Cellular FLIP long isoform transgenic mice overcome inherent Th2-biased immune responses to efficiently resolve Leishmania major infection

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Abstract

c-FLIPL expression in T cells is required for mounting effective T cell responses and can also be critical for effector T cell differentiation, as has recently been shown by a number of in vivo studies in conditional knockout and transgenic mouse systems. Available data supports therefore a novel immunomodulatory role of this anti-apoptotic protein besides its traditionally proposed function in homeostatic maintenance of T cell populations. In this study, the responses to infection with Leishmania major of mice over-expressing FLIPL specifically in the T cell compartment (TgFLIPL) are assessed. Although previous studies have shown that FLIPL drives T cells towards a Th2 differentiation programme in various autoimmune and allergic paradigms, in this study, we show that TgFLIPL are able to overcome this Th2 bias in a dermal L. major infection model to mount a robust Th1 response to pathogen and effectively clear infection. Our results suggest that vaccination protocols designed to enhance FLIPL expression in T cells may be useful for the treatment of autoimmune diseases like multiple sclerosis, without necessarily compromising immune responses towards infectious agents.

Introduction

CD4+ T helper lymphocytes are responsible for orchestrating appropriate immune responses to a diverse array of antigens ranging from self- to pathogen-derived antigens. Following antigen encounter, naive CD4+ T cells show substantial plasticity and have the potential to differentiate towards distinct effector or regulatory cell lineages depending upon the antigen and concomitant signals from cells of the innate immune system. To date, three types of CD4+ effector T cell populations have been described, namely Th1, Th2 and Th17 (1, 2). Th1 cells produce high levels of IFN-γ and tumour necrosis factor (TNF)-α and are involved in the immunity against intracellular pathogens and autoimmunity, Th2 cells produce IL-4, IL-5 and IL-13 and control parasitic infections and allergy and recently described Th17 cells produce high levels of IL-17, IL-17F and IL-6 and are involved in pathogenesis of experimental autoimmune diseases. A major goal in the field of T cell differentiation is to identify the factors responsible for directing different effector T cell responses.

Specific activation signals transmitted by the TCR, co-stimulatory signals from members of the CD28 or TNF receptor families and cytokine stimuli, interplay to specify the fate of the naive T cell and to shape the resulting immune response (3). Co-stimulatory molecules, with CD28 being the best characterized, have been shown to play a critical role in the development of Th1,2 effector cells (4), particularly in the presence of weak TCR signals (5). Several lines of evidence indicate that death receptors (DRs) of the TNF receptor family, further to their role in mediating apoptosis in activated T cells, may also modulate TCR signalling and control T cell differentiation. For example, studies in gene knockout mice have shown that the receptor adaptor Fas-associated death domain (FADD) (6), caspase 8 (7, 8) and c-FLIP (9, 10) are essential for pathways that instruct thymocyte development and T cell maturation and effector function. In addition, the modulation of DR signalling by the over-expression of c-FLIP long isoform in T cells of transgenic mice (TgFLIPL) resulted in diminished Th1 profiles and the preferential expansion of the Th1,2 pool in several in vivo immune paradigms including experimental autoimmune encephalomyelitis (EAE) (11) and airway hypersensitivity (12). Infection by the intracellular pathogen Leishmania major in mice represents a prototypic model for studying the
mechanisms underlying CD4⁺ Tε effector cell differentiation in vivo. To further understand the role of FLIPL in shaping of T cell immunity, we tested the susceptibility of TgFLIPL (C57BL/6) mice in a dermal L. major infection model and determined their ability to mount a parasite-specific T\textsubscript{H}1 immune response and to clear infection. Disease resolution in genetically resistant strains (e.g. C57BL/6) correlates with a higher level of activation of T\textsubscript{H}1 cells that produce IFN-γ, whereas disease progression in genetically susceptible strains (e.g. BALB/c) is associated with a more prevalent T\textsubscript{H}2 response (13). Overreaction of homeostatic mechanisms involving IL-10 in genetically resistant strains (e.g. C57BL/6) correlates with allergy and development of an adequate Th1 effector cell response.

Infection with L. major

Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). lysate. Total protein determination was carried out using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). 

Infection with L. major

Mice received a subcutaneous (s.c.) immunization in the footpad with 2 x 10⁶ stationary stage promastigotes in 40 µl volume PBS. Footpad swelling was determined thereafter at 1 week intervals by subtracting footpad thickness of the non-immunized footpad from the thickness of the L. major-immunized footpad. Footpad thickness was determined using an electronic caliper gauge (Kori Seiki MFG Ltd, Tokyo, Japan). For secondary infection, mice received an identical challenge with L. major in the opposite footpad from the one that received the primary immunization.

In vitro T cell proliferation assays

Spleens and lymph nodes from L. major-infected mice were aseptically excised and used to prepare single-cell suspensions in RPMI-1640 supplemented with 10% foetal bovine serum (FBS), 10 mM HEPES, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Gibco, Invitrogen Corporation, Paisley, UK). Cell viability was >95% as determined by trypan blue exclusion. Cells were cultured in triplicate in round-bottom 96-well plates (Costar, Cambridge, MA, USA) at a concentration of 1 x 10⁶ cells per ml, for proliferation assays, or 24-well flat-bottom plates at 5 x 10⁵ cells per ml for supernatant collection. Splenocytes were stimulated with crude soluble promastigote lysate at a concentration range of 5–20 µg ml⁻¹ or Con A (Sigma, Munich, Germany) at a range of 2.5–10 µg ml⁻¹. In lymphoproliferation experiments, cultures were incubated in 5% CO₂ at 37°C for 96 h and pulsed with 0.2 µCi ml⁻¹ [³H]-thymidine (Amersham Co, Buckinghamshire, UK) during the final 18 h of culture. Cells were harvested and [³H]-thymidine incorporation was assessed by liquid scintillation counting (Wallac, Turku, Finland). Results are expressed as the stimulation index (ratio between radioactivity counts of cells cultured in presence of antigen and cells cultured with medium alone).

Measurement of cytokine production

IL-4 and IFN-γ ELISA kits (Endogen, Woburn, MA, USA) and a mouse IL-17 ELISA set (R&D Systems, Wiesbaden-Nordenstadt, Germany) were used to measure cytokine secretion from splenocyte and draining lymph node culture supernatants according to the manufacturer’s instructions. The detection limit for IL-4 was 10 pg ml⁻¹ and for IFN-γ 50 pg ml⁻¹. In addition, the mouse T\textsubscript{H}1/T\textsubscript{H}2 cytokine cytometric bead array kit (BD Biosciences, San Jose, CA, USA) was used to measure cytokine levels in culture supernatants according to the manufacturer’s instructions. The sensitivity of the assay for the different cytokines is the following: IL-2, 5 pg ml⁻¹; IL-4, 5 pg ml⁻¹; IL-5, 5 pg ml⁻¹; IFN-γ, 2.5 pg ml⁻¹ and TNF-α, 6.3 pg ml⁻¹. For intracellular cytokine staining, draining lymph node cells were isolated from wt and TgFLIPL 4 weeks after footpad infection with L. major. After 3 days in culture with or without a mixture of L. major antigens (used at a concentration of 20 µg ml⁻¹), cells were re-stimulated with phorbol myristate acetate (PMA)/ionomycin (Sigma) for 5 h and brefeldin A (5 µg ml⁻¹, Sigma) was added for the last 3 h of culture. Cells were then washed and fixed with 2% formaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.5% saponin in PBS/BSA/azide and stained with APC-conjugated anti-CD4 (L3T4) and PE-conjugated anti-IFN-γ (XMG1.2), anti-IL-10 (JES5-16E3) and anti-IL-4 (11B11) (all antibodies were from BD Pharmingen, San Jose, CA, USA). Cytometric analysis was performed using a FACSCalibur and the CellQuest software (BD Biosciences).

Infection and determination of tissue parasitism

Parasite burden was determined using a sensitive microtitration assay as published (15). Rabbit blood agar was used to...
support the growth of parasites. Tissue samples were homogenized through serial passage from 21GA11/2 and 30GA1/2 needles and adjusted to 100 \mu g/ml in RPMI-1640 supplemented with 10% FBS. The number of wells positive for parasite growth was scored using an inverted microscope.

**Antibody levels**

Serum parasite-specific IgG antibodies and specific IgG isotypes were determined by using a standard sandwich ELISA method. Briefly, microtitre plates (Nunc Maxisorp, Roskilde, Denmark) were coated overnight with 10 \mu g/ml of a mixture of L. major antigens in 0.05 M carbonate–bicarbonate coating buffer (pH 9.6). Uncoated sites were blocked with 1% BSA in PBS. Serum samples from immunized transgenic and control littermate mice were plated and HRP-conjugated rat anti-mouse IgG1 and IgG2a mAbs (BD PharMingen) were used to detect bound antibodies. The enzyme-labelled complexes were detected by a reaction with 0.01% TMB substrate (Sigma) and 0.02% hydrogen peroxide in 0.18 M Na-acetate buffer (pH 4.0). The reaction was stopped with 2N H2SO4 and optical density at 450 nm was measured using a microplate reader (Dynatech MR 5000, Dynatech Laboratories, Chantilly, VA, USA).

**Statistical analysis**

Data were evaluated using Student’s t-test and \( P < 0.05 \) was considered significant.

**Results**

**TgFLIP\(_{L}\) efficiently resolve infection by L. major**

TgFLIP\(_{L}\) and non-transgenic littermate control mice, in the C57BL/6 genetic background, were inoculated with \( 2 \times 10^6 \) stationary phase L. major promastigotes s.c. in one hind footpad, and subsequently monitored for lesion development by measuring the difference in thickness between injected and non-injected footpads. wt mice developed increasing swelling from the first week post-inoculation with maximum swelling observed between 6 and 10 weeks. Lesions were completely resolved by 17–18 weeks after inoculation (Fig. 1A). TgFLIP\(_{L}\) showed a similar clinical course with no significant difference in initiation or resolution phases and overall disease pattern, although a significant increase of swelling was seen at the peak of disease (Fig. 1A). Measurement of tissue parasite burden using a sensitive microtitration assay showed that TgFLIP\(_{L}\) had equivalent levels of parasite infestation as wt mice at 3 weeks after primary infection (Fig. 1B). By 17 weeks after L. major inoculation, parasites were cleared from the site of infection, since no L. major promastigotes could be recovered from the footpad of either mouse strain (data not shown). These results show that TgFLIP\(_{L}\) efficiently resolve infection by L. major with similar kinetics as wt mice.

**TgFLIP\(_{L}\) mount a normal memory response to secondary infection with L. major**

To examine the memory response of TgFLIP\(_{L}\) to L. major infection, TgFLIP\(_{L}\) and wt mice were re-infected by s.c. inoculation of \( 2 \times 10^6 \) stationary stage L. major promastigotes.
25 weeks after the primary infection. Re-infection induced rapid intense footpad swelling in both groups of mice indicating the presence of an efficient and equivalent memory cellular immune response. In both groups of mice, footpad swelling reached its peak during the first week and lesions were resolved by 5 weeks after parasite inoculation (Fig. 2). These results show that TgFLIP<sub>L</sub> mount a normal memory response to <i>L. major</i> infection.

**Priming and recall responses of lymphocytes to <i>L. major</i> are normal in TgFLIP<sub>L</sub>**

To analyze the immune response of TgFLIP<sub>L</sub> to <i>L. major</i>, TgFLIP<sub>L</sub> and wt mice were inoculated with parasite and analyzed for in vitro recall responses to a mixture of antigens prepared from <i>L. major</i> soluble promastigote lysate, as described in Methods. As predicted from the observation of efficient lesion resolution in TgFLIP<sub>L</sub>, splenocytes and lymph node cells showed normal proliferative responses at 3 weeks (progressive phase of infection) (Fig. 3A) and 17 weeks (resolution phase) (Fig. 3B and C) after infection. Moreover, memory T cell proliferation at 3 weeks after secondary challenge with parasites was also normal in TgFLIP<sub>L</sub> when compared with wt animals (Fig. 3D). Collectively, these results demonstrate that TgFLIP<sub>L</sub> respond to <i>L. major</i> infection with a normal lymphocyte proliferation response which effectively limits and clears parasitic infection.

**Antibody responses are normal in TgFLIP<sub>L</sub> following primary infection but show enhanced IgG1 production following secondary infection with <i>L. major</i>**

A combination of T<sub>H</sub>1 cell-mediated and humoral immune responses is considered important for controlling <i>L. major</i> infection in C57BL/6 mice. To determine whether TgFLIP<sub>L</sub> are able to develop a normal antibody immune response to <i>L. major</i> that is typical of the C57BL/6 strain, we measured the titers of <i>L. major</i>-specific IgG isotypes in sera sampled from TgFLIP<sub>L</sub> and wt mice at 3 and 17 weeks after primary infection and at 3 weeks after secondary infection (i.e. at 28 weeks) by ELISA (Fig. 4). During the course of primary infection, both groups of mice produced statistically equivalent amounts of IgG1 and IgG2a. Upon re-infection, however, TgFLIP<sub>L</sub> produced significantly increased levels of the T<sub>H</sub>2 priming IgG isotype IgG1 at 3 weeks after re-infection. No significant differences were detected between the groups in the production of IgG2a, the T<sub>H</sub>1 priming antibody isotype. These results show that TgFLIP<sub>L</sub> develop a normal T<sub>H</sub>1 response following primary and secondary infection and an enhanced T<sub>H</sub>2-driven memory response to secondary infection.

**Cytokine production following infection with <i>L. major</i> or in vivo priming with a mixture of parasite antigens**

We next measured the levels of IFN-γ produced by splenocytes isolated from TgFLIP<sub>L</sub> and wt mice after primary and
secondary infection with L. major and re-stimulated in vitro with L. major antigens using a sandwich ELISA. Splenocytes from both strains showed similar expression of IFN-γ at both 3 (Fig. 5A) and 17 (data not shown) weeks time points following primary infection. In reflection of the enhanced IgG1 antibody isotype production profiles, IFN-γ production in the memory phase after re-infection with L. major showed a trend towards lower levels in the TgFLIPₜ (Fig. 5B), but this difference did not reach significance levels. IL-4 production by splenocytes from both strains was undetectable at all time points studied by ELISA assay (data not shown). We also measured IL-10 production in these cells since it has been described to be the cytokine responsible for immunosuppression and parasite persistence in cutaneous leishmaniasis (16–18). TgFLIPₜ showed comparable IL-10 production to wt mice (Fig. 5E and F).

**Discussion**

Using a cutaneous L. major infection model in conventionally resistant C57BL/6 mice, we have further investigated the role of T cell-specific FLIPₜ transgene over-expression in the specification of Tₘₜ fate. In comparison to previous findings, where down modulation of Tₘₜ-mediated immune function and Tₘₜ skewing of T cell responses resulted in the suppression of myelin oligodendrocyte glycoprotein 35–55 peptide (MOG₃₅–₅₅)-induced EAE (11) and exacerbation of ovalbumin peptide-induced asthma (12), we show here that in the L. major infection model, TgFLIPₜ exhibited a robust Tₘₜ adaptive immune response to the pathogen, which is typical of the C57BL/6 strain, and cleared pathogen as effectively as normal mice. Furthermore, even though memory responses induced by secondary infection with the same parasite were characterized by reduced Tₘₜ-polarized immunity, as indicated by significantly increased IgG1 production, mice were still able to clear re-infection normally. These observations confirm that FLIPₜ plays an important role in shaping T cell responses to a wide range of antigens, now including those derived from the parasite L. major, and has functional consequences in several autoimmune and allergic immune paradigms. They also suggest that during an infection, for example by L. major parasites, additional signals are delivered to T cells by the innate immune environment that overrides the otherwise Tₘₜ-polarizing effects of T cell-specific FLIPₜ transgene expression.

It has become clear that apoptosis-related mediators, such as caspase 8 and FLIPₜ, play important roles in the control of thymocyte development and the maturation and effector function of mature T cells (6–9, 19). Inactivation of flip selectively in the T cell lineage of conditional FLIP knockout mice enhanced the sensitivity of CD4⁺ and CD8⁺ single-positive thymocytes to TCR/CD3 and Fas-induced apoptosis resulting in severely reduced numbers of mature T cells in the periphery (9, 10). Further, the over-expression of FLIPₜ in T cells of transgenic mice resulted in increased CD3- and
antigen-induced proliferation (20). We have seen that TgFLIP\(_L\) (C57BL/6) T cells are capable of differentiating towards both the Th1 and Th2 cellular fates, but show a markedly lower production of Th1 cytokines when compared with wt cells when stimulated \textit{ex vivo} or \textit{in vitro} with various peptide antigens or polyclonal activators (V. Tseveleki and L. Probert, unpublished data). As mentioned above, \textit{in vivo} priming and \textit{in vitro} recall stimulation of TgFLIP\(_L\) cells with a wide range of purified antigens resulted in reduced Th1 and enhanced Th2 cytokine responses, and in several disease paradigms, this effect translated into markedly altered clinical signs (11, 12). The mechanism by which FLIP\(_L\) alters Th differentiation is not known but biochemical studies have shown that FLIP\(_L\) expression in cell lines promotes nuclear factor-kappaB and extracellular signal-regulated kinase activation (21) and inhibits c-jun N-terminal kinase activation (22). It remains to be determined whether the Th2 skewing effect of FLIP\(_L\) in T cell pools is due to altered signalling in T cells which affects their differentiation or whether the Th2 population is selectively protected from apoptosis leading to its aberrant expansion. Our finding, that T cell FLIP\(_L\) does not lead to a Th2 bias when mice are infected with the intracellular parasite \textit{L. major}, suggests that although it might be important for physiological T cell functioning, for example for tolerance or memory formation, it might not play such a critical role during host defence reactions, where additional

Fig. 5. Measurement of IFN-\(\gamma\) production by splenocytes isolated from TgFLIP\(_L\) (grey bars, \(n = 3\)) and wt (black bars, \(n = 3\)) mice 3 weeks following primary (A) or 3 weeks following secondary (B) infection with \textit{Leishmania major}, and re-stimulated \textit{ex vivo} with \textit{L. major} antigens, using an ELISA assay. Similar levels of IFN-\(\gamma\) were secreted by lymphocytes of both strains of mice following primary and secondary infection. The production of Th1/Th2 cytokines by splenocytes isolated from TgFLIP\(_L\) (grey bars, \(n = 3\)) and wt (black bars, \(n = 3\)) mice that have been immunized with a mixture of \textit{L. major} antigens, and re-stimulated \textit{ex vivo} with the same antigens were measured by a flow cytometric bead assay. TgFLIP\(_L\) cells showed normal production of both TNF-\(\alpha\) (C) and IFN-\(\gamma\) (D) and levels of IL-4 and IL-5 were undetectable using this assay. Intracellular IFN-\(\gamma\), IL-10 and IL-4 production by CD4\(^+\) T lymphocytes was measured for PMA/ionomycin re-stimulated (E) and for 72-h \textit{L. major} antigen stimulated prior to PMA/ionomycin re-stimulation (F), draining lymph node cells isolated from wt (black bars, \(n = 4\)) and TgFLIP\(_L\) (grey bars, \(n = 4\)) mice 4 weeks following parasitic infection. The \(P\) values for the cytokine measurements were calculated by paired Student’s \(t\)-test and were as follows: (A) \(P < 0.45\), (B) \(P < 0.12\), (C) \(P < 0.15\), (D) \(P < 0.25\) (E) \(P > 0.05\) and (F) \(P > 0.05\).
infection-specific signals are delivered to T cells and robust Th1 responses are required. However, we cannot exclude the alternative possibility that differences in TCR signal strength delivered by various antigens determine whether or not FLIpL can be involved in shaping T cell responses.

A major pursuit in the field of T cell biology is to apply basic knowledge to the development of improved therapeutic vaccines and vaccination strategies for the treatment of autoimmune diseases. Several of the current approaches that involve the prolonged use of immunosuppressants for the treatment of autoimmune diseases have been linked with significant adverse effects such as increased susceptibility to opportunistic infections that can be fatal (23). For example, there are multiple Food and Drug Administration, USA (FDA) warnings associating the use of the TNF-α blocking agents with the occurrence of serious infections, including sepsis and disseminated tuberculosis. It is tempting to envisage vaccination strategies that would be able to boost FLIpL expression in T cells for the treatment of autoimmune disorders without necessarily compromising host defence mechanisms to pathogens.

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Abbreviations
DR death receptor
EAE experimental autoimmune encephalomyelitis
FBS foetal bovine serum
PMA phorbol myristate acetate
s.c. subcutaneous
TgFLIPL CD2-FLIP transgenic mice
TNF tumour necrosis factor
Wt wild type

References