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Research paper

A novel tool to identify the relative contribution of lymphoid cell types that contribute to IL-10 production during the infection with *Schistosoma mansoni*: The TIGER index

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ABSTRACT

Infection with the trematode helminth *Schistosoma mansoni* affects more than 200 million people worldwide. Infected patients are thought to show a decreased incidence of asthma and autoimmune diseases, which is, among others, considered a result of an increased production of the immunoregulatory cytokine IL-10. However, the location and the type of cell that is responsible for the highest production of IL-10 in vivo are still unknown.

Here, we describe the use of the IL-10 reporter mouse TIGER for the study of murine schistosomiasis and introduce a novel tool, which we have called the TIGER index (TI). This index combines data from flow cytometric measurements and cell count analysis and allows identifying the cell type with the highest contribution of IL-10 during the course of infection in the secondary lymphoid organs, sites of extensive immunoregulatory activity in schistosomiasis. The strength of the TI is the possibility to analyze IL-10 production in a long term experiment without the need of an external standard between each time point of analysis. In this paper we have calculated the TI for the mesenteric lymph nodes and the spleen in the course of a chronic infection with *S. mansoni*.

Using the TI, we identified CD4^{pos} CD25^{pos} and CD4^{pos} CD25^{neg} cell populations as the highest producers of IL-10 in the mesenteric lymph node and the spleen in chronic schistosomiasis, respectively, whereas B cells, NK cells and NKT cells showed a lower contribution to IL-10 production throughout the infection.

The TI is thus a highly useful tool to measure the relative contribution of different cell types, which are responsible for the *in vivo* production of IL-10 in the secondary lymphoid organs during the infection with *S. mansoni*.

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1. Introduction

Infection with the trematode helminth *Schistosoma mansoni* affects more than 200 million people worldwide. Infection occurs when cercariae of *S. mansoni*, produced by freshwater snails that function as intermediate hosts, penetrate the skin of

the definitive host. They then pass through the lung at week 1 55
after entry. The developing schistosomulae travel to the portal 56
system where male and female meet and mate at weeks 4-5 57
after infection. The reproductive cycle in the mammalian host 58
and egg deposition start at weeks 5-6. The eggs travel through 59
the gut wall, inducing a strong inflammatory reaction. Not 60
all eggs make it into the gut. Instead they are flushed into the 61
liver where they cause a host response resulting in granuloma 62
formation. In response to the induced inflammation the 63
host initiates an immunoregulatory response to decrease the 64

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deleterious effects of proinflammatory cytokines. Among the regulatory cytokines induced, interleukin 10 (IL-10) is the most prominent and best described cytokine. IL-10 was initially described as a cytokine with potent anti-inflammatory properties and was thus considered a Th2-associated cytokine. Subsequently it was discovered, that regulatory T cells (Tregs) are a major source for IL-10 (Powrie et al., 1993; Roncarolo et al., 1997; Sher et al., 1991).

The receptor for IL-10 is a tetramer consisting of two heterodimers with the subunits IL-10R1 (IL-10R α) and IL-10R2 (IL-10R β). The engagement of IL-10R results in the activation of the signal transducer and activator of transcription-3 (STAT3). STAT3 binds to STAT-binding elements (SBEs) in the promoter region of various IL-10-responsive genes. One of these is the suppressor of cytokine signaling (SOCS) 3, a gene involved in the inhibition of many lipopolysaccharide (LPS)-inducible genes such as TNF- α or IL-6. Thus, IL-10 is a potent regulator of pro-inflammatory cytokines (Donnelly et al., 1999).

The *S. mansoni* induced the production of IL-10 interferes in various stages with the host: early in the infection, IL-10 is able to block the development of resistance to reinfection (Wilson et al., 2011), whereas in the later stages, IL-10 deficiency was also associated with increased mortality (Hoffmann et al., 2000; Powrie et al., 1993; Roncarolo et al., 1997; Sher et al., 1991). Thus, IL-10 has an important role in the immunoregulation during *S. mansoni* infection (Donnelly et al., 1999; Duell et al., 2012). However, it is still unknown whether Tregs are the main cells responsible for IL-10 production during the infection or whether other cell-types are involved at certain time points during the infection.

The cellular source of IL-10 can be studied reliably and in detail with the help of the reporter mouse TIGER (interleukin ten IRES enhanced GFP reporter), which expresses the enhanced green fluorescence protein (eGFP) under the same transcriptional control as IL-10 through the use of an IRES sequence 3' of the IL-10 gene. As shown before, the levels of eGFP expression correlate with the level of IL-10 expression and also with the level of IL-10 production. Thus, the quantity of IL-10 production of individual cells can be acquired by flow cytometry analysis. Here, the parameter of the mean fluorescence intensity (MFI) is the factor for the strength of IL-10 expression (Kamanaka et al., 2006; Wilson et al., 2011). However, the acquisition of the MFI parameter at different time points of an infection is subject to changes in the flow cytometer setup and therefore, either an internal or external standard is required to allow the comparison of IL-10 production in reporter mice at different days of analysis.

Information about the strength of IL-10-production in each individual cell type at each time point of the infection should result in a better understanding of their influence in the course of the disease. Here, we describe a novel tool to identify the cell type with the highest contribution to the production of IL-10 in the mesenteric lymph nodes (mLN) and the spleen (SP) of mice in the course of the infection with *S. mansoni*.

2. Materials and methods

2.1. Mice

TIGER (Kamanaka et al., 2006) and FIR (Wan and Flavell, 2005) mice on C57BL/6J background were bred and kept

in the SPF animal facility of the MPI-IE. Animal experimentation was performed after the approval of the animal ethics committee of the local authority (Regierungspraesidium Freiburg, Germany). All mice were monitored on a daily basis to identify ailing mice. Mice were euthanized by cervical dislocation at the time point of analysis. Control animals (week 0) represent uninfected animals at 10 weeks of age. Untreated aged mice did not show increased TIGER indices in steady state (data not shown).

2.2. Infection with *S. mansoni*

An Omani human isolate of *S. mansoni* was maintained using *Biomphalaria glabrata* and *Biomphalaria pfeifferi* as intermediate hosts. The fresh-water snails were kept at 24 °C and at a fixed photoperiod (light/dark: 12 h/12 h). Mice were anesthetized with Ketamine/Xylazine (0.1 mg resp. 0.5 μ g/mouse) and exposed to 80–90 cercariae via the shaved belly method (Standen, 1953). Infection efficiency was generally ~75% (number of cercariae left after 1 h of exposure/number of input cercariae) throughout all experiments. Mice were infected with *S. mansoni* at 10–11 weeks of age.

2.3. Flow cytometry

Single-cell suspensions of organs were treated with Gey's solution where appropriate and washed with PBS/3% FCS. The cells were preincubated with 20 μ g anti-CD16/32 antibody (clone 2.4G2), washed and stained with a combination of fluorochrome-labeled monoclonal antibodies (all steps at 4 °C). Antibodies were from BD Biosciences {name (clone)} {TCR β (H57-597); CD4 (RM4-5 or GK1.5), CD8 (53-6.7), CD19 (1D3); CD138 (281-2); NK1.1 (PK136)} and eBioscience {CD3e (145-2C11), CD25 (PC61.5)}. Samples were processed on a LSR II flow cytometer equipped for 10-parameter acquisition and analyzed with the TreeStar FlowJo software (version 9.5).

2.4. ELISPOT analysis

Splenocytes were prepared as in 2.3. CD4^{pos} GFP^{pos} cells were directly sorted into wells of an ELISPOT plate (Mouse IL-10 ELISPOT Ready-Set-Go, eBioscience), incubated for 24 h in the presence of ConA and analyzed according to the manufacturer's protocol.

3. Results

3.1. Using the TIGER mice in murine schistosomiasis

TIGER mice on a C57BL/6J background were infected via the natural (shaved belly) infection route with 80–90 cercariae/mouse and analyzed weekly for the induction of the IL-10 production of lymphocytes in the mesenteric lymph nodes and spleen.

The initial report on the TIGER mouse verified the correlation between IL-10 protein and eGFP expression (Kamanaka et al., 2006). However, to confirm, that this correlation was also found under our test conditions, i.e. whether cells positive for eGFP expression also secrete IL-10, we sorted eGFP-positive T cells and analyzed them in an ELISPOT assay for IL-10 production. Indeed, more than 80% of

the sorted T cells from the spleen were positive in the ELISPOT assay (Supplementary Fig. 1).

As detailed in the introduction, CD4^{pos} Tregs are considered the major cell type producing IL-10. Tregs are characterized, but not exclusively, by the expression of the alpha chain of the IL-2 receptor (CD25). We used the expression of the Foxp3 transcription factor (FIR mice) to verify the authenticity of CD4^{pos} CD25^{pos} cells as Tregs. Approximately 80% of CD4^{pos} CD25^{pos} cells was also positive for Foxp3, thus were *bona fide* Tregs (Supplementary Fig. 2).

3.2. Frequency of eGFP^{pos} cells

Without infection, about 11% of the CD4^{pos} T cells, 2% of CD8^{pos} T cells, 25% of NKT cells, 2% of NK cells and 4% of B cells in the mLN were positive for eGFP (Fig. 1). Early in the infection, before the onset of egg deposition, the percentage of eGFP-positive CD4^{pos}-T cells decreased, as did the fraction of eGFP-positive CD4^{pos} CD25^{pos} cells. In the chronic, late phase of infection the percentage of eGFP-positive CD4^{pos}-T cells increased again, as did the fraction of eGFP-positive CD4^{pos} CD25^{pos} cells. It is noteworthy that late in the infection, the production of eGFP was not increased in CD4^{pos} CD25^{neg} T cells (Fig. 1, week 12 compared to control). CD8^{pos}-T cells and NKT cells lost most of their eGFP expression in the course of the infection, whereas more NK and B cells expressed eGFP in the later stages of infection. Based on these data, the increase of IL-10 production is most prominent in CD4^{pos} CD25^{pos} T cells and NK cells throughout the infection.

The fraction of eGFP^{pos} lymphocytes was increased significantly between 2 and 3-fold at week 3 of infection in both mLN and spleen, but was not increased in the later stages of the infection, neither in the mLN, nor in the spleen (Supplementary Fig. 3).

3.3. The MFI of eGFP^{pos} cells

The simple classification of eGFP expression in positive and negative in the individual types of lymphocytes does not per se depict the contribution to the production of IL-10 because the amount of IL-10 produced by each cell type is also prominently influenced by the level of expression (and thus, see above, by the secretion rate). This notion is reflected in the measurement of the mean fluorescence intensity (MFI); the higher the MFI, the higher the expression of eGFP and thus of IL-10. Therefore, both the MFI and the numbers of eGFP-positive cells of a certain type in the secondary lymphoid organs are predictive of the contribution of a cell type to the overall IL-10 secretion.

3.4. The relative MFI (rMFI)

Remarkably, the MFI of the different cell types was not generally increased in the late stages of the infection in the mLN. Late in infection (after week 10) the MFI was lower in CD138^{pos} B cells (B cells with a plasma cell phenotype), NK cells, NKT cells, CD8^{pos} T cells and most interestingly and significantly, also in CD4^{pos} CD25^{pos} T cells, than that before infection and in the early stages of infection. (Fig. 2A). The data in Fig. 2A represent cumulated data, in which the value at each time point after infection was obtained from data in

several individual experiments. Reflective of the natural process of infection, the cell types in each individual experiment differed considerably – in some instances significantly – in the measured MFI (Fig. 2B). Similar data were obtained in experiments with spleen lymphocytes (data not shown). Thus, using only the MFI can produce inconsistent results between experiments and time points of analysis and therefore, each experiment will require an internal standard. Measurement of the MFI can also be influenced by the hardware set-up or type of the cytometer, making comparisons of the contribution of each cell type to IL-10 production between experiments difficult or impossible. We therefore introduced a relative MFI (rMFI). This index uses the MFI of the cell type with the highest MFI within the experiment as a standard, which is set to 100%. The MFIs of the other cell types are hence calculated as a fraction of this value (Supplementary Fig. 4B). Therewith, the values for each cell line become comparable between different time points, experiments or set-up of the cytometer, which makes this tool highly useful for long-term experiments.

3.5. Fraction of lymphocyte subsets that contribute to IL-10 production

To identify the contribution by each cell type to the IL-10 production in the secondary lymphoid organs, it is necessary to identify the contribution of each eGFP^{pos} cell type to all eGFP^{pos} lymphocytes at individual time points. During the infection with *S. mansoni*, CD4^{pos} T cells contributed most to the pool of eGFP-positive cells in the mLN (% GFP^{pos} cells of all analyzed lymphocytes), with an almost equal contribution of CD4^{pos} CD25^{pos} and CD4^{pos} CD25^{neg} cells, respectively (Fig. 3A). In the spleen, the situation is different: the CD4^{pos} CD25^{pos} T cell population was not among the major contributors, B cells, in particular the CD138^{neg} B cell population (B cells without plasma cells characteristics), were important early in the infection, later their contribution decreased and that of CD4^{pos} cells prevailed, both in the CD25^{pos} and in the CD25^{neg} subpopulations, the latter being the most important contributor to the analyzed eGFP^{pos} lymphocytes (Fig. 3B).

Regarding the other parameters, the rMFI, we found, that the population with the highest rMFI throughout the infection was that of CD4^{pos} CD25^{pos} T cells in the mLN and in the spleen. Further, compared to CD4^{pos} CD25^{pos} T cells, the rMFIs of CD4^{pos} CD25^{neg} T cells are significantly higher in the spleen than in the mLN (Supplementary Fig. 5). The rMFI of NKT cells increases in the mLN whereas it decreases in the spleen later in the infection (Fig. 3C, D).

Thus, both the rMFI and the fraction of eGFP^{pos} cells in each lymphocyte subset were independent entities and did not allow identifying the highest contributor to the production of IL-10 per se.

3.6. Absolute number of cells in the secondary lymphoid organs

A last factor contributing significantly to the amount of IL-10 produced during the course of the infection is the absolute number of producing cells. This analysis revealed, that both in the mLN and in the spleen the number of lymphocytes significantly increased after the onset of egg deposition in week 6. Consequently, more lymphocytes

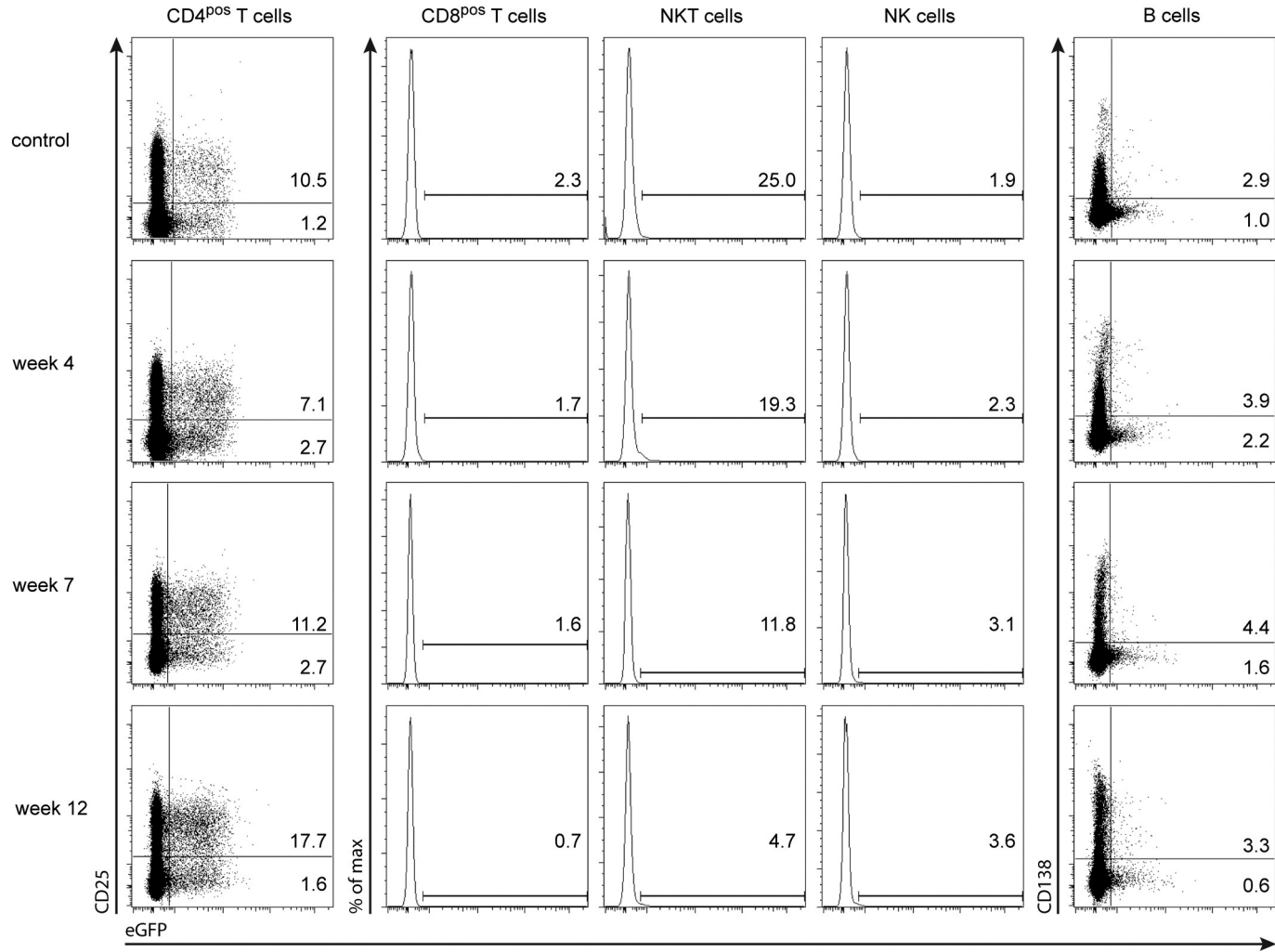


Fig. 1. Analysis of lymphocyte populations expressing IL-10 in the course of the infection with *S. mansoni*. Flow cytometry analysis for eGFP production (IL-10) of indicated lymphocyte populations in the mLN of uninfected control mice and after 4, 7 and 12 weeks post infection with *S. mansoni*. The plots show representative plots from 8, 6, 7 and 6 mice in 4, 3, 4 and 4 experiments, respectively.

produce IL-10 in the late stages of infection compared to the early stage of infection (until week 6; Fig. 3E).

3.7. The TIGER index (TI)

Thus, the ranked identification of the contributors to the production of IL-10 during the infection with *S. mansoni* can be achieved by combining a) the contribution of the individual cell type to all eGFP^{pos} lymphocytes, b) the MFI of each individual cell type compared to all other eGFP^{pos} (and thus IL-10-producing) lymphocytes (rMFI) and c) the absolute number of cell in the secondary lymphoid organ at each individual time point. The combined factors at each time point of the analysis result in a novel index: the TIGER index (TI; Figs. 4 and 5A, B, significances are depicted in Supplementary Fig. 6; an example for the calculation can be found in Supplementary Fig. 4).

Using the tool of TI, we identified the population of CD4^{pos} CD25^{pos} T cells as the highest contributors to the IL-10 production in the mLN, whereas in the spleen the CD4^{pos} CD25^{neg} T cells were the most prominent producers of IL-10. Leaving the absolute cell number out of the equation the contribution of each cell type without the aspect of the time point of infection to the TI is demonstrated (reduced TIGER index; redTI) (Fig. 5C, D). The redTI revealed that the CD4^{pos} CD25^{neg} T cells were constant contributors to the IL-10 production in the mLN, independent from the status of infection, whereas the CD4^{pos} CD25^{pos} T cells increased the production of IL-10 on a per cell basis after the onset of egg deposition in week 6. All other analyzed lymphocytes only marginally contributed to the IL-10 production throughout the infection (Fig. 5C). Remarkably, in the spleen the CD4^{pos} CD25^{neg} T cells increased the IL-10 production on a per cell basis even before the onset of egg deposition, just after the arrival of the adolescent worms in the portal vein system (Fig. 5D). The contribution of the CD4^{pos} CD25^{pos} T cells is not prominent, as it does not significantly increase until week 11 of the infection. Contrasting to their contribution in the mLN, in the spleen CD138^{neg} B cells, NKT cells, CD4^{pos} CD25^{pos} and CD4^{pos} CD25^{neg} T cells are equal contributors (on a per cell basis) also in the non-infected mouse, ranging from 7 to 13%. Their contribution get slightly lower late in infection (Fig. 5D).

Both TIGER indices are prominently visualizing the influence of CD4^{pos} T cells on IL-10 production in the late stages of infection (Fig. 5). In both the mLN and the spleen, the CD4^{pos} CD25^{pos} T cells had indices over a value of 7500, whereas in the spleen the highest contributor, the CD4^{pos} CD25^{neg} T cells, had an index of more than 18,000 (Fig. 5A, B). As for the redTI, this indicator was highest for CD4^{pos} CD25^{pos} T cells in the mLN, whereas in spleen, CD4^{pos} CD25^{neg} T cells scored highest for this index.

4. Discussion

Although previous reports described various cell types that are able to produce IL-10 in response to *S. mansoni* (Hesse et al., 2004; Sadler et al., 2003), the exact contribution of the various lymphoid cells to the production of IL-10 in the secondary lymphoid organs in vivo remained largely unknown. In the present study, we used combined data from flow cytometry analysis and cell count analysis to identify the highest IL-10-producing lymphocytes in the mLN and spleen during an infection with *S. mansoni* and their contribution to the overall IL-10 production. Even though the previous reports took advantage of the factors MFI and the frequency of a given cell type to describe their contribution in the overall cytokine production (Darrah et al., 2007), we here describe a universally applicable way to analyze the contribution of each cell type from a specific in vivo source, which allows the analysis of the contribution of each cell type over an extended period of time in individual experiments without the use of an external standard. The normalization of the MFI at each time point of analysis is necessary to overcome potential differences in the measurement and to allow comparison at each time point of analysis between individual experiments. Thereto, we took advantage of the IL-10 reporter mouse TIGER, which expresses the enhanced green fluorescence protein (eGFP) under the same transcriptional control as IL-10 through the use of an IRES sequence 3' of the IL-10 gene. Because IL-10 and eGFP expression highly correlate in the TIGER mouse line ((Kamanaka et al., 2006) and this paper), it allowed us to use flow-cytometric analysis to improve the detection and description of IL-10-producing cells, in terms of the producing cell type, the amount produced, the location and the time

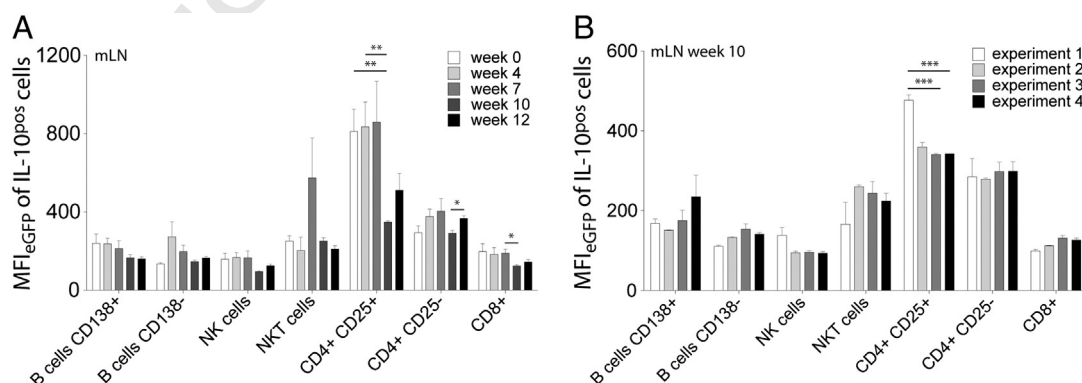


Fig. 2. Comparison of the MFI of IL-10^{pos} lymphocytes in the mLN at different time points and experiments. Mean fluorescence intensity of IL-10^{pos} lymphocyte populations in the mLN (A) after indicated time points post infection with *S. mansoni* and in (B) 4 individual experiments (week 10). Bars represent 5, 8, 6, 7 and 6 (weeks 10, 0, 4, 7, 12 respectively) mice in 2, 4, 3, 4 and 4 experiments, respectively $\pm$ SEM. Significant differences are marked: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

of production. The mean fluorescence intensity for eGFP of individual cells was used as an indicator of the amount of IL-10 produced per cell. The similarity in the half lives of IL-10 and eGFP (less than one week) makes it possible to use the signal for eGFP as a readout for IL-10 production at each time point (each week) (Corish and Tyler-Smith, 1999; Li et al., 2004).

The TIGER index – as presented in this study – allows the ranking of each individual cell type regarding its IL-10 production (redTI) and its contribution to the total amount of IL-10 produced in a lymphoid organ at a certain time point during an infection. The TI can be extended to allow for comparisons between the absolute amounts of IL-10 pro-

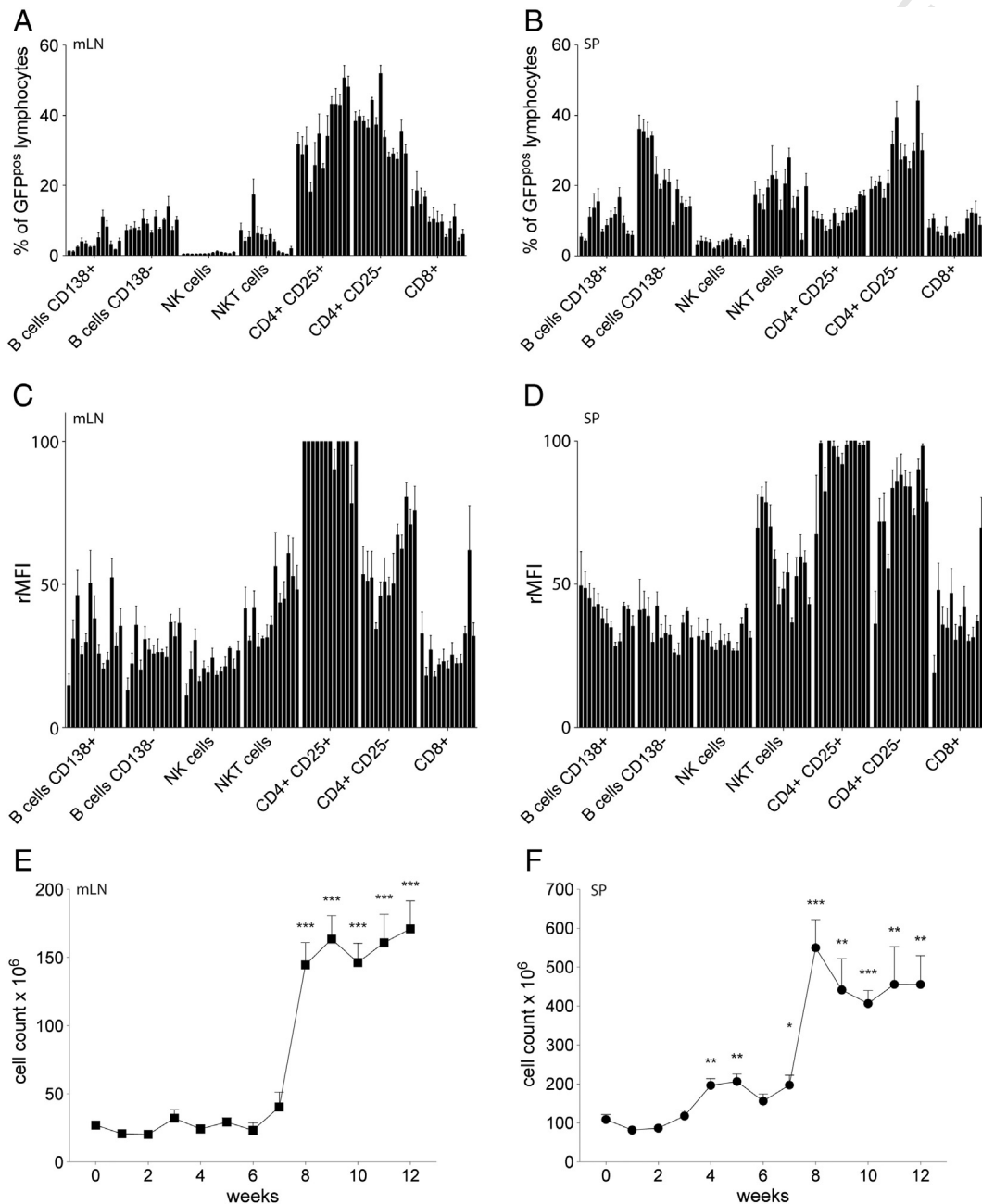


Fig. 3. Analysis of the highest contributing lymphocyte population to all eGFP^{pos} lymphocytes. (A, B) Contribution of indicated cell types to all analyzed eGFP^{pos} lymphocytes in A the mesenteric lymph nodes (mLN) and B in the spleen. (C, D) Ratio of individual cell types to the cell type with the highest mean fluorescence intensity (rMFI) C in the mLN and D the spleen. (E, F) Cell count (lymphocytes) of mLN (E) and spleen (F) at indicated time points after infection. Total numbers of a certain GFP^{pos} cell type can be calculated by multiplication of the individual percentage to the total number of lymphocytes. Data represent cumulative data from 4 to 9 individual mice in 2–4 individual experiments $\pm$ SEM. Significant differences between each time point and control mice (week 0) are marked: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

$$TI_{(x)} = \frac{x_{eGFP^{pos}}}{lym_{eGFP^{pos}}} \cdot \frac{MFI_x}{MFI_{x_{max}}} \cdot N$$

Fig. 4. Formula to calculate the TIGER index. TI: TIGER index; x: individual lymphocyte population; lym: all analyzed lymphocytes; MFI: mean fluorescence intensity; N: cell count in organ.

duced by different cell types in mice in different experiments or at different time points.

With the help of the TI, the importance of T cells as the prominent contributors to the IL-10 production was confirmed. However, not only CD4^{pos} CD25^{pos} T cells contributed: in the spleen CD4^{pos} CD25^{neg} T cells contributed most to the IL-10 production. A similar observation was previously also made in mice infected with *Leishmania major* (Anderson et al., 2007; Hesse et al., 2004). Also in other conditions involving parasites Tregs were not among the most conspicuous cell types producing IL-10, e.g.: during infection with *Toxoplasma gondii* conventional T-bet^{pos} Foxp3^{neg} Th1 cells were shown to be the major source of IL-10 (Jankovic et al., 2007). The previous reports mention follicular B cells (CD19^{pos} CD138^{pos/neg} CD23^{neg} CD43^{pos}) and plasma cells/plasmablasts (Powrie et al., 1993; Roncarolo et al., 1997; Scapini et al., 2011; Sher et al., 1991), plasma cells (CD19^{pos} CD138^{pos}) (Donnelly et al., 1999; Neves et al., 2010) or B10

cells (CD19^{pos} CD5^{pos} CD23^{pos}) (DiLillo et al., 2012; Wilson et al., 2011) as high producers of IL-10 in various sites or disease and infection models, they were only minor contributors to the production of IL-10 (low ranking in the TI) in the spleens and mLN of chronically *S. mansoni*-infected mice.

In the present study we did not offer a complete analysis of all IL-10 producing cells; we have analyzed the prominent lymphocyte subsets in two lymphoid tissues. We cannot exclude that other cell types like DCs, may contribute significantly to the IL-10 production in vivo, because these cells were underrepresented in the way we isolated the lymphocytes. This was further underlined by summing up the percentage of cells we analyzed and compared these numbers to the total GFP^{pos} lymphocytes. Here, we did not find more than approximately 1% of unspecified cells (data not shown). We further did not analyze cells outside of secondary lymphoid organs and therefore have no information about IL-10-producing cells in these sites. However, the

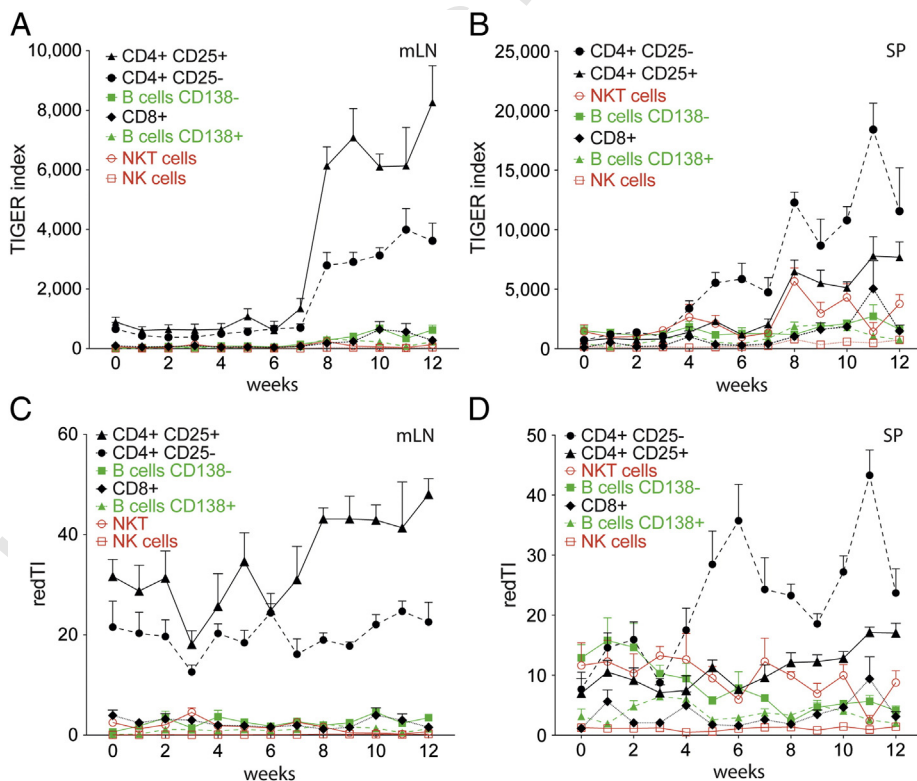


Fig. 5. TIGER indices of individual lymphocytes in the mLN and spleen. Calculated contribution of the main lymphocytes in the mLN (A, C) and spleen (B, D) of control mice (week 0) and at indicated time points after the infection with *S. mansoni*. (A, B) TIGER index representing the relative amount of IL-10 production and the contribution of each cell type. (C, D) TIGER index without the contribution of the lymphocyte number: reduced TIGER index (redTI). Significances are listed in Supplementary Fig. 6. Fig. 5, Supplementary Fig. 6 intended for color reproduction on the Web and in print.

TI represents an important tool to ensure consistency in data analysis throughout a long-term experiment. Also, the use of reporter mice without the necessity of ex vivo stimulation experiments makes the TI highly suitable to image the in vivo situation during the course of the infection with *S. mansoni*.

To our knowledge, we show for the first time that a combination of the values for the MFI (strength of expression), the respective percentage of the expressing lymphocyte population and the overall cell number – the TIGER index – is an elegant tool to visualize the ranking of IL-10 producers in the secondary lymphoid organs without the need of an external standard to compare between different time points and represent most likely the in vivo contribution to immunoregulation by IL-10 of the analyzed lymphocytes in the course of an infection with *S. mansoni*.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2014.03.008>.

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