TCR- and IL-1-mediated co-stimulation reveals an IL-4-independent way of Th2 cell proliferation

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Keywords: IL-4-independence, IL-1, ‘proliferative block’, Th2 cells

Abstract

Previously, it has been shown that Th1 cells, when triggered solely via their TCR, are blocked from proliferation in response to IL-2. Herein, we describe a similar characteristic for Th2 cells in that immobilized mAb directed to the TCR blocked proliferation of Th2 cells in response to IL-4. This ‘proliferative block’ was observed in all four Th2 cell clones tested, but not in a subline of one of the clones which has been cultured in vitro for several years. Addition of IL-1 neutralized the proliferative block in all four Th2 cell clones. Surprisingly, blocking experiments with sIL-4R and anti-IL-4 mAb revealed that in three out of four Th2 cell clones this effect of IL-1 was IL-4-independent and could also not be blocked by cyclosporin A (CsA). In contrast, the proliferation of one Th2 cell clone in response to the TCR- and IL-1-mediated signals was indeed inhibited by sIL-4R, anti-IL-4 mAb and CsA. Thus, our data illustrate that in addition to the well-known IL-4-dependent proliferation, there also exists an IL-4-independent, IL-1-mediated way of Th2 cell proliferation.

Introduction

T cell precursors that react with endogenous antigens in the thymus are usually eliminated (1,2). However, not all potential autoantigens are available for thymic antigen-presenting cells (APC) to present them to T cells. Therefore, potentially autoreactive T cells are delivered into the peripheral lymphoid organs. In order to inactivate such T cells, the immune system has learnt to bring them in an unresponsive state in which they are either non-functional and/or unable to proliferate (3–5).

When stimulated by foreign antigen, peripheral CD4+ T cells usually are not inactivated, but rather acquire effector functions and differentiate into one of mainly two subtypes termed Th1 and Th2 (6,7). Under certain circumstances, however, even differentiated T cells are still susceptible to the induction of unresponsiveness. Two types of unresponsiveness have been demonstrated for Th1 cells. In one experimental system, this state is created by fixed APC presenting the antigen to Th1 cells (8). This treatment leads to anergy, i.e. an incapability of the Th1 cells to produce IL-2 upon subsequent re-stimulation with antigen and viable APC (8).

Co-stimulation of Th1 cells with anti-CD28 antibodies together with fixed APC and antigen during the induction period avoids the appearance of anergy (9).

We and others have reported on a different type of Th1 cell unresponsiveness (10–13): Th1 cells are unable to proliferate in response to exogenous IL-2, when they are simultaneously stimulated by solid-phase coupled anti-TCR antibodies. We have termed this second type of unresponsiveness ‘proliferative block’ (14) and have shown that, in contrast to anergy, it is not influenced by anti-CD28 (14).

Most interestingly, Th2 cells, which have been implicated as causative agents in some autoimmune disorders (e.g. 15) and even AIDS (16), were shown to be refractory to the induction of unresponsiveness: stimulation of Th2 cells with anti-TCR antibodies has been reported not to influence the proliferation of these cells in response to IL-2 (10,12); similarly, Th2 cells generally are resistant to the induction of anergy (17), although one exception to this rule has been reported (18). It might appear therefore that, in vivo, a Th2 response is less controlled than a comparable Th1 response.
In this report, however, we show that a single signal delivered via the TCR suppresses proliferation of T<sub>h</sub> cells in response to IL-4 to a similar degree, as it does in T<sub>h</sub> cells in response to IL-2. The signal that overcomes the proliferative block in T<sub>h</sub> cells can be specified to be IL-1, a cytokine with known proliferative function on T<sub>h</sub> cells (19-21) and produced by T<sub>h</sub> cells themselves after a combined stimulus via the TCR and CD28 (22). Furthermore, we demonstrate that IL-1, together with the signal mediated via TCR, co-stimulates IL-4-independent proliferation in three out of four T<sub>h</sub> cell clones tested.

**Methods**

**Cell lines**

The characteristics and propagation of the BALB/c-derived *Leishmania major*-specific T<sub>h</sub> cell clones L1/1 and BEL-16, as well as of the T<sub>h</sub> cell clone D10.G4.1, have been described elsewhere (23-25). The purified protein derivative-specific T<sub>h</sub> cell line LNC-4 was provided by Dr E. Schmitt (Institute for Immunology, Mainz, Germany) (11). A subline of the L1/1 T cell clone (termed L1/1def.) with characteristics different from the parental clone (see below) has been raised by continuous *in vitro* culture for more than 10 years. All T cells were used between 2 and 4 weeks after their last antigenic re-stimulation.

**Lymphokines, antibodies and reagents**

Recombinant (r) human (h) IL-1β was provided by Dr D. Boraski (Sclavo, Siena, Italy). Recombinant murine (m) IL-2 and IL-4 were applied as crude culture supernatant of the myeloma cells X63Ag8-653-mIL-2 and X63Ag8-653-mIL-4 that had been transfected with the genes for mIL-2 and mIL-4 respectively, and were kindly provided by Dr F. Melchers (Basel, Switzerland) (26). In some experiments, affinity-purified mIL-4 was used, obtained by passage of supernatant of X63Ag8-653-mIL-2 and X63Ag8-653-mIL-4 that had been transfected with the genes for mIL-2 and mIL-4 respectively, and were kindly provided by Dr F. Melchers (Basel, Switzerland) (26). In some experiments, affinity-purified mIL-4 was used, obtained by passage of supernatant of X63Ag8-653-mIL-2 and X63Ag8-653-mIL-4 that had been transfected with the genes for mIL-2 and mIL-4 respectively, and were kindly provided by Dr F. Melchers (Basel, Switzerland) (26). In some experiments, affinity-purified mIL-4 was used, obtained by passage of supernatant of X63Ag8-653-mIL-2 and X63Ag8-653-mIL-4 that had been transfected with the genes for mIL-2 and mIL-4 respectively, and were kindly provided by Dr F. Melchers (Basel, Switzerland) (26).

**Assay for T cell proliferation and IL-4 synthesis**

The 96-well microtiter plates (Greiner, Nürtlingen, Germany) were coated with anti-TCR antibodies, as described (28). Thereafter, T cells (2 × 10<sup>4</sup>/well) were added with or without lymphokines in a total volume of 200 µl of Click's medium, containing 10% FCS and supplemented, as described (29). The T cells were incubated at 37°C, 5% CO<sub>2</sub> for 48 h. Then, each well was pulsed with [³H]thymidine (1.85 × 10<sup>4</sup> Bq/well), harvested and processed for β-scintillation counting. In some experiments, culture supernatant was harvested after 48 h and tested for IL-4 using a purchased (PharMingen, San Diego, CA) ELISA, as specified by the manufacturer.

**Results**

**The proliferative block of T<sub>h</sub>2 cells**

Cells of four different T<sub>h</sub>2 cell clones (BEL-16, D10.G4.1, L1/1 and LNC-4) were used to show (Fig. 1A) that T<sub>h</sub>2 cells proliferate strongly in response to IL-4 when tested between 2 and 4 weeks after their final antigenic re-stimulation. However, when the same cells were exposed to anti-CD3 mAb immobilized on plastic, they were dose-dependently rendered incapable to further proliferate in response to IL-4 (Fig. 1A). The cells nevertheless remained viable, as indicated by Trypan blue staining and by the fact that this same anti-CD3 treatment was capable to trigger IL-4 production by these cells (11 and data not shown). The proliferative block was also induced by a panel of mAb reactive to different epitopes of the TCR (TCRα, TCRβ, TCRγ and TCRδ) demonstrating that the signal leading to the proliferative block can be initiated by different parts of the TCR (data not shown). The proliferation of a subline of one of the clones (L1/1def.), which has been cultured *in vitro* for 10 years, was not altered by anti-CD3 (Fig. 1A), although this subline produced similar amounts of IL-4 as the parental L1/1 cell clone after anti-CD3-stimulation (data not shown). Since IL-1 is known to be a co-stimulus for T<sub>h</sub>2 cell proliferation (19-21), we next tested for an ability of IL-1 to overcome the proliferative block of T<sub>h</sub>2 cells. Figure 1(B) demonstrates that IL-1 indeed restored the IL-4-induced proliferation of T<sub>h</sub>2 cells, which in the absence of IL-1 was blocked by anti-CD3 mAb, as before.

**IL-1 co-stimulates IL-4-independent proliferation in the majority of T<sub>h</sub>2 cell clones**

The most direct explanation for the effect of IL-1 was that, in the presence of IL-1, T<sub>h</sub>2 cells would gain the capacity to use IL-4 to proliferate. To test this hypothesis, T<sub>h</sub>2 cells were stimulated with anti-CD3 mAb and IL-1, and the endogenously produced IL-4 was neutralized by increasing amounts of sIL-4R. The results of this set of experiments were surprising: proliferation could be blocked almost completely in L1/1 cells, while proliferation of three other T<sub>h</sub>2 cell clones was not altered (Fig. 2). This was true, although sIL-4R equally blocked free IL-4 in the supernatant of all four T<sub>h</sub>2 clones to levels below 10 pg, as evidenced by ELISA (data not shown); sIL-4R was not limiting in these experiments, because clone BEL-16 which was non-responding to sIL-4R, produced <1/10th (0.37 ng/ml) of the IL-4 of clone L1/1 (6 ng/ml) which was responding.

The same result was also obtained when anti-IL-4 mAb was used instead of sIL-4R to neutralize endogenous IL-4 (Table 1). As an important control, anti-IL-4 mAb efficiently blocked the proliferation induced by amounts of exogenous IL-4 as big as those endogenously produced in response to anti-CD3 mAb (Table 1) in the same experiment.

Therefore, while in the presence of the TCR signal IL-1 was necessary for proliferation of all four T<sub>h</sub>2 cell clones, it induced IL-4-independent proliferation in three of them. None of these
A.

B.

Fig. 1. IL-1 neutralizes the proliferate block of Th2 cells. (A) Cells (2 x 10^4) of the indicated Th2 cell clones were cultured in 200 µl medium containing 1 ng/ml rmIL-4 in 96-well culture plates coated with the indicated amounts of anti-CD3 mAb 145-2C-11. After 48 h of culture, the cells were pulsed with [3H]thymidine (1.85 x 10^4 Bq/well), further incubated for 16 h and processed for β-scintillation counting. Data represent c.p.m ± SD of triplicate cultures. (B) Cells (2 x 10^4) of the indicated Th2 cell clones were cultured in 200 µl medium. Where indicated, the wells also received 1 ng/ml rmIL-4, 300 U/ml rhIL-1β or were coated with 1 µg/ml of anti-CD3 145-2C-11 mAb. The test was performed as described in (A).

clones secreted IL-2 as tested by ELISA, and their proliferation in response to anti-CD3 plus IL-1 could also not be blocked, when sIL-4R was combined with anti-IL-2 (data not shown). We also tested whether IL-1 and anti-CD3 cooperated to induce the synthesis of an autocrine soluble factor different from IL-2 and IL-4 that induced proliferation of these three clones. However, when supernatants from these cells were harvested after stimulation with immobilized anti-CD3 mAb and IL-1, and tested on the same cells in the absence of anti-CD3 mAb, all proliferation-inducing activity could be neutralized by sIL-4R or by anti-IL-4 (data not shown).

The IL-4-independent Th2 cell proliferation is insensitive to CsA. As shown above, the activity of IL-1 on L1/1 Th2 cells was dependent on endogenous IL-4, while its activity on three other Th2 cell clones (including D10.G4.1 cells) was not. Therefore, we next tested if proliferation of these clones could be influenced when the synthesis of endogenous IL-4 was blocked by the immunosuppressive drug CsA. After stimulation with anti-CD3 mAb and IL-1, L1/1 and D10.G4.1 Th2 cells proliferated, as anticipated (Fig. 3A). Addition of CsA dose-dependently blocked proliferation of L1/1 cells, but not of D10.G4.1 cells. This was true, although equal concentrations of CsA were sufficient to suppress secretion of endogenous IL-4 in both clones (Fig. 3B). The effect of CsA on the proliferation of L1/1 cells was not toxic, because the same concentrations of CsA showed no effect when proliferation of L1/1 cells was triggered by exogenous IL-2. Therefore, the activity of IL-1 that operated independently of IL-4 was also uninfluenced by CsA. At the same time, the activity of IL-1 that was dependent on IL-4 was completely blocked by CsA.

Discussion
The immune system has developed mainly two ways to deal with autoreactive T cells: they are either deleted (1,2) or
IL-4-independent proliferation of T<sub>H</sub>2 cells

Fig. 2. Neutralization of free IL-4 inhibits anti-CD3 + IL-1β-induced proliferation of only one out of four T<sub>H</sub>2 cell clones. Cells (2 x 10<sup>4</sup>) of the indicated T<sub>H</sub>2 cell clones were cultured in 200 μl medium containing 300 U/ml rhIL-1β in 96-well culture plates coated with 1 μg/ml of anti-CD3 145-2C-11. Cultures also contained the indicated amounts of sIL-4R. The test was performed, as described in the legend for Fig 1.

Table 1. Anti-CD3 + IL-1β-induced proliferation of D10. G4. 1 cells is IL-4-independent

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>[H]Thymidine incorporation (cpm ± SD)</th>
<th>Free IL-4 in culture supernatant (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3</td>
<td>913 ± 99</td>
<td>14 40 ± 0.7</td>
</tr>
<tr>
<td>Anti-CD3 + IL-1β</td>
<td>14891 ± 2247</td>
<td>10.54 ± 1.9</td>
</tr>
<tr>
<td>Anti-CD3 + IL-1β + anti-IL-4</td>
<td>14304 ± 1546</td>
<td>≤0.01</td>
</tr>
<tr>
<td>IL-4 + IL-1β</td>
<td>30610 ± 1815</td>
<td>15.00 ± 1.7</td>
</tr>
<tr>
<td>IL-4 + IL-1β + anti-IL-4</td>
<td>2543 ± 245</td>
<td>≤0.01</td>
</tr>
</tbody>
</table>

D10. G4. 1 T<sub>H</sub>2 cells (2 x 10<sup>4</sup>) were cultured in 200 ml medium. Where indicated, the wells also received rmIL-4, 300 U/ml rhIL-1β, anti-IL-4 mAb (11B11) ascites 1:100 or were coated with 1 μg/ml of anti-CD3 145-2C-11. A proliferation test was performed, as described in the legend for Fig 1, and 48 h supernatants were tested for free IL-4 by ELISA.

functionally inactivated. Functional inactivation, in turn, also exists in different types of manifestation. The T cells may be in a state in which a trigger does no longer stimulate the T cells to acquire the functions that they usually get after triggering, e.g. cytolytic or helper functions (4,5,8). Alternatively, the T cells are blocked from proliferation (3,10-14). Possibilities to inactivate T cells have been described for almost all known types of T cells including γδ T cells (3-5,8,30,31).

So far, there appeared to be a controversy about T<sub>H2</sub> cell tolerization. In one report, T<sub>H2</sub> cells were described as resistant to the induction of anergy by fixed APC and antigen, a procedure that leads to defective IL-2 production in T<sub>H1</sub> cells (8). In a different report, this treatment indeed led to T<sub>H2</sub> cell anergy (18). In addition, T<sub>H2</sub> cells also were not blocked from their IL-2-triggered proliferation by solid-phase coupled anti-CD3 mAb (10,12). Again, this was in contrast to the behaviour of T<sub>H1</sub> cells.

Therefore, it became relevant to confirm that T<sub>H2</sub> cells indeed follow the general rules of T cell inactivation. Herein, we show that after triggering via the TCR in the absence of co-signals, T<sub>H2</sub> cells develop a proliferative block towards IL-4. Thus, T<sub>H2</sub> cells in this regard behave like T<sub>H1</sub> cells: each of the two cell types is blocked towards the activity of its own autocrine growth factor, i.e. IL-2 for T<sub>H1</sub> cells and IL-4 for T<sub>H2</sub> cells. It could be argued that the proliferative block of T<sub>H2</sub> cells represents an artefact due to the experimental conditions. However, this is rather unlikely, because we have isolated a mutant T cell line from one of the tested T<sub>H2</sub> cell clones that is insensitive to the induction of the proliferative block,
IL-4-independent proliferation of T\textsubscript{h}2 cells

Proliferation

- **A.**
  - D10.G4.1 (αCD3+IL-1β)
  - L1/1 (αCD3+IL-1β)
  - L1/1 (+IL-2)

  - CsA (μg/ml)
    - 0.01
    - 0.03
    - 0.1
    - 0.3
    - 3
  - CPM
    - 50000
    - 40000
    - 30000
    - 20000
    - 10000
    - 5000

  - Fig. 3. Anti-CD3 + IL-1β-induced proliferation of D10.G4.1 but not of L1/1-T2 cell clones is resistant to CsA. Cells (2 x 10^4) of the indicated T\textsubscript{h}2 cell clones were cultured in 200 μl medium containing 300 U/ml rmIL-1β in 96-well culture plates coated with 1 μg/ml of anti-CD3 145-2C-11 mAb. Cultures also contained the indicated amounts of CsA and, where indicated, 1 ng/ml rmIL-2. (A) A proliferation test was performed, as described in the legend for Fig. 1. (B) The 48 h supernatants were tested for free IL-4 by ELISA.

B. IL-4 production

- **A.**
  - D10.G4.1 (αCD3+IL-1β)
  - L1/1 (αCD3+IL-1β)

  - CsA (μg/ml)
    - 0.01
    - 0.03
    - 0.1
    - 0.3
    - 3
  - IL-4 (ng/ml)
    - 25
    - 20
    - 15
    - 10
    - 5

although it still grows strictly antigen-dependent and can be perfectly triggered by solid-phase coupled anti-CD3 mAb, as evidenced by the production of high quantities of IL-4. Therefore, the proliferative block is not caused solely by the experimental conditions, e.g. by stickiness to plastic, but instead by a feedback control mechanism present in T\textsubscript{h}2 cells and lost in the mutant T cell line during 10 years of continuous in vitro culture.

The co-signal necessary to overcome the proliferative block in T\textsubscript{h}2 cells can be specified and identified to be IL-1. While in T\textsubscript{h}1 cells, a signal mediated via CD28 is insufficient to overcome the proliferative block (14), CD28 has been shown to induce IL-1 synthesis in T\textsubscript{h}2 cells (22) and may therefore equip T\textsubscript{h}2 cells with the capacity to overcome this block themselves, as long as they find APC that carry B7 and deliver the signal via CD28.

In one report mentioned above (18), T\textsubscript{h}2 cell clones have been shown to secrete IL-4, but to be blocked from proliferation when triggered by fixed APC plus antigen. At first glance, therefore, it would appear that the proliferative block created by triggering either with anti-CD3 mAb or with fixed APC plus antigen are the same states of T\textsubscript{h}2 cell unresponsiveness. However, in the cited paper heterogeneity between T\textsubscript{h}2 cell clones was noted with regard to their susceptibility to the proliferative block induced by fixed APC plus antigen. Specifically, the commonly used D10.G4.1 cell line was refractory to the induction of this type of unresponsiveness. In contrast, D10.G4.1 cells, as well as three other T\textsubscript{h}2 cell clones, uniformly are accessible to the herein described proliferative block created by immobilized anti-CD3 mAb. Therefore, we would like to hypothesize that unresponsiveness created by fixed APC plus antigen still differs from that induced by anti-CD3 mAb. Possibly, fixed APC deliver a co-signal to D10.G4.1 cells that avoids unresponsiveness and is missing after stimulation with anti-CD3 mAb in the absence of APC. Further experiments are necessary to clarify this point.

In all four T\textsubscript{h}2 cell clones tested by us, addition of IL-1 neutralized the proliferative block. Surprisingly, in three of these (including the commonly used D10.G4.1 cell clone), endogenous IL-4 synthesized in response to the TCR signal...
and IL-1 could be neutralized without any loss of cell proliferation. Obviously, here the proliferation observed in the presence of the TCR signal plus IL-1 was mediated via a different growth factor. This factor was neither IL-2 nor IL-9. In addition, culture supernatants of Th2 cells stimulated via the TCR plus IL-1, when tested for their proliferation inducing capacity on Th2 cells that had been removed from the TCR signal, contained no new activity that could not be neutralized by anti-IL-4 mAb. We also tested the hypothesis that a putative, newly induced growth factor might be a membrane-integrated molecule and that the IL-4-independent IL-1 activity was depending on cell contact. However, we were unable to block the TCR plus IL-1-induced proliferation with mAb directed to LFA-1, CD2, CD5, CD40L, CD44 and Ly6, as well as with CTLA-4-lg. From all these results we like to conclude that the putative factor is presumably IL-1 itself.

IL-1, therefore, in conjunction with the TCR-mediated signal probably acted directly as an IL-4-independent growth factor for the majority of our Th2 cell clones. In contrast, IL-1-co-stimulated proliferation of one Th2 cell clone (L1/1) was dependent on IL-4. Since IL-4 has been reported to induce IL-1R expression on T cells (32,33), it is possible that IL-1R is not constitutively expressed on L1/1 cells and these cells require IL-4 for the induction of IL-1R expression to proliferate in response to IL-1. Studies are in progress in our laboratory to determine the IL-1R expression and the regulation of IL-1R expression by IL-4 on L1/1 cells. Alternatively, IL-1 has two qualitatively different effects on Th2 cells: firstly, together with the signal mediated via TCR, it co-stimulates Th2 cell proliferation in the absence of IL-4; secondly, it helps Th2 cells to use endogenous IL-4 to proliferate in the continuous presence of the TCR-mediated signal. At present, it is unclear whether or not these potential distinct activities of IL-1 can be attributed to the presence of different IL-1 receptors (34) or different components of one receptor (35). The induction of these potential two activities of IL-1 apparently requires extensive cross-linking of the TCR, because autocrine proliferation of D10.G4.1 cells can indeed be blocked by anti-IL-4 mAb if anti-CD3 mAb is applied in soluble rather than immobilized form (19 and our unpublished results).

The proliferative block of Th2 cells to IL-4 appears to follow the rules of the two-signal theory originally put forward by Bretscher and Cohn (36). This theory claims that two signals are necessary for full activation of T cells and that stimulation of T cells with signal one in the absence of signal two leads to a down-regulation of T cell function. We have recently extended this hypothesis by introducing the possibility that different co-signals may exist for different functions. In this regard, we have shown that triggering of Th1 cells via the TCR (signal one) plus either CD28 or CD44 (co-signal 2A) leads to lymphokine production, but not to proliferation of Th1 cells (14,37). Co-signal 2B required for proliferation of Th1 cells might be IL-12, as suggested by earlier studies (38-40). Th2 cells also follow this model: again, co-signal 2A for lymphokine production can be delivered via CD28 (2), while co-signal 2B for proliferation is mediated by IL-1.

The existence of an IL-4-independent pathway for Th2 cell proliferation may have important applications for the in vivo situation, because in diseases with an established Th2 profile, such as murine leishmaniasis, treatment solely with IL-4-neutralizing agents had no influence on the course of the disease, although Th2 cells have been shown to be critical for the development of this disease (41). In view of our results it may be worthwhile to repeat these therapeutic studies using a combination of IL-4- as well as IL-1-neutralizing agents. These studies are under way in our laboratory.

Acknowledgements

The authors are especially grateful to Dr Enssle, Behringwerke, Marburg, for providing IL-4R and Dr E. Schmitt for donation of T cell clones. We also thank Ms Martina Bach and Susanne Bischof for perfect technical assistance. This work was supported by the SFB 263 of the Deutsche Forschungsgeminschaft. A. R. and W. M completed this work in partial fulfilment of the requirements for the doctoral thesis.

Abbreviations

- APC: antigen-presenting cell
- CsA: cyclosporin A
- h: human
- m: murine
- PMA: phorbol myristate acetate
- r: recombinant
- siL-4R: soluble IL-4 receptor

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