Lack of IL-4 receptor expression on T helper cells reduces T helper 2 cell polyfunctionality and confers resistance in allergic bronchopulmonary mycosis

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T helper (Th)1 and Th2 cells play decisive roles in the regulation of resistance vs. susceptibility to pulmonary cryptococcosis. To study the function of interleukin (IL)-4 receptor (IL-4R) on Th cells in pulmonary cryptococcosis, we infected mice specifically lacking IL-4Rα on CD4$^+$ T cells (Lck^{Cre}IL-4Rα$^{-/lox}$ mice) and IL-4Rα$^{-/lox}$ controls. Lck^{Cre} IL-4Rα$^{-/lox}$ mice developed enhanced resistance accompanied by reduced pulmonary allergic inflammation and diminished production of the Th2 cytokines IL-4, IL-5, and IL-13 as compared with IL-4Rα$^{-/lox}$ mice. Polyfunctional antigen-specific Th2 cells producing simultaneously two or three Th2 cytokines were reduced in infected Lck^{Cre} IL-4Rα$^{-/lox}$ mice, pointing to a critical role of polyfunctional Th2 cells for disease progression. Reduced Th2 polyfunctionality was associated with fewer pulmonary alternatively activated macrophages. This work is the first direct evidence for a critical contribution of the IL-4R on Th cells to Th2-dependent susceptibility during allergic bronchopulmonary mycosis. Moreover, the data demonstrate that the quality of the Th2 response has an impact on type 2 inflammation. The analysis of polyfunctional Th2 cells may be useful for monitoring the course of the disease.

INTRODUCTION

Cryptococcal infection is a threat for immunocompromised patients, especially in underdeveloped countries.[1,2] The missing or misguided immune response can lead to the dissemination of formerly inhaled cryptococcal yeasts or spores[3] from the lung to other organs, especially the brain.[4,5] Thus, every year, more than half a million HIV-infected people in sub-Saharan Africa succumb to cryptococcal meningitis.[6] In the healthy individual a T helper (Th)1/Th17 response can control the fungal infection,[7–10] but in most cases this immune response does not lead to sterile elimination of the pathogen. The control of cryptococcal growth results in a dormancy state, which is terminated when cryptococci are re-activated by a breakdown of immune defense mechanisms.[11]

In murine pulmonary cryptococcosis, interleukin (IL)-4 is a critical determinant for fatal Th2 cell development.[8,12,13] Very recently we demonstrated that, in addition to Th2 cells, eosinophils are able to produce IL-4 in cryptococcosis, enhancing Th2 cell development.[14] Th2 cells secreting IL-4, IL-13, and IL-5 can orchestrate a pulmonary immune response, which is predominately associated with production of immunoglobulin (Ig)G1 and IgE, and recruitment of eosinophils and basophils to the site of infection,[15] alternative activation of macrophages (aaMphs),[16,17] and goblet cell metaplasia with elevated mucus production.[18] Th2-dependent effector mechanisms are efficient in the control of extracellular pathogens, e.g., helminths, but can be detrimental in the case of (facultative) intracellular organisms, e.g., *Cryptococcus neoformans*.

IL-4R complexes that are ubiquitously expressed and bind to IL-4 and IL-13 are determining factors during Th2 responses.[19] Different IL-4R complexes exist: (i) the type I receptor IL-4R

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(IL-4Rα with common γ chain (γc)), which can only bind to IL-4, and (ii) the type II receptor IL-4R (IL-4Rα with the IL-13Rα1 chain), which can bind to IL-4 and IL-13.[20,21] Signal transduction of the type I and type II IL-4 receptor complexes is mediated via the STAT6 pathway.[22] It is noteworthy that murine T cells only express the IL-4R type I on their surface, allowing them to respond only to IL-4 but not to IL-13.[23]

As we showed in recent studies, IL-4R is a major factor determining the susceptibility in pulmonary cryptococcosis. In the presence of IL-4R, impaired pulmonary cryptococcal growth control, elevated serum IgE levels, enhanced goblet cell mucus production, eosinophil recruitment to the lung, and enhanced dissemination to the central nervous system were observed.[24,25] Interestingly, a gene-dosage effect for IL-4R expression was shown by us to result in gradual Th2-dependent pathology.[25] However, the critical IL-4R-expressing cell type in pulmonary cryptococcosis remained unclear. In the light of the finding that Th cells play an important role in resistance[26] but, on the other hand, can induce pathogenic Th2 responses,[27] it was of interest to study Th cell-specific IL-4Rα-deficient mice on a susceptible BALB/c background. As shown earlier, Th cell-specific IL-4Rα-deficient mice (i.e., $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice) generated by the Cre lox technique[28] lack the IL-4R specifically on Th cells,[29] whereas all other cells including cytotoxic T cells are heterozygous for the IL-4R, and are therefore ideally suited to elucidate the role of IL-4-promoted Th2 cells in susceptibility to pulmonary cryptococcosis. In wild type mice pulmonary cryptococcosis leads to an allergic bronchopulmonary mycosis[27] associated with enhanced airway hyperreactivity.[30] In a rat model of chronic pulmonary cryptococcosis evidence was provided that this infection is even able to favor development of asthma.[31]

To study the potential contribution of Th cell IL-4R expression to susceptibility in pulmonary cryptococcosis, we aimed to characterize both the quantity and quality of Th2 cells in infected $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ vs. $IL\text{-}4R\alpha^{-/lox}$ mice. Therefore, we hypothesized that the quality of Th2 cells relies on the different combinations of the Th2 cytokines IL-4, IL-5, and IL-13 expressed at the single-cell level, as has been described previously for polyfunctional (also termed multifunctional) Th1 cells producing interferon (IFN)-γ, tumor necrosis factor-α, and IL-2.[32] For Th1 cells it has been shown that the frequency of polyfunctional Th cells expressing multiple combinations of IFN-γ, tumor necrosis factor-α, and IL-2 correlates with their potential to protect against murine leishmaniasis.[33] Polyfunctional Th1 cells appeared to secrete higher amounts of IFN-γ and tumor necrosis factor-α on a cellular basis.[33] As of yet similar data for the role of Th2 cell polyfunctionality in an infection model are not available. Therefore, we were interested in studying the role of polyfunctional Th2 cells in murine pulmonary cryptococcosis. The data of the present study (summarized in a simplified scheme in **Figure 8**) demonstrate that (i) polyfunctional antigen-specific Th2 cells are more frequent when the IL-4R is present on Th cells and (ii) polyfunctional Th2 cells are associated with disease progression during cryptococcosis.

RESULTS

Enhanced resistance in murine pulmonary cryptococcosis in the absence of IL-4R on Th cells

IL-4 and IL-13 are factors determining susceptibility in pulmonary cryptococcosis.[13,24,25,27,30] Both, IL-4 and IL-13, are involved in Th2 responses and act by binding to IL-4Rα chain-bearing receptors,[20,21] with IL-4 as the main promoter of Th2 differentiation. Recently, we showed that global $IL\text{-}4R\alpha^{-/-}$ mice have higher survival rates after intranasal (i.n.) infection with *C. neoformans* than either $IL\text{-}4^{-/-}$ or $IL\text{-}13^{-/-}$ mice.[25] Therefore, it was of interest to characterize the IL-4R-expressing cell type determining susceptibility to infection with *C. neoformans*. Based on the central regulatory role of Th cells in cryptococcosis,[26,27] a likely candidate for specific deletion of the IL-4Rα chain were the Th cells. In this context, it is noteworthy that owing to a gene-dosage effect of the IL-4Rα chain even a mild reduction of IL-4R expression as induced by heterozygous expression (i.e. $IL\text{-}4R\alpha^{+/-}$) leads to partial resistance in pulmonary cryptococcosis.[25]

IL-4R heterozygous mice specifically deficient in Th cell expression of the IL-4Rα chain ($Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice) were previously generated and found to completely lack IL-4R expression on $CD4^+$ cells, and hence showed impaired IL-4-induced $CD4^+$ cell proliferation and Th2 differentiation. Other T-cell subpopulations such as $CD8^+$ or NK T cells showed residual expression and non-T cells showed normal IL-4Rα levels.[29] Th2 cells have a significant role in development of susceptibility in pulmonary cryptococcosis and therefore allergic bronchopulmonary cryptococcosis.[16,25,27,34] Thus, we were interested in analyzing $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice in this infection model. $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice were infected i.n. with *C. neoformans* strain 1841 and analyzed for survival and pulmonary cryptococcal growth control. $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice proved to be highly resistant against pulmonary cryptococcal infection, showing a survival rate of 90% (**Figure 1a**). In contrast, littermate control mice (i.e., $IL\text{-}4R\alpha^{-/lox}$ mice) had a significantly lower resistance to *C. neoformans* infection (survival rate 61% at 150 d.p.i., see **Figure 1a**). As mentioned above, intermediate levels of IL-4R expression found in $IL\text{-}4R\alpha^{+/-}$ mice translate into gradual gain of resistance in pulmonary cryptococcosis.[25] This explains why the $IL\text{-}4R\alpha^{-/lox}$ littermate control mice present with a basal resistance here (see **Figure 1a**). It is noteworthy that in spite of the relatively resistant heterozygous $IL\text{-}4R\alpha^{+/-}$ background, a significant contribution of Th cell IL-4Rα expression to susceptibility is evident.

Consistent with enhanced survival in $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice, fungal organ burden in the lung at 70 d.p.i. was significantly lower than in $IL\text{-}4R\alpha^{-/lox}$ mice (**Figure 1b**) and as low as in global $IL\text{-}4R\alpha^{-/-}$ mice (data not shown). This strikingly demonstrates that IL-4R expression on Th cells is sufficient to counteract fungal growth control.

Reduced Th2-associated pulmonary allergic inflammatory response in the absence of IL-4R on Th cells

In order to unravel the mechanism of the enhanced resistance observed in $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice, we first analyzed the

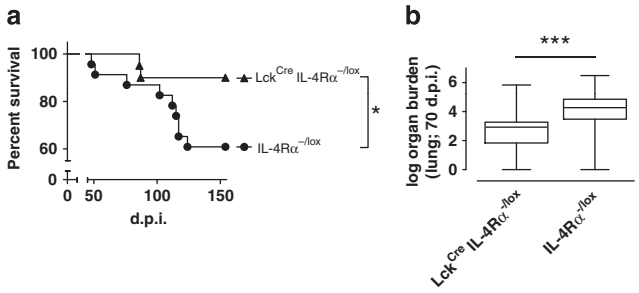


Figure 1 Enhanced survival rate and lower lung burden of mice with T helper (Th)-specific interleukin (IL)-4R deficiency in contrast to littermate controls. Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice and their littermate controls (IL-4R$\alpha^{-/lox}$) were compared for survival rate (**a**) and lung burden (**b**) in pulmonary cryptococcosis on day 70 p.i. (**a**) To examine survival rate infected mice were checked daily for signs of morbidity until 150 dpi. The data for organ burden of the lung (70 d.p.i.) were pooled from eight independent experiments and the survival analysis from four independent studies. (survival: in total 20–23 mice per genotype were used; organ burden: in total 30–36 mice were studied per genotype) The significances were determined by using log-rank test for survival and Mann-Whitney test for organ burden; $^{*}P<0.05$; $^{***}P<0.001$.

hallmarks of the pulmonary inflammatory response typically associated with IL-4R-dependent Th2-mediated allergic inflammation, i.e., eosinophil recruitment and goblet cell mucus production.[25] Pulmonary eosinophils characterized by SSC^{high} and $CD11c^{-/dim}SiglecF^{+}$ surface expression were significantly reduced in the absence of IL-4R on Th cells (**Figure 2a,c,e**). The frequency of alveolar macrophages and pulmonary dendritic cells did not differ between infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice (data not shown). However, in contrast to Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice with only single eosinophils, IL-4R$\alpha^{-/lox}$ mice showed small clusters of eosinophils in their lungs (**Figure 2d,f**). Similar to the difference in eosinophil numbers, goblet cell mucus production was significantly reduced in the absence of expression of IL-4Rα on Th cell (**Figure 2b**). Ample mucus production in the epithelial lining of small bronchi could be detected in the lung sections of infected IL-4R$\alpha^{-/lox}$ mice stained with Periodic acid Schiff (PAS), whereas PAS^{+} goblet cells were rarely found in the lungs of infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice (**Figure 2g,h**). Taken together, the hallmarks of allergic inflammation such as eosinophilic infiltration and mucus production typically found in mice susceptible to pulmonary cryptococcosis are significantly reduced in the absence of IL-4R expression on Th cells.

Reduced but not absent Th2-associated pulmonary cytokine production of Th cells in the absence of IL-4R on Th cells

As eosinophil recruitment and goblet cell mucus production rely on the Th2 cytokines IL-5 and IL-13,[35,36] respectively, and both parameters were reduced in infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice, the IL-4R expressed by Th cells appears to regulate Th2 cytokine production responsible for allergic pulmonary inflammation in cryptococcosis. To characterize the Th cytokine profile in the presence or absence of IL-4R expression, pulmonary leukocytes from mice infected for 70 days were isolated and their immune response was measured after *ex vivo* re-stimulation with cryptococcal antigen. It is noteworthy that the absolute numbers of pulmonary leukocytes were comparable between Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice (mean and s.e.m.: $7.48\times10^{6}\pm1.14\times10^{6}$ vs. $7.54\times10^{6}\pm1.11\times10^{6}$). The secretion of cytokines by lung leukocytes was analyzed by sandwich ELISA (**Figure 3a**) and a clear shift towards a Th2 phenotype in the more susceptible littermate controls (i.e., IL-4R$\alpha^{-/lox}$ mice) could be observed. In contrast, the production of Th2 cytokines such as IL-4, IL-5, or IL-13 was diminished in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice. Moreover, differences in the secretion of IL-10, a regulator of the Th1 response, could be seen with reduced levels of IL-10 produced in the absence of IL-4Rα expression on Th cells (**Figure 3b**), although the observed reduction was not significant. Interestingly, *ex vivo* production of the Th1 cytokine IFN-γ did not differ between infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice, suggesting that the reduced levels of Th2 cytokines may better reflect the course of the disease.

To analyze more directly *in vivo* Th2 development in the absence of IL-4R expression on Th cells, we took advantage of a surface marker characteristically expressed on Th2 cells, i.e., the IL-33 receptor (also termed T1/ST2).[37,38] Consistent with the reduced Th2 cytokine production found in pulmonary leukocytes of infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice (**Figure 3a**), the proportion of $IL\text{-}33R^{+}$ Th cells from freshly isolated pulmonary leukocytes was reduced in mice lacking the IL-4R on Th cells (**Figure 3c**).

Reduced antigen-specific Th2 cells in the absence of IL-4R on Th cells

Quantitative analysis of the Th2 profile shown in **Figure 3** clearly demonstrates residual IL-4R-independent Th2 responses. It has been described earlier that Th2 cells *in vivo* can develop in an IL-4R-dependent and IL-4R-independent manner.[39–41] To specifically distinguish the quality of the cytokine profile from either Th2 subset in infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice, we applied multiparameter flow cytometry for cytokine determination at the single-cell level.[32] Following *ex vivo* re-stimulation of isolated pulmonary leukocytes with cryptococcal antigen, $CD4^{+}$ T lymphocytes were analyzed for intracellular cytokine production by multicolor flow cytometry. Consistent with the ELISA data shown in **Figure 3a**, the proportions especially of $IL\text{-}4^{+}$ Th cells and $IL\text{-}5^{+}$ Th cells were reduced in Lck^{Cre}IL-4R$\alpha^{-/lox}$ vs. littermate control mice (**Figure 4a**). Interestingly, the proportion of $IFN\text{-}\gamma^{+}$ Th cells was clearly higher than that of any of the Th2 cytokine-producing Th cells. Additionally, in contrast to the IFN-γ level found in the supernatant of pulmonary leukocytes by ELISA (see **Figure 3a**), the proportion of $IFN\text{-}\gamma^{+}$ Th cells was higher in the absence of IL-4R on Th cells, pointing to the presence of low-level IFN-γ producers in pulmonary leukocytes of infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice (median fluorescence intensity (MFI) for antigen-specific Th cells from Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice: 8,827 vs. IL-4R$\alpha^{-/lox}$ mice: 12,023).

Next, we wished to study selectively the cytokine pattern of antigen-specific Th cells. To this end, we took advantage of CD40L (i.e., CD154) expression in Th cells. Expression of

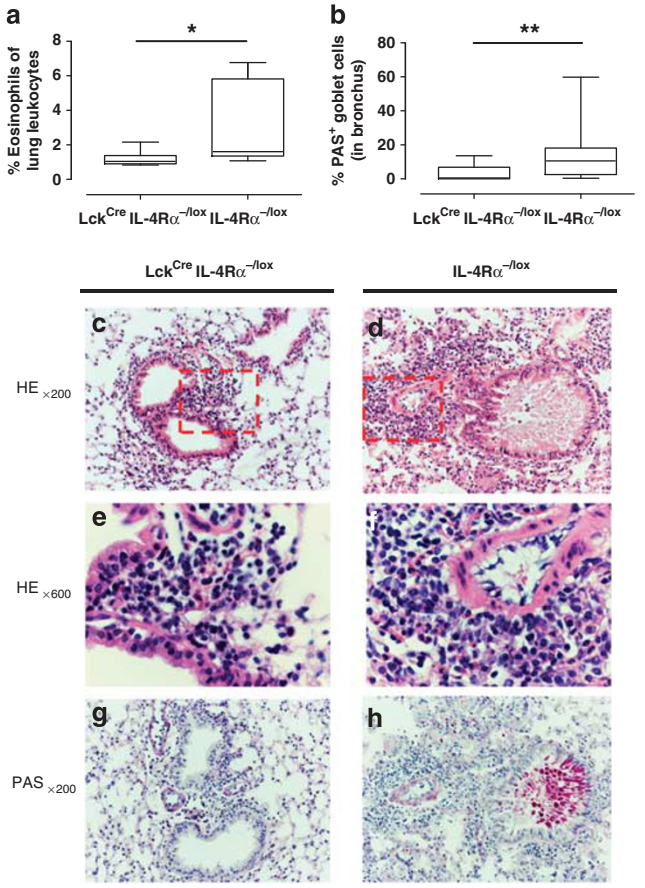


Figure 2 Diminished eosinophil recruitment and goblet cell mucus production in mice with T-helper (Th)-specific IL-4R deficiency in contrast to littermate controls. Comparison of eosinophil proportions and mucus production in the lung of infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice and littermate controls (IL-4R$\alpha^{-/lox}$) on day 70 p.i. (**a**) Fluorescence-activated cell sorting (FACS) analysis of surface markers on lung cells: the proportion of eosinophils was detected by gating for Siglec-$F^+CD11c^{-/dim}$ cells. (**b**) Mucus-producing cells (PAS^+) were determined by counting PAS^+ and PAS^- bronchial epithelial cells in 10 bronchi per mouse (with 18–22 mice per group). (**c**–**h**) Histopathology of the lung revealed similar findings by H&E staining for the recruitment of eosinophils (**c**–**f**) and by PAS staining for mucus production by goblet cells (**g**, **h**). Panels **e** and **f** are derived from marked areas (dashed box) in panels **c** and **d**. Single eosinophils in the lung of infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice are depicted, whereas a cluster of eosinophils is shown in littermate controls (IL-4R$\alpha^{-/lox}$). Two independent experiments were pooled for the analysis. The significances were determined by using Mann-Whitney test; $^*P<0.05$; $^{**}P<0.01$. H&E, hematoxylin & eosin; IL, interleukin; Lck^{Cre}IL-4R$\alpha^{-/lox}$, lacking IL-4Rα on $CD4^+$ T cells; IL-4R$\alpha^{-/lox}$, littermate controls; PAS, periodic acid Schiff.

CD154 enables one to discriminate between antigen-specific Th cells and bystander cells because it has been shown that CD154 is selectively expressed in Th cells activated in an antigen-specific manner.[42,43] Thus, *ex vivo* re-stimulation of isolated pulmonary leukocytes with cryptococcal antigen was done and intracellular cytokine expression was selectively analyzed in $CD4^+CD154^+$ lung lymphocytes. In pulmonary leukocytes of infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice, we found similar proportions of antigen-specific Th cells (i.e., $CD4^+$ $CD154^+$ cells) (**Figure 4b**) with equal absolute numbers of lung Th cells (median 4.3×10^5 Th cells/lung for Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice). When we then compared the cytokine profiles of only these antigen-specific Th cell population, it was obvious that the more resistant Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice show reduced proportions of antigen-specific Th2 cells producing IL-4, IL-5, or IL-13. On the other hand, an elevated proportion of Th1 cells producing IFN-γ was observed in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice (**Figure 4c**). In the more susceptible littermate control mice with IL-4R expression on Th cells, a more profound portion of Th2 cytokine-producing Th2 cells was found (**Figure 4c**). This indicates that the IL-4R-dependent Th2 subset is critical for the phenotypic difference between infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice. We also examined the involvement of Th bystander cells (i.e., $CD154^-CD4^+$) and non-Th bystander cells (i.e., $CD154^-CD4^-$) in especially Th2 cytokine production, but these proportions were negligible (data not shown).

Although there are significant limitations in acquiring sufficient numbers of antigen-specific Th2 cells for multiparameter flow cytometry analysis, we believe that the data derived from cells of 3–4 pooled mice are meaningful. By using PMA/ionomycin for *ex vivo* re-stimulation instead of cryptococcal antigen, we gained a considerably higher rate of activated Th cells with an elevated higher frequency of cytokine producers but essentially similar differences between infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice as with antigen (data not shown). In contrast to stimulation with cryptococcal antigen or PMA/ionomycin, pulmonary leukocytes incubated for the same time period in medium showed a similar pattern with only lower cytokine levels (data not shown).

Reduced induction of polyfunctional Th2 cells in the absence of IL-4R on Th cells

To further characterize differences in the Th2 cytokine profile between infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice, we analyzed the pattern of Th2 cytokines expressed at a single-cell level. To this end, we subdivided the antigen-specific Th cells by analyzing double (**Figure 5a**) and triple cytokine-producing cells (**Figure 5b**). Strong mucus production by pulmonary goblet cells contributing to elevated airway hyperreactivity has been demonstrated earlier to be IL-13-dependent.[30] It was now possible to link the aforementioned differences in pulmonary allergic inflammation to *Cryptococcus*-specific Th cells that express IL-13 together with other Th2 cytokines or together with the Th1 cytokine IFN-γ. Analysis of both bi- and tri-functional Th cells reveals that infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice differ most in the proportion of polyfunctional antigen-specific Th cells secreting IL-13 together with IL-4, IL-5, or even IFN-γ (**Figure 5**). This is intriguing in light of the lung pathology found in infected IL-4R$\alpha^{-/lox}$ mice (see **Figure 2b**). Even the Th1 and Th2 cytokine combinations (e.g., IL-4^+/IFN-γ^+, IL-5^+/IFN-γ^+) could be detected (**Figure 5a,b**)), extending the conventional view on separate Th1 or Th2 cytokine profiles. Interestingly, IL-4^+/IL-5^+/IL-13^+ $CD154^+$ Th cells were almost exclusively detectable in the presence of IL-4R on Th cells (0.001% in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice vs. 0.187% in IL-4R$\alpha^{-/lox}$ mice containing *all* antigen-specific Th cells independent of their cytokine profile), while IL-4^+ IL-13^+IFN-γ^+ cells were comparable between the two groups.

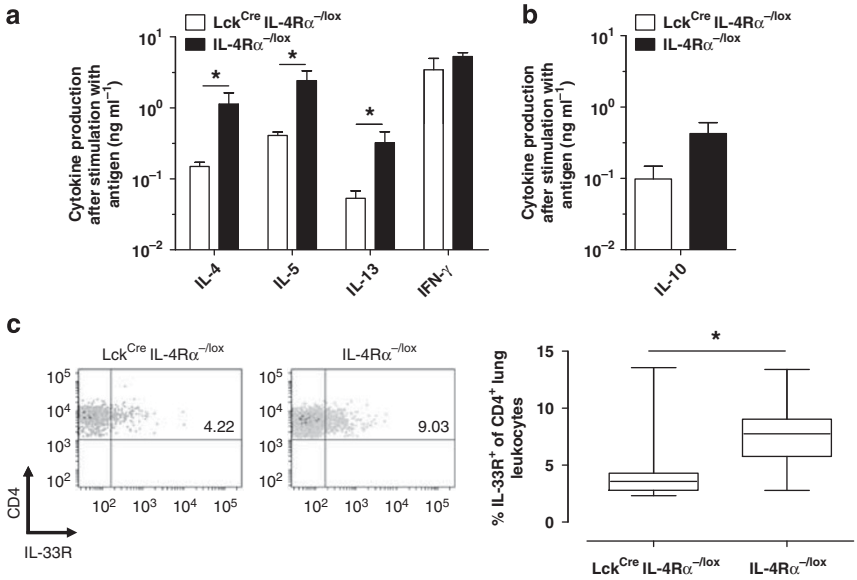


Figure 3 Reduced T helper (Th)2 cytokine production and reduced Th2 cell proportion in mice with Th-specific IL-4R deficiency in contrast to littermate controls. Comparison of the cytokine profile after re-stimulation of purified lung leukocytes with cryptococcal antigen. Cytokine production and Th2 cell proportions of Lck^{Cre}IL-4Rα$^{-/lox}$ mice and littermate controls (IL-4Rα$^{-/lox}$) were analyzed (70 d.p.i.). (**a**, **b**) Pulmonary leukocytes were re-stimulated for 22 h with cryptococcal antigen and the secreted cytokines were determined in the supernatant by sandwich ELISA. (**c**) The proportions of Th2 cells in the lung of infected mice (right panel) were analyzed by studying freshly isolated pulmonary leukocytes by flow cytometry using CD4 and IL-33R as markers (left panel: representative fluorescence-activated cell sorting (FACS) plots gated for living CD4$^+$ cells, the upper right quadrant contains the IL-33R$^+$CD4$^+$ cells). Pooled data from two independent experiments are shown (see the Methods section for a detailed description). The significances were determined by using Mann-Whitney test; $^*P<0.05$. IL, interleukin; Lck^{Cre}IL-4Rα$^{-/lox}$, lacking IL-4Rα on CD4$^+$ T cells; IL-4Rα$^{-/lox}$, littermate controls; Th, T helper.

To further extend the comparison of the cytokine profiles between Lck^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice, we analyzed the proportion of mono-, bi-, and tri-functional Th2 cells that produce IL-4, IL-5, and/or IL-13 (**Figure 6a**). The fraction of antigen-specific Th2 cytokine-producing cells was lower in Lck^{Cre}IL-4Rα$^{-/lox}$ mice, with only 4.20% of CD4$^+$CD154$^+$ cells, whereas in the littermate control an average proportion of 10.24% was found by applying Boolean gating as described in the Methods section. This is consistent with the data depicted in **Figure 4c** and fits nicely to the differences in secreted protein detected in the supernatant of antigen-restimulated lung leukocytes (**Figure 3a**). In addition, in Lck^{Cre}IL-4Rα$^{-/lox}$ mice 75.4% of the antigen-specific Th2 cells (i.e.,producing IL-4, IL-5, or IL-13) were mono-functional, 23.8% bi-functional, and only 0.8% tri-functional, but in IL-4Rα$^{-/lox}$ mice only 68.1% were mono-functional, 29.7% bi-functional, and more than 2.2% tri-functional (i.e., producing IL-4, IL-5, and IL-13) (**Figure 6a**). By analysing the MFI for these cytokines by flow cytometry, we were able to assess quantitatively the relative strength of Th2 cytokine production on a per cell basis. It has been shown that integrated MFIs as a measure of cytokine MFI multiplied by the frequency of this cytokine-expressing Th subset can be a simple predictive parameter for the course of the disease.[32,33] Indeed, when we calculated integrated MFIs by considering the percentage of antigen-specific Th cells producing IL-4, IL-5, or IL-13, we found marked differences between infected Lck^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice (**Figure 6b–d**). The largest difference was observed for IL-13 (**Figure 6d**). As Lck^{Cre}IL-4Rα$^{-/lox}$ mice almost completely lack tri-functional Th2 cells, determination of the integrated MFIs for IL-4, IL-5, and IL-13 was not possible in these mice.

These findings emphasize the role of polyfunctional Th2 cells in *Cryptococcus*-induced pathology (summarized in **Figure 8**). Taken together, a quantitative and qualitative comparison of antigen-specific Th2 cells from *Cryptococcus*-infected Lck^{Cre}IL-4Rα$^{-/lox}$ vs. IL-4Rα$^{-/lox}$ mice reveals two different Th2 subpopulations: IL-4R-independent as well as IL-4R-dependent Th2 subsets. IL-4R-dependent Th2 cells display a higher degree of polyfunctionality and a higher per cell production of individual Th2 cytokines than IL-4R-independent Th2 cells.

Reduced aaMph in the absence of IL-4R on Th cells

Considerable reduction without complete abrogation of Th2 development in the absence of IL-4R on Th cells led us to ask whether effector mechanisms initiated by Th2 development could be critical for the observed disease phenotype. A major effector mechanism for control of *C. neoformans* is the killing ability of macrophages that harbor cryptococci.[44] The activation status of pulmonary macrophages is crucial for the outcome of pulmonary cryptococcosis. Classically activated macrophages are a hallmark in the control of cryptococcal infection and survival of mice, whereas aaMph are strongly correlated with the susceptibility of infected mice.[16,30] Very recently, even intermediate classical/alternative activation states of single macrophages were described and associated with steady-stage cryptococcal infection.[45] To study the status

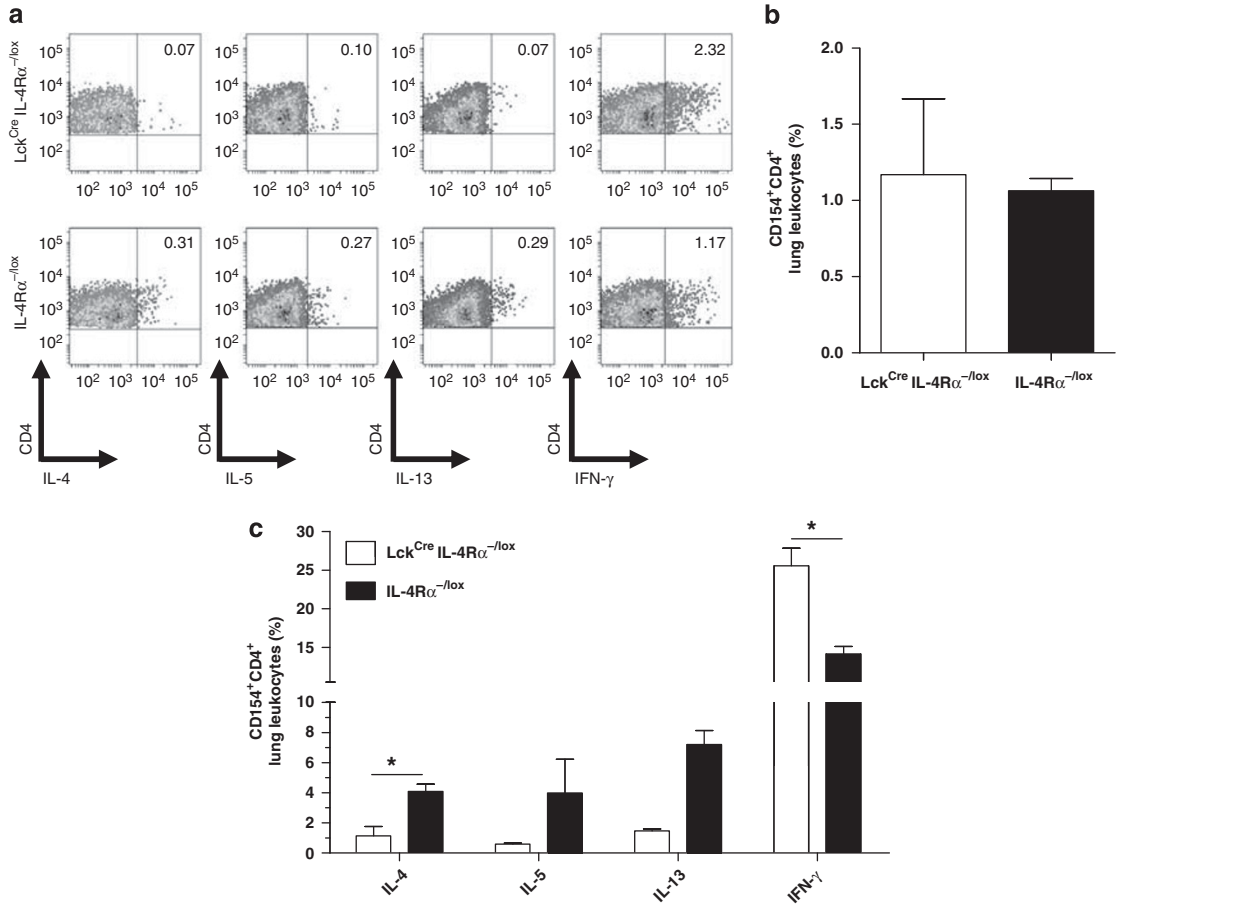


Figure 4 Diminished proportions of T helper (Th)2 cells in mice with Th-specific IL-4R deficiency in contrast to littermate controls. Comparison of total ($CD4^+$) and antigen-specific Th cell proportions ($CD4^+CD154^+$) and their cytokine profile in $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice vs. littermate controls ($IL\text{-}4R\alpha^{-/lox}$) (70 d.p.i.). (**a**) Using multiparameter flow cytometry for detection of intracellular cytokine staining, the proportions of Th cells producing interferon (IFN)-γ, interleukin (IL)-4, IL-5, or IL-13 were determined after re-stimulation of isolated pulmonary leukocytes with cryptococcal antigen in two independent experiments (representative fluorescence-activated cell sorting (FACS) plots gated for living antigen-stimulated $CD4^+$ cells, depicted are the proportions of cytokine-positive $CD4^+$ cells). (**b**) The proportion of antigen-specific (i.e., $CD154^+$) Th cells was analyzed by flow cytometry. (**c**) Using an intracellular cytokine staining assay, the proportions of Th cells producing IFN-γ, IL-4, IL-5, or IL-13, after stimulation with cryptococcal antigen in two independent experiments was analyzed. Pooled data are shown (see the Methods section for a detailed description). The significances were determined by using Mann-Whitney test; $^*P<0.05$. $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$, lacking IL-4Rα on $CD4^+$ T cells. $IL\text{-}4R\alpha^{-/lox}$, littermate controls.

of macrophage activation in the presence or absence of IL-4R on Th cells, lungs were analyzed by immunohistochemistry for expression of arginase-1, chitinase 3-like 3 (YM1), and macrophage mannose receptor (CD206).[30] Lungs of resistant $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice were found to harbor only a few scattered small macrophages, which expressed markers of alternative activation such as arginase-1 (**Figure 7a**), YM1 (**Figure 7b**), and CD206 (**Figure 7c**). The remaining $CD11b^+$ alveolar macrophages that were stained in the pulmonary parenchyma did not show significant expression of the aforementioned markers. In lungs of susceptible $IL\text{-}4R\alpha^{-/lox}$ mice, however, most $CD11b^+$ macrophages expressed markers of alternative activation (**Figure 7d–f**). In addition, these macrophages were large, appeared foamy (see arrows in **Figure 7a–f**), and showed a variable number of ingested fungi or fungal remnants. The frequency of alveolar macrophages, as mentioned above, did not differ between both genotypes. Even macrophages from susceptible $IL\text{-}4R\alpha^{-/lox}$ mice were found to express iNOS similar to the more resistant $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice (data not shown) reflecting similar levels of IFN-γ found in both groups (**Figure 3a**). This implies that the observed difference in the aaMphs is based on pronounced Th2 cytokine production in $IL\text{-}4R\alpha^{-/lox}$ mice vs. $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$, extending previous reports at the level of macrophage activation.[12,30] Therefore, in pulmonary cryptococcosis IL-4R expression on Th cells is required for aaMphs (**Figure 7g–i**). This indicates that IL-4 and/or IL-13 produced by $IL\text{-}4R^+$ Th2 cells are essential for induction of aaMph. Moreover, low-level Th2 cytokine production by IL-4R-deficient mono- and bi-functional Th2 cells allows only for a low level of aaMph (**Figure 7g–i**).

DISCUSSION

The IL-4R is a central regulator in immunity to *C. neoformans*.[25] We and others have previously identified a gene-dosage

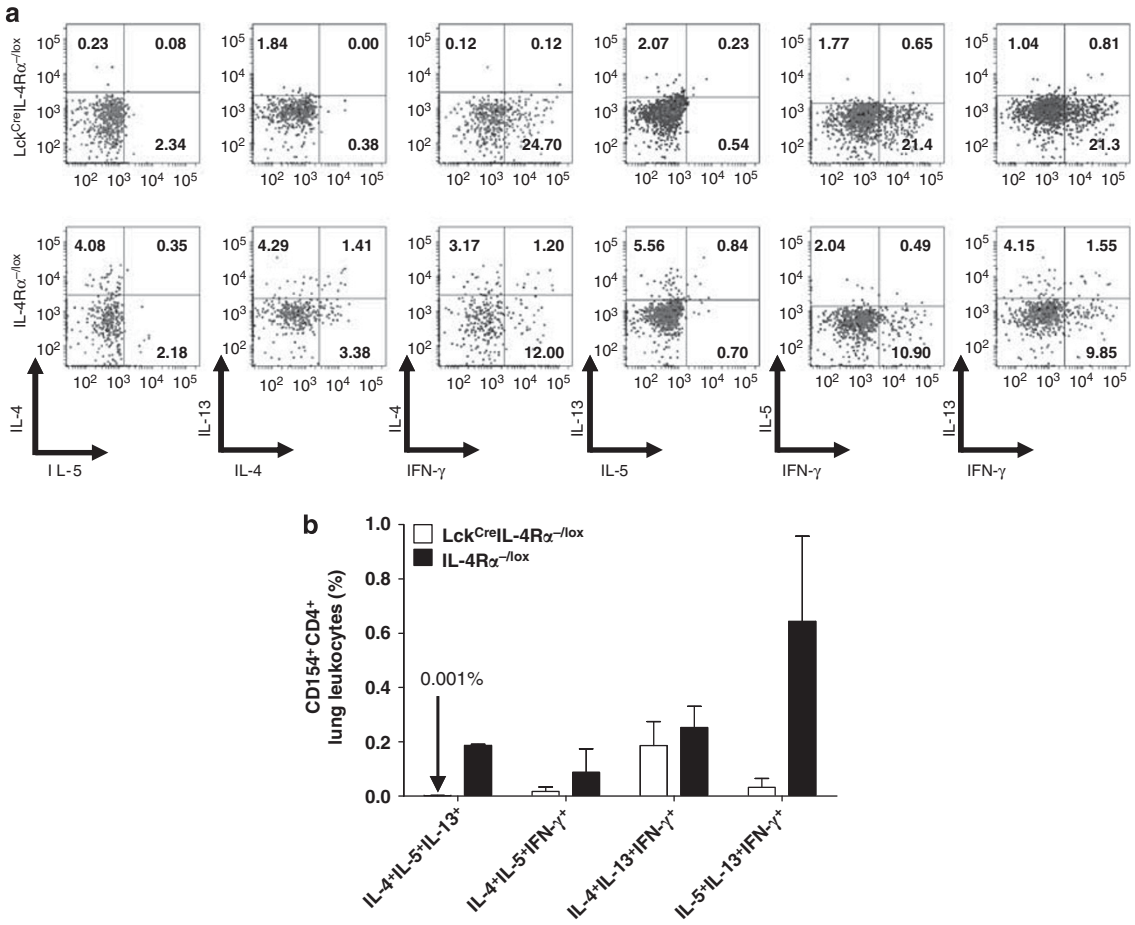


Figure 5 Reduced polyfunctional antigen-specific Th2 cell response in mice with T helper (Th)-specific interleukin (IL)-4R deficiency in contrast to littermate controls. Comparison of antigen-specific Th cell proportions ($CD4^+CD154^+$) and their polyfunctional cytokine profile ($Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice vs. littermate controls ($IL\text{-}4R\alpha^{-/lox}$)) (70 d.p.i.). (**a**) Representative fluorescence-activated cell sorting (FACS) plots gated for living $CD154^+CD4^+$ Th cells are shown. Proportions of (**b**) tri-functional $CD154^+CD4^+$ Th cells were analyzed after re-stimulation of isolated pulmonary leukocytes with cryptococcal antigen. Pooled data are shown (see Methods for a detailed description). $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$, lacking IL-4Rα on $CD4^+$ T cells; $IL\text{-}4R\alpha^{-/lox}$, littermate controls.

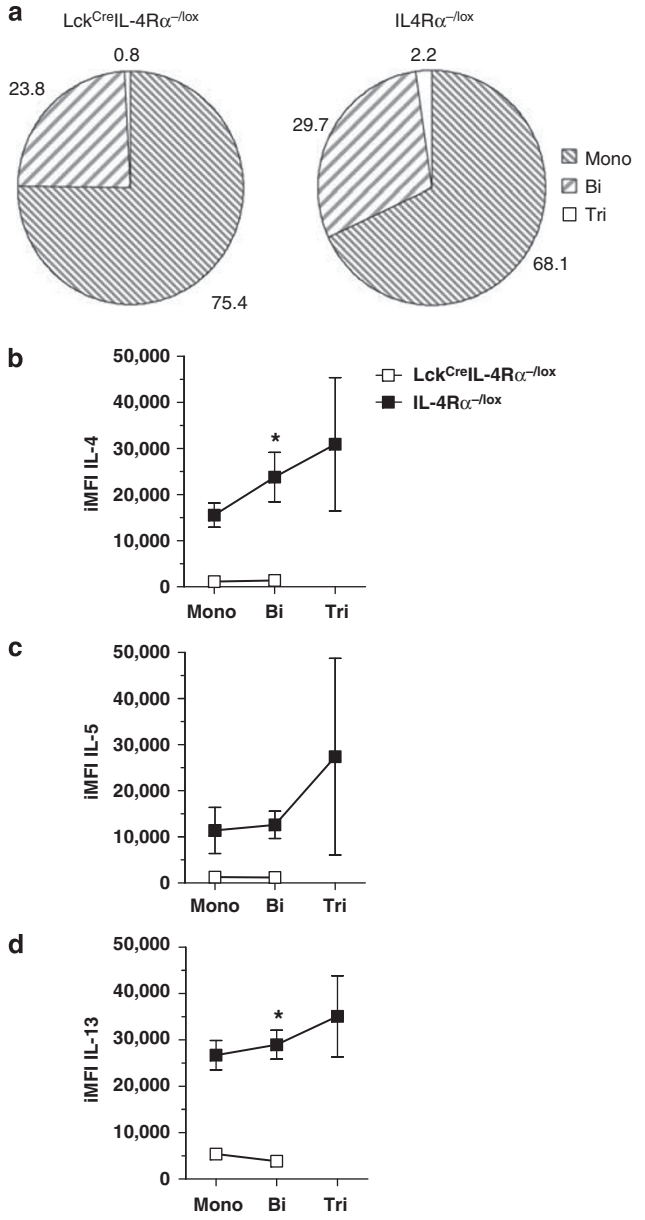
effect for expression of IL-4Rα.[25,46] Interestingly, gradual expression of IL-4Rα resulted in gradual susceptibility to infection with *C. neoformans*.[25] This argues for a sensitive low-level interaction of IL-4/IL-13 with IL-4R in our model of pulmonary cryptococcosis.[25] To deepen the functional analysis of the IL-4R, we now undertook a further study to define the cell type responsible for IL-4R-dependent susceptibility. As type 1 IL-4R is expressed ubiquitously[19] and Th cells are central regulators in immunity to *C. neoformans*,[26] we chose to analyze mice lacking the IL-4R on Th cells. Our study in a murine model of pulmonary cryptococcosis reveals that the IL-4R on Th cells (i) is able to mediate susceptibility by allowing for pulmonary allergic inflammation and for aaMphs, and (ii) is capable of upregulating Th2 development, leading to a pronounced Th2 cytokine profile, especially by the differentiation of antigen-specific Th cells to polyfunctional Th2 cells (**Figure 8**). It was intriguing to observe that the lack of IL-4R on Th cells resulted in a significant increase of resistance against infection with *C. neoformans* despite a residual Th2 response. It is noteworthy that even in the absence of IL-4R on Th cells Th2 cells can develop. However, already the reduction (but not complete loss) in the frequency of antigen-specific Th2 cells together with the altered quality of the Th2 cytokine profile occurring in the absence of IL-4R on Th cells is sufficient to significantly impact Th2-dependent disease development (**Figure 1**) and Th2-dependent allergic inflammation (**Figure 2**).

Murine T cells do not respond to IL-13 but to IL-4,[23] because they only express the type 1 IL-4R. Thus, our data with IL-4Rα-deficient Th cells shed light on the effect of IL-4 on Th cells, and reveal an IL-4/IL-4R-independent pathway to induce antigen-specific Th2 cells *in vivo*. However, it is noteworthy that there is a difference in the quantity and quality of Th2 induction/maintenance in the presence vs. absence of IL-4R on Th cells. This difference apparently has major functional consequences for the outcome of pulmonary cryptococcosis. IL-4R-independent Th2 development has been shown previously *in vivo* but not *in vitro*.[40,41] Remarkably, certain Th2-dependent effector functions (e.g., production of IgE by B cells, eosinophil recruitment, and goblet cell

activation for mucus production) depend on IL-4/IL-4R-induced Th2 generation providing sufficient and longlasting levels of IL-4, IL-5, and IL-13.[47] There are various reports describing IL-4/IL-4R/STAT6-independent Th2 induction *in vivo* by alternative mechanisms including Notch Delta/Jagged, IL-25, IL-33, and TSLP.[48]

In our study, we not only analyzed the amount of secreted Th2 cytokines in *ex vivo* antigen-stimulated cultures of isolated pulmonary leukocytes, but also characterized the proportions of (i) antigen-specific Th cells, and (ii) mono-functional and polyfunctional antigen-specific Th2 cells producing one, two, or three Th2 cytokine(s). Using multiparameter flow cytometry, we were able to combine the analysis of quantitative and qualitative aspects of novel Th2 subpopulations. In vaccination and infection studies, it has been shown that polyfunctional Th1 cells provide a better correlate of protection than mono-functional Th1 cells.[32,33] In these models higher per cell cytokine production (especially of IFN-γ) appears to be a characteristic of polyfunctional T cells. In this context, it is interesting that the tri-functional IL-4/IL-5/IL-13-producing Th2 cells that we found in the lung of mice infected with *C. neoformans* are almost exclusively present in mice expressing the IL-4R on Th cells (**Figures 5b** and **6a**). Such IL-4/IL-4R-dependent polyfuntional Th2 cells may have a central role in immunopathology underlying susceptibility to *C. neoformans*. Presently, we can only provide indirect evidence for polyfunctional Th2 cells conferring pathology as live sorting of polyfunctional Th2 cytokine producers is technically not feasible, making transfer studies with sorted mono-, bi- or tri-functional Th2 impossible. Potentially the quantity of IL-4-dependent polyfunctional Th2 cells can be utilized as diagnostic or prognostic parameters in allergic bronchopulmonary mycosis. Moreover, investigation of polyfunctional Th2 cells in humans is needed.

The analysis of Th cell-specific Lck^{Cre}IL-4Rα$^{-/lox}$ mice infected with *C. neoformans* provides evidence for the IL-4R-dependent Th2 cells being the essential regulators of aaMph. We have previously shown that aaMph in pulmonary and cerebral cryptococcosis is IL-4/IL-13-dependent and associated with mortality.[24,30] Our present data point to a close link between IL-4R-dependent Th2 cells and macrophages. A critical threshold level of IL-4/IL-13 production by IL-4R-expressing Th2 cells may be required to induce aaMphs. This may be especially relevant in our model of pulmonary cryptococcosis, where no IL-4R upregulation can be found in response to infection.[25] Then IL-4/IL-13 becomes limiting and reduced expression of IL-4R in heterozygous (i.e., IL-4Rα$^{+/-}$) mice translates into reduced aaMphs allowing for elevated resistance.[25]



METHODS

Mice. Female C.Cg-Il4ra^{tm1Fbb}/Il4ratm2Fbb-Tg(Lck-Cre)/J mice[29] and their non-Cre-transgenic IL-4Rα$^{-/lox}$ littermates on a BALB/c

Figure 6 Reduced levels of bi- and tri-functional T helper (Th)2 cells associated with reduced Th2 cytokine iMFIs in mice with Th-specific interleukin (IL)-4R deficiency in contrast to littermate controls. A comparison of mono-, bi-, and tri-functional antigen-specific CD4$^+$CD154$^+$ Th2 cell proportions (i.e., producing IL-4, IL-5, or IL-13) of Lck^{Cre}IL-4Rα$^{-/lox}$ mice vs. littermate controls (IL-4Rα$^{-/lox}$) on day 70 p.i. was done following re-stimulation of isolated pulmonary leukocytes with cryptococcal antigen. The gating strategy is described in Materials and Methods in detail. Mono-functional: production of IL-4, IL-5, or IL-13; bi-functional: production of IL4/IL-5, IL-4/IL-13, or IL-5/IL-13; tri-functional: production of IL-4/IL-5/IL-13. (**a**) Average proportions of mono-, bi-, and tri-functional CD4$^+$CD154$^+$ lung leukocytes in Lck^{Cre}IL-4Rα$^{-/lox}$ vs. IL-4Rα$^{-/lox}$ mice. (**b**–**d**) The integrated median fluorescence intensities (iMFI) of mono-, bi-, and tri-functional antigen-specific Th cells for IL-4, IL-5, and IL-13 are shown. In Lck^{Cre}IL-4Rα$^{-/lox}$ mice tri-functional Th2 cells were almost absent; therefore no iMFIs could be determined. Pooled data from two independent experiments are shown (see Methods for a detailed description). The significances were determined by using Mann-Whitney test; *$P < 0.05$. Lck^{Cre}IL-4Rα$^{-/lox}$, lacking IL-4Rα on CD4$^+$ T cells; IL-4Rα$^{-/lox}$, littermate controls.

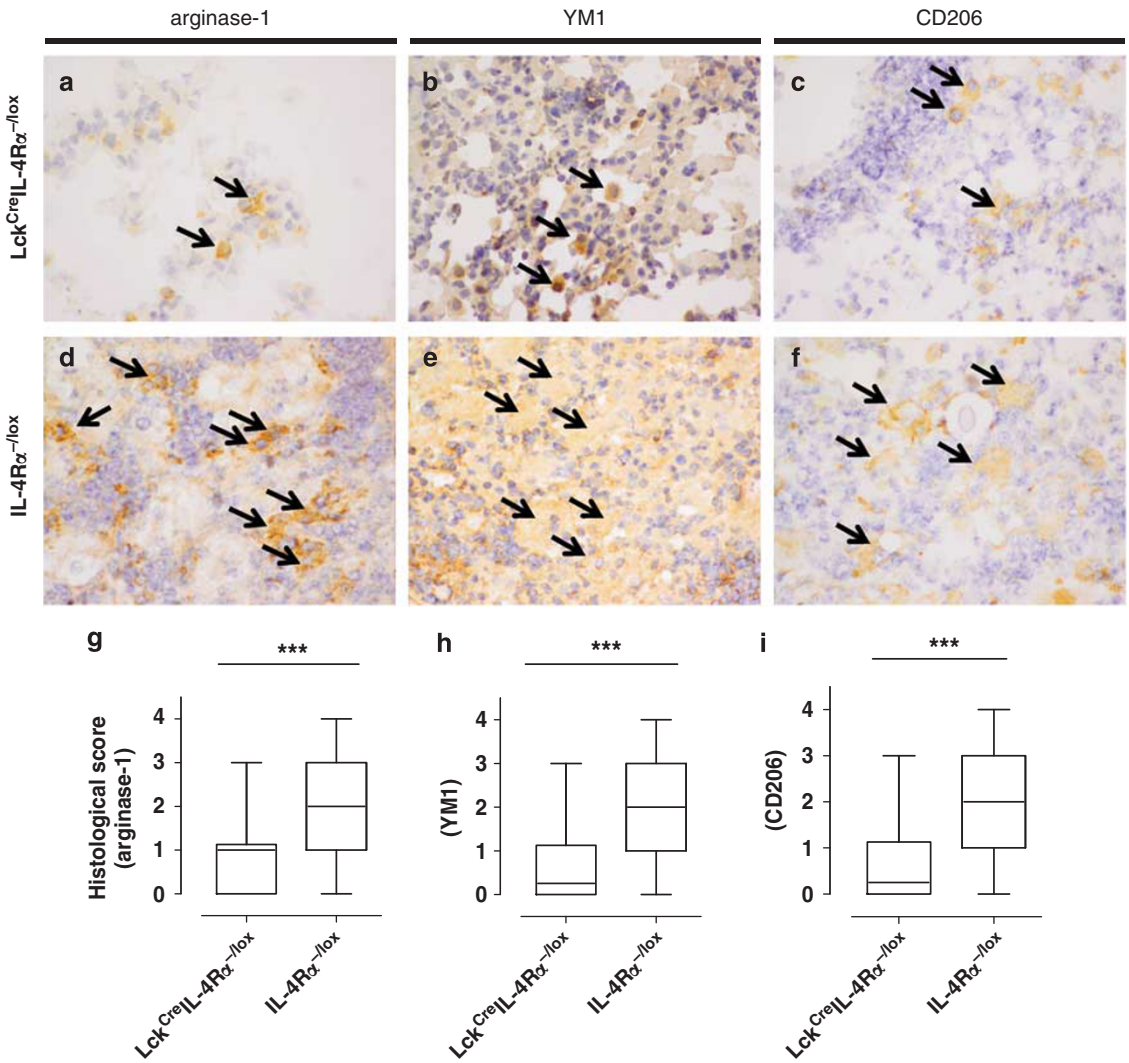


Figure 7 Diminished alternative activation of pulmonary macrophages in mice with T-helper (Th)-specific interleukin (IL)-4R deficiency in contrast to littermate controls. Analysis of the alternative activation status of pulmonary macrophages in lungs of *C. neoformans*-infected Lck^{Cre}IL-4Rα$^{-/lox}$ mice and littermate controls (IL-4Rα$^{-/lox}$) on day 70 p.i. 10 HPF of immunohistochemistry-stained lung slices were analyzed for signs of aaMph at 70 d.p.i. In (**a**–**f**) representative samples are shown for Lck^{Cre}IL-4Rα$^{-/lox}$ (**a** (arginase-1), **b** (YM1), **c** (CD206)) and IL-4Rα$^{-/lox}$ mice (**d** (arginase-1), **e** (YM1), **f** (CD206)). Black arrows point at large foamy macrophages (magnification = original ×600). A semiquantitative score based on immunohistochemical stainings for the respective markers of alternative activation was performed to quantify expression of arginase-1 (**g**), YM1 (**h**), and macrophage mannose receptor (CD206) (**i**). Significances were determined by using Mann-Whitney test; ***$P<0.001$. In total, data from seven independent experiments (in total 17–23 mice per genotype) was used for scoring. Few small macrophages stained positive for arginase-1 (**a**), YM1 (**b**) and CD206 (**c**) in Lck^{Cre}IL-4Rα$^{-/lox}$ mice, whereas in littermate controls macrophages with a large cytoplasm and a foamy appearance were strongly positive for arginase-1, YM1, and CD206 (**d**–**f**). HPF, high-power fields; Lck^{Cre}IL-4Rα$^{-/lox}$, lacking IL-4Rα on CD4$^+$ T cells; IL-4Rα$^{-/lox}$, littermate controls.

background with an age from 6 to 12 weeks were used for the experiments. The Lck/Cre-transgenic mice are CD4$^+$ T cell-specific IL-4Rα-deficient and are named Lck^{Cre}IL-4Rα$^{-/lox}$ in this publication. These mice and their non-Cre-transgenic IL-4Rα$^{-/lox}$ (i.e., heterozygous for the IL-4R) littermates were kept under specific pathogen free conditions and in accordance with the guidelines approved by the Animal Care and Usage Committee of the Landesdirektion Leipzig. Sterile food and water were given *ad libitum*.

***C. neoformans* and infection of mice.** Encapsulated *C. neoformans*, strain 1841, serotype D was stored in 10% skimmed milk at −80°C and was grown in Sabouraud dextrose medium (2% glucose, 1% peptone, Sigma, Deisenhofen, Germany) for 15 h on a shaker at 30°C. Infection was performed as described before.[14]

Determination of survival rate and organ burden. Survival and morbidity of infected mice were monitored daily. Organ burdens were determined by plated samples from homogenized organs (lung, brain) removed sterilely from killed mice. Serial dilutions of the homogenates were analyzed, using the Sabouraud dextrose agar plates. Colonies were counted after an incubation period of 72 h at 30°C. The organ burdens were plotted in a box-plot scheme; the "in box" samples (i.e., the box depicts the median with the upper and lower quartiles and the whiskers show the minimum and maximum values) were used for histological and immunohistological analyses.

Histopathological analysis and scoring. The protocol for immunohistochemical analysis for markers of aaMph in *C. neoformans*-infected Lck^{Cre}IL-4Rα$^{-/lox}$ mice and their non-Cre-transgenic

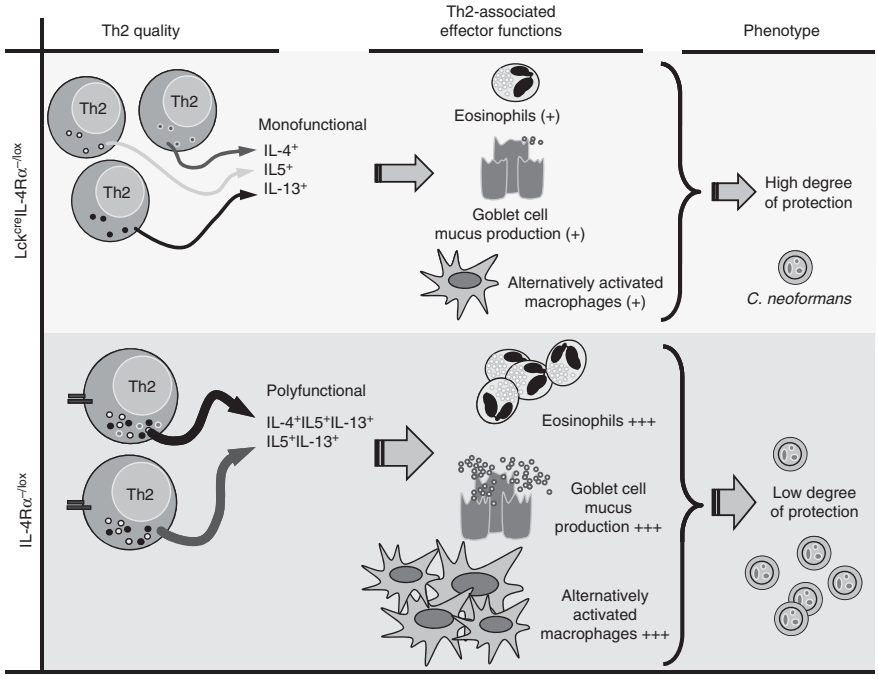


Figure 8 Polyfunctional T-helper (Th)2 cells in pathology of pulmonary cryptococcosis — a simplified scheme. Interleukin (IL)-4R expression on Th cells determines the quality (mono-functional vs. polyfunctional) and the magnitude of the Th2 response. IL-4R-dependent polyfunctional Th2 cells lead to a higher degree of eosinophil recruitment, goblet cell mucus production, and development of aaMph as compared with IL-4R-independent primarily mono-functional Th2 cells. Together, IL-4R expression on Th cells induces a stronger Th2 profile, resulting in effector functions that mediate susceptibility in pulmonary cryptococcosis.

littermates was published before.[24] Immunohistochemistry was performed by the use of the Vectastain Elite ABC-Kit (Vector, Burlingame, CA) as published elsewhere.[24] Negative controls without application of the primary antibody confirmed the specificity of the reactions. Here, 10 µm frozen sections were prepared in a serial fashion (30 transversal sections on 6 consecutive levels per lung). CD206 (mannose receptor) rat anti-mouse antibody (Serono, Unterschleißheim, Germany) and YM1 (ECF-L) goat anti-mouse antibody (R&D Systems, Minneapolis, MN) were used. In addition, the mouse anti-arginase-1 antibody (BD Biosciences, Heidelberg, Germany), in combination with the DAKO ARK Peroxidase kit (DAKO, Hamburg, Germany), was used according to the manufacturer's protocol[24] for staining of aaMph.

For immunohistological scorings 20 high-power fields (i.e., 20×0.16 mm^2) per "in box" (samples within the box plot for lung colony-forming units) samples were scored by two independent investigators. Histopathological alterations were microscopically evaluated on hematoxylin and eosin and immunostained lung sections with a scoring system reaching from 0 (no infiltrates) to 4 (maximum of macrophage infiltrates).

Another part of the lung and of the other organs was fixed in 4% buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin to characterize the extent and morphology of the inflammatory response in the various organs, or with PAS to detect cryptococci in the tissue by staining their polysaccharide capsule and to stain mucus production by goblet cells in the lung. The percentage of PAS-positive goblet cells in bronchial tissue was determined by counting PAS-positive and PAS-negative bronchial epithelial cells of a total of 10 bronchi per lung in serial sections.

Digestion of lung tissue and analysis of lung leukocytes. To check the recruitment of cells into the lung during cryptococcal infection, dissected lung tissue was prepared as described elsewhere.[14]

For cell recruitment analyses by flow cytometry (FACS Canto II; BD), total lung cells were used, termed unpurified. For surface staining 10^5 lung cells were used per staining. The cells were stained for leukocytes (leukocyte common antigen CD45 FITC; clone 30-F11; BD), Th cells (CD4 PE; clone H129.19; BD), Th2 cells ($CD4^+$ PE; IL-$33R^+$ ($T1/ST2^+$) FITC, clone DJ8 (MD Biosciences, Zürich, Switzerland)), eosinophils (Siglec-F^+, clone E50-2440 (BD Biosciences); $CD11c^-$ and a small proportion of $CD11c^{dim}$ clone N418 (eBioscience, Frankfurt, Germany)), and alveolar macrophages (Siglec-F^+ PE; $CD11c^+$ APC; $F4/80^+$ FITC, clone BM8 (eBioscience)) in FACS buffer (3% heat-inactivated fetal calf serum, 0.1% Na-azide in phosphate-buffered saline). Exclusion of dead cells was performed by LIVE/DEAD Fixable Dead Cell Stain Kit near-IR fluorescent reactive dye (Invitrogen, Darmstadt, Germany). Lung leukocytes were purified as described elsewhere.[14]

For intracellular staining of cytokines at least 10^6 purified pulmonary leukocytes were adjusted to $5\times10^6\ ml^{-1}$ in Iscove's modified Dulbecco's medium. Owing to the small numbers of recovered cells it was not possible to do re-stimulation with the cells from individual mice. Therefore, cells pooled from 3–4 mice of one genotype were used for re-stimulation. This resulted in up to two pools per genotype in one experiment. The stimulation protocol was published before.[14] Prior to fixation, a LIVE/DEAD Fixable Dead Cell Stain Kit, aqua fluorescent reactive dye (Invitrogen), was used to stain dead cells. For specific stainings the following antibodies were used: anti-CD4 APC-Cy7 (clone GK1.5 (BD)), anti-IL-4 PE-Cy7 (clone 11B11 (Biolegend, Fell, Germany)), anti-IL-17 Pacific Blue (clone eBio17B7 (eBioscience)), anti-IFN-γ PerCP (clone XMG1.2 (eBioscience)), anti-IL-13 Alexa Fluor 488 (clone eBio13A (eBioscience)), anti-IL-5 PE (clone TRFK5 (eBioscience)), and anti-CD154 APC (clone MR1 (Miltenyi Biotec, Bergisch Gladbach, Germany)).

Appropriate isotype antibodies for surface and intracellular staining were all from eBioscience, except for anti-IL-4 APC, clone 11B11 (Biolegend). For detection of cytokine-producing cells the cells were acquired on a FACS LSRII (BD).

Analysis of flow cytometry data was done using Weasel 2.7.4 (Walter and Eliza Hall Institute, Parkville, Australia) and FlowJo 7.6 (Treestar Inc., Ashland, OR) software.

Gating strategies for ICS analyses. To determine cytokine-producing Th cells, the following gating strategy was used: (i) gating for intact cells without debris, (ii) gating for vital cells (i.e., negative for aqua fluorescent reactive dye), and (iii) gating for $CD4^+$ cells. These cells were plotted in dot plots for two cytokines. The percentages of cytokine-producing cells were identified by using quadrant statistics for single and double producers.

Antigen-specific (i.e., $CD154^+CD4^+$) Th cells were analyzed using the same scheme described above, with additional gating for $CD154^+$ Th cells (termed also $CD40L^+$ $CD4^+$ cells). Triple producers were detected by an additional gating of double producers and analyzing the proportion of cells also positive for a third cytokine.

A Boolean gating strategy was used to examine the proportions of single, double, and triple Th2 cytokine-producing cells. As described above, living antigen-specific Th cells were gated. Then, $IL\text{-}4^+$ and $IL\text{-}5^+$ cells were plotted in a dot plot and three gates were used: (a) $IL\text{-}4^+IL\text{-}5^-$, (b) $IL\text{-}4^+IL\text{-}5^+$, and (c) $IL\text{-}4^-IL\text{-}5^+$. For gate (a) IL-13/IL-4 were analyzed indicating IL-4 single producers and IL-4/IL-13 double producers, for gate (b) IL-13/IL-4 analyses indicated IL-4/IL-5 double producers and IL-4/IL-5/IL-13 triple producers, and for gate (c) IL-13/IL-5 analyses indicated IL-5 single producers and IL-5/IL-13 double producers. To examine the proportion of IL-13 single producers, IL-13 and IL-4 were plotted and gate (d) $IL\text{-}4^-IL\text{-}13^+$ cells was determined. Gate (d) was used on a plot of IL-13 with IL-5 indicating IL-13 single producers.

The MFI was determined from the Boolean-gated cells for IL-4, IL-5, and IL-13. Integrated MFI values were calculated by multiplying individual cytokine MFIs with the percentage of antigen-specific Th cells producing IL-4, IL-5, or IL-13.

Cytokine analysis. To determine the concentrations of cytokines in the supernatant of purified lung leukocytes stimulated with cryptococcal antigen (heat inactivated acapsular *C. neoformans* CAP67), sandwich ELISAs were performed, as described elsewhere.[25]

Statistical analysis. The statistical significance of differences between experimental groups of animals was determined using the log-rank test for survival analysis, the one-tailed Mann-Whitney test for organ burden, cytokine analysis of pulmonary leukocyte re-stimulations, and FACS analysis (including surface staining and intracellular cytokine staining), and the two-tailed Mann-Whitney test for scoring.

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DISCLOSURE

The authors declared no conflict of interest.

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