated that IL-2 could act as a growth factor for both Th1 and Th2 cells, a function which IL-4 on its own could not perform. However, the addition of IL-4 to IL-2 could provide enhanced growth of Th1 cells compared to that provided by IL-2 alone, while IL-4 and IL-1 combined could promote the growth of Th2 cells. We next investigated whether the varying proliferation characteristics of the two sets of clones were reflected by a difference in the expression profiles of the corresponding IL receptors. Although neither the Th1 nor Th2 cells proliferated to IL-4 by itself, they both expressed IL-4R at a high density. Upon prolonged incubation with Con A plus IL-4, the level of expression of IL-4R increased significantly for representative clones of both Th1 (WEP 999) and Th2 (WEP 988) CD4+ subsets (Table 1) ($P < 0.05$), seen as a rise in both the percentage of IL-4R+ cells and their intensity of fluorescence between 7 and 28 days incubation. However, the expression of IL-2R was relatively unaffected by exposure to exogenous IL-4 ($P > 0.05$), remaining at a very high level throughout, and thereby suggesting that IL-2R is expressed constitutively by Th1 and Th2 cells. For WEP 988, extended culturing in the presence of IL-4 led to a significant rise in the density of expression of IL-1R ($P < 0.05$), reflecting the co-stimulatory activity of IL-4 and IL-1 on Th2 cell growth.

There was a striking, and in some cases, complete, difference in expression of IL-1R between the Th1 and Th2 cells. While WEP 988 (Table 1 and Fig. 3) and other Th2 clones (data not shown) all expressed IL-1R at a high level, there was virtually no detectable expression of this receptor by any of the Th1 clones examined (shown in Table 1 and Fig. 3 for WEP 999). Residual

Table 1. Time-course of alterations in the expression of IL receptors by WEP 999 (Th1) and WEP 988 (Th2) CD4⁺ clones

	WEP 999 (Th1)			WEP 988 (Th2)		
Culture period (days)	IL-2R	IL-4R	IL-1R	IL-2R	IL-4R	IL-1R
Positive fluorescence (%)						
7	95·9±2·6	75·6±3·7	0	94·3±1·7	84·3±5·6	91·2±3·3
14	93·5±3·1	89·4±4·2	0	92·6±4·0	93·8±4·2	96·0±2·7
21	97·5±2·1	95·0±2·2	0·04±0·06	95·8±3·6	97·5±1·9	96·9±2·8
28	96·1±1·5	96·8±3·5	0	95·6±1·2	98·0±1·5	97·6±1·8
MFI ratio						
7	376·9±20·6	241·2±11·5	6·2±0·8	383·4±17·2	226·3±18·3	244·0±8·9
14	372·7±14·5	275·3±16·2	7·8±1·3	390·0±24·1	255·4±6·7	272·1±17·2
21	392·1±12·4	283·2±7·4	10·5±2·4	392·7±16·6	277·0±11·4	307·7±23·0
28	388·6±7·9	290·1±15·8	8·5±3·9	377·3±20·4	281·8±10·6	315·8±14·3

Cells were incubated with 1 μg/ml Con A plus 100 U/ml IL-4 for the times indicated. Values given represent the means ± SD of three separate experiments.

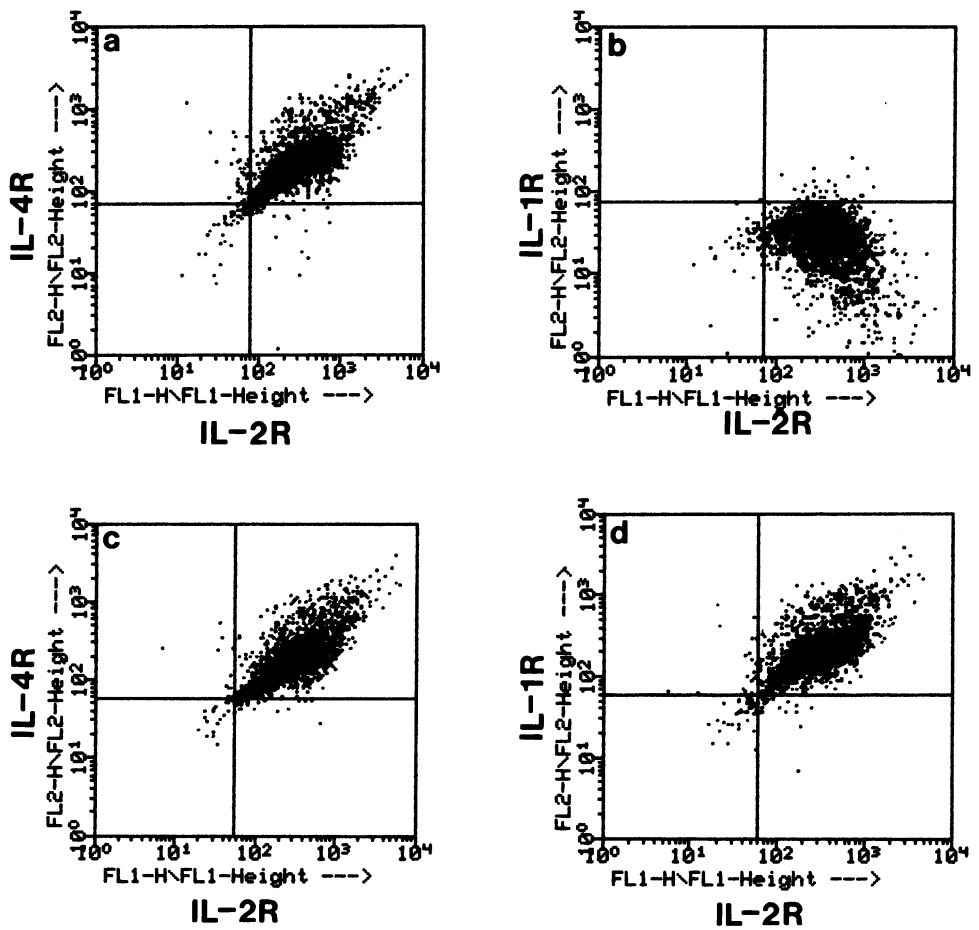

Figure 3. Two-colour analysis of two representative CD4⁺ clones, WEP 999 (Th1) cells (a, b), and WEP 988 (Th2) cells (c, d) for surface expression of IL receptors. Cells were stained with FITC-anti-IL-2R, together with either PE-anti-IL-4R (a, c) or PE-anti-IL-1R (b, d). The level of co-expression of IL-2R with either IL-4R (a, c) or IL-1R (b, d) is shown in the upper right quadrant of each profile. Similar results were attained for Th1 and Th2 cells for all clones examined.

co-expression of IL-2R and IL-1R (< 0·5% of cells) was seen for two of four Th1 clones, WEP 997 and WEP 999 (Fig. 3). For WEP 996 and WEP 998, however, all cells tested were IL-1R$^-$. Lack of IL-1R expression by the Th1 cells was observed at all times, even after prolonged incubation of WEP 999 with Con A plus IL-4 and IL-1 (Table 1). It is probable that the absence of IL-1R correlates directly with the inability of each Th1 clone to respond to IL-1 as a co-factor for IL-4-regulated proliferation.

DISCUSSION

In this report, a panel of murine CD4$^+$ T-cell clones specific for the malaria parasite *P. chabaudi* was analysed with regard to the cytokine requirements for proliferation and cytokine receptor expression of each clone. We found that whereas each Th2 clone expressed constitutively IL-1R, this molecule was not present at all on the Th1 clones. Thus, on the basis of the absolute expression of IL-1R, each of the cloned cell lines examined could be assigned to the particular CD4$^+$ subset appropriate to its cytokine secretion profile. The IL-1 dependence for enhanced proliferation of the Th2 clones supports the finding of Lichtman *et al.*[8] that Th2 cells require exogenous IL-1 (in this case macrophage-derived IL-1) for optimal proliferation *in vitro*. This is not the case for Th1 cells, as shown here and indicated by the inhibition of proliferative responses of Th2 but not Th1 cells with an IL-1R antagonist.[13]

The finding of IL-1R on Th2 but not Th1 clones shown here by flow cytometry is largely in agreement with studies in which binding assays indicated that Th2 clones express much higher levels of IL-1R.[7,8] The discrepancy between our finding of an absolute difference in IL-1R expression between Th1 and Th2 cells and the quantitative difference reported previously[7,8] may reflect the state of activation of the individual clones concerned. For example, for Th2 cells the requirement for IL-1 and the commensurate up-regulation of IL-1R expression appears to increase with time after antigenic or mitogenic stimulation, and results may vary according to the length of the incubation period. These observations have all been made using cloned lines of CD4$^+$ T cells that were maintained long-term *in vitro* and may result from a tissue culture artefact. It could be reasoned, for instance, that extended incubation *in vitro* selects for a subpopulation of Th2 cells the growth of which is activated by IL-1, while under the same conditions, Th1 cells rapidly lose expression of IL-1R and become insensitive to IL-1. All the clones analysed herein provide a memory response to malarial antigen *in vitro* as they were originally stimulated *in vivo* by exposure to malaria infection.[11] Virgin, unstimulated Th2 cells may also be IL-1R$^+$. However, if IL-1R is a marker of T-cell activation, as has been postulated,[8] naive T cells may be expected to be IL-1R$^-$ in much the same way as the Pgp-1 glycoprotein activation marker is expressed on memory but not virgin T cells.[14,15]

It has recently been shown that two receptors for IL-1 exist on T cells,[16] now termed type I and type II, the binding of IL-1 to which activates signal transduction pathways involving cyclic AMP and protein kinase C, respectively. In the present study, a mAb to the type I IL-1R was used and the possibility can not be excluded that type II IL-1R may have a different distribution on the CD4$^+$ clones examined. Currently, whether or not differential expression of IL-1R between Th subsets extends to the same functional populations freshly isolated from normal mice can not be investigated as there are no markers available to distinguish between Th1 and Th2 cells in the mouse. However, these data suggest the possibility that a mAb to IL-1R may allow the dissection of these two functionally different subsets *in vivo*.

Notwithstanding the qualifications outlined above, a qualitative difference in the expression of IL-1R on the surface membrane of Th1 and Th2 CD4$^+$ T cells confirms earlier suggestions[7,8] that IL-1R may provide a simple lineage marker for Th2 cells. If it transpires that IL-1R is associated uniquely with this one lineage, it may provide as useful a marker of Th2 cells as CD4 and CD8 have done for helper and cytotoxic T cells. This discrimination would be beneficial to studies involving the phenotyping or quantification of Th1 and Th2 cells, and the positive or negative selection or depletion of Th2 cells in mixed or uncloned populations of CD4$^+$ T cells.

Our interest lies in the fact that each of the Th1 and Th2 clones used here is protective against *P. chabaudi* infection.[11,12] The discovery of a cell-surface marker which discriminates Th2 from Th1 clones will facilitate a clearer understanding of the mechanisms by which, and where, each clone confers protective immunity *in vivo*. Although the sequestration patterns of various lymphocyte populations during malaria infection have been documented,[17,18] the altered migration of malaria-specific lymphocytes or of CD4$^+$ T-cell subsets remains to be determined. It is known that several weeks after clearance of *P. chabaudi* infection, levels of specific lymphocytes remain low,[19] but whether they are sequestered in the liver and spleen for this time is not clear. In this specific context, the finding of expression of IL-1R by Th2 but not by Th1 CD4$^+$ clones reactive to *P. chabaudi* may prove very useful. This distinction should enable the kinetics of recirculation of these protective, specific cells between the peripheral blood and the lymphoid tissues to be followed.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust. We thank E. Galloway (Department of Immunology, University of Glasgow) for guidance with the flow cytometric analysis and Malcolm Kennedy (WLEP) for critical appraisal of the manuscript.

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