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Interleukin (IL)-4, IL-13 und der IL-4-Rezeptor – molekulare Schrittmacher der Immunpathologie in der Kryptokokkose

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in memoriam

Jens-Uwe Müller

für

Moreen, Niklas & Daniel

„Die Erforschung der Krankheiten hat so große Fortschritte gemacht, dass es immer schwerer wird, einen Menschen zu finden, der völlig gesund ist.“

Aldous Huxley

Die vorliegende Arbeit wurde publiziert:

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Abkürzungsverzeichnis

AIDS	*aquired immunodeficiency syndrome* (erworbenes Immundefizienzsyndrom)
C3	Komplementfaktor 3
CR	Komplementrezeptor
CD	*cluster of differentiation*
DC	Dendritische Zelle
ELISA	*enzyme linked immunosorbent assay* (Enzym-gekoppelter Immunabsorptionstest)
FcR	Fc-Rezeptor
GFP	*green fluorescent protein* (grün-fluoreszierendes Protein)
GXM	Glucuronoxylomannan
HIV	Humanes Immundefizienzvirus
IFN-γ	Interferon gamma
Ig	Immunglobulin
IL-	Interleukin-
IL-4R	Interleukin-4-Rezeptor
IL-4Rα	Interkeukin-4-Rezeptor alpha-Kette
JAK	Januskinase
KbE	Koloniebildende Einheiten
MMR	Makrophagen-Mannoserezeptor
NK	Natürliche Killerzelle
PAS	*periodic acid-Schiff stain*
STAT	Transkriptionsfaktor (*signal transducer and activator of transcription*)
Th	T-Helferzelle
Th1	T-Helferzellantwort vom Typ 1
Th2	T-Helferzellantwort vom Typ 2
TNF-α	Tumornekrosefaktor alpha
Tyk	Tyrosinkinase
ZNS	Zentralnervensystem

1 Einleitung und Zielstellung

Zur Abwehr von Pathogenen besitzen wir ein Immunsystem, welches durch ein Zusammenspiel humoraler und zellulärer Faktoren in der Mehrheit der Fälle Infektionen effektiv bekämpft und damit Infektionskrankheiten vorbeugt. Dabei ist es wichtig, dass für den jeweiligen Erreger die adäquaten Abwehrmechanismen induziert werden. Sonst kann es zu immunpathologischen Effekten und zu einer mangelnden Erregerkontrolle führen, mit fatalen Folgen für das betroffene Individuum (1). Aber auch eine Suppression der Immunantwort durch Infektionen (z.B. HIV) oder immunmodulierende Therapien (z.B. nach einer Organtransplantation) können es Erregern erlauben, sich im Körper auszubreiten und lebensbedrohliche Komplikationen hervorzurufen. Mikroorganismen, denen eine Infektion durch ein unterdrücktes Immunsystem erst möglich wird, werden Opportunisten genannt. Zu dieser Gruppe von Pathogenen gehört *Cryptococcus neoformans*.

Der einzellige bekapselte Pilz *Cryptococcus neoformans* ist ein Vertreter der Basidiomyceten und kommt ubiquitär in der Umwelt vor (2). In urbanen Gebieten sind besonders Tauben als Vektoren von großer Bedeutung, da sie *C. neoformans* mit dem Kot ausscheiden können. Aufgrund ihrer hohen Körpertemperatur erkranken Vögel nur äußerst selten an einer Kryptokokkose. Als Opportunist kann der Pilz immungeschwächte Individuen infizieren und Pneumonien hervorrufen. Des Weiteren kommt es durch den Neurotropismus des Pilzes zu potentiell tödlich verlaufenden Meningitiden. In menschlichen Patienten treten Kryptokokkosen vor allem im Kontext von AIDS-Erkrankungen sowie bei Patienten, die eine Therapie mit Immunsuppressiva erhalten (z.B. Transplantatempfänger) auf (3). Jährlich infizieren sich weltweit mehr als eine Millionen immunsupprimierte Personen neu mit *C. neoformans* und über 600.000 sterben jedes Jahr an einer Kryptokokken-Meningitis (4). Epidemiologisch von großem Interesse ist darüber hinaus, dass die Art *C. neoformans* in der Lage ist, immunkompetente Individuen persistent zu infizieren (5), in diesen jedoch nicht zu einer Erkrankung führt. Kryptokokken besitzen ein großes Spektrum potentieller Wirte und können neben dem Menschen eine Vielzahl von Tierarten befallen. Kryptokokkeninfektionen konnten bei einer Vielzahl von Haustieren beschrieben werden. Neben Katzen (6) und Hunden (6) sind Infektionen u.a. auch bei Pferden (7), Schafen (8) und Rindern (9) bekannt. Bei Katzen und Hunden sind vor allem kutane und pulmonale Infektionen mit entsprechender Symptomatik beschrieben, bei Pferden können pulmonale und zentralnervöse Infektionen und bei Rindern u.a. Mastitiden auftreten.

In der vorliegenden Arbeit wurde mit Hilfe von Mausmodellen die Wechselwirkung zwischen *Cryptococcus neoformans* und dem Immunsystem des Säugetierwirts in pulmonalen Infektionen untersucht.

Aufbauend auf Ergebnissen der Arbeitsgruppe von Prof. Dr. Gottfried Alber und anderer Labore zur Pathogenese in der Kryptokokkose ergaben sich Fragen nach molekularen und zellulären Mechanismen

der Immunpathologie in der pulmonalen Kryptokokkose, die im Rahmen der vorliegenden Arbeit beantwortet werden sollten.

Als geeigneter Modellorganismus hat sich dabei die Maus erwiesen, da man in ihr gut die pulmonale Infektion und Dissemination in periphere Organe untersuchen kann. Wichtige wegweisende Erkenntnisse zur Bedeutung von Immunzellen und Zytokinen in der Kryptokokkose wurden mithilfe von Mausmodellen erhoben. Um die Fragen zu wichtigen Immunzellen, Botenstoffen und Rezeptoren in der pulmonalen Kryptokokkose beantworten zu können, wurde aus diesem Grunde ein intranasales Infektionsmodell in der Maus etabliert, welches dem natürlichen Infektionsweg sehr nahe kommt. Die Mäuse werden anästhesiert und ihnen wird ein kleines Inokulum Kryptokokken (500 KbE) in die Nasenlöcher geträufelt. Der Balb/c-Mausstamm erwies sich als gut geeigneter Modellorganismus. Balb/c-Mäuse sind empfänglich für die Infektion mit einem virulenten Kryptokokkenstamm und entwickeln starke Pathologien, die entzündliche Reaktionen in der Lunge und im Zentralnervensystem (ZNS) mit einschließen, ähnlich dem, was man auch in menschlichen Patienten beobachten kann. Die Maus ist ideal für diese Forschung, da es für die zu untersuchenden Aspekte transgene Mauslinien gibt, die es erlauben, die gestellten Fragen zu beantworten. Wie aus vorangegangenen Arbeiten bekannt ist, spielt Interleukin-(IL-)4 bei der Pathogenese eine bedeutende Rolle. Auf welche Weise und auf welche Zelltypen das IL-4 aber wirkt und ob auch andere Th2-Zytokine an der Pathologie beteiligt sind, war bis dato unbekannt. Aus diesem Grunde wurden Untersuchungen zu dem Th2-Zytokin IL-13 angestellt und die Bedeutung der Typ I und Typ II IL-4 Rezeptoren (IL-4R) untersucht. Insbesondere war von Interesse, wie IL-4, IL-13 und deren Rezeptorkomplex an den Entzündungsreaktionen im Gehirn während einer Kryptokokkose beteiligt sind. T-Helferzellen spielen eine wichtige Rolle in der Pathogenese der pulmonalen Kryptokokkose. Aus diesem Grunde war es Ziel der vorliegenden Arbeit die Rolle des IL-4R auf T-Helferzellen aufzuklären. Aus diesen Forschungsergebnissen ergaben sich zudem Fragen, welche Zelltypen bedeutend für die Induktion und Aufrechterhaltung einer Th2-Antwort sind. Die Fragen zur Bedeutung von IL-13 konnten mit IL-13-defizienten und IL-13-transgenen (überexprimierenden) Mäusen ermittelt werden. Für die Untersuchungen zur Rolle der Typ I und Typ II IL-4R wurden IL-4Rα-defiziente Mäuse sowie heterozygote IL-4Rα-Mäuse und Wildtyp-Tiere verwendet. IL-4Rα ist eine gemeinsame Untereinheit der Typ I und Typ II IL-4R. Die Dissemination in das ZNS wurde untersucht. Hierzu wurden Immunreaktionen und Pilzverteilungen im Gehirn von IL-4-, IL-13- und IL-4Rα-defizienten Mäusen im Vergleich zu Wildtyptieren in der pulmonalen Krytpokokkose ermittelt. Die aus diesen Untersuchungen resultierenden Fragen zu den bedeutendsten IL-4-Produzenten konnten an Mäusen untersucht werden, deren Zellen grün leuchten. Die Tiere sind transgen für das Gen des *green fluorescent protein* (GFP), welches unter dem Einfluss des IL-4-Promotors steht. Sobald die Zellen in diesen Tieren IL-4 bilden, beginnen sie grün zu leuchten. Mit Hilfe von dblGATA-Mäusen, die keine eosinophilen Granulozyten bilden können, konnte die Bedeutung der eosinophilen Granulozyten in der Pathologie der

pulmonalen Kryptokokkose bestimmt werden. Um letztendlich festzustellen, wie zentral die Rolle der T-Helferzellen in der Pathologie ist, wurden T-Helferzell-spezifische IL-4Rα-defiziente Tiere verwendet. Wichtige Analysepunkte sind für die verschiedenen transgenen Mauslinien die Bestimmung der Überlebensrate sowie die Organlast in der Lunge und den peripheren Organen, vor allem im Gehirn. Des Weiteren wurden pathologische Veränderungen in der Lunge untersucht, wie z.B. die Hyperplasie von Becherzellen (Nachweis der Schleimbildung durch eine PAS-Färbung und Auszählung der positiven Zellen in den Bronchien), die alternative Aktivierung von Makrophagen (Nachweis durch immunhistologische Untersuchungen zu wichtigen Markern der alternativen Aktivierung) und die Rekrutierung von Th2-assoziierten Zellen (durchflusszytometrische Analyse der Leukozytenproportionen). Zudem wurden Untersuchungen zur Hyperreagibilität der Bronchien vorgenommen. Für die Untersuchung zur Zytokinbildung wurden Zytokine mittels mRNA-Untersuchungen (qRT-PCR), Proteinnachweis (ELISA) und intrazellulärer Multiparameter-Durchflusszytometrie bestimmt und somit auch Hinweise auf Zytokin-Multiproduzenten erhalten, die in der Diagnostik eine bedeutende Rolle für die Vorhersage der Schwere der Erkrankung haben könnten.

1.1 Zielsetzung

Insbesondere ergaben sich im Rahmen der vorliegenden Arbeit folgende Fragen:

- **Welche Zytokine fördern die Pathologie (neben IL-4) und welche Auswirkungen haben diese in der Kryptokokkose? Publikation 1**
- **Welches sind die zellulären Quellen des Th2-induzierenden Zytokins IL-4 in der pulmonalen Kryptokokkose? Publikation 2**
- **Welche Rolle spielt der IL-4-Rezeptor (IL-4R) in der Pathologie der pulmonalen Kryptokokkose, welche Rolle spielt der IL-4R bei der zerebralen Kryptokokkose und welche Zelltypen werden insbesondere durch den Rezeptor aktiviert? Publikationen 3, 4, 5**

Die Antworten auf diese wichtigen Fragen in der Pathogenese der pulmonalen Kryptokokkose, die durch die vorliegende Arbeit gegeben werden können, helfen dabei, die zugrunde liegenden Mechanismen besser zu verstehen. Die Befunde bieten darüber hinaus Optionen für therapeutische Ansätze, die über das Infektionsgeschehen der pulmonalen Kryptokokkose weit hinausgehen. So sind vielfältige Gemeinsamkeiten zur Asthmapathologie aufgedeckt worden.

2 Literaturübersicht

2.1 Infektionen mit *C. neoformans*

Der bekapselte Basidiomycet *Cryptococcus neoformans* wurde ursprünglich in vier Serotypen eingeteilt (10), wobei die Einteilung aufgrund der Struktur der Polysaccharidkapsel erfolgte. Man unterschied die Serotypen A, B, C und D. Aufgrund der geografischen Verbreitung der Serotypen und des Phänotyps der von den jeweiligen Serotypen hervorgerufenen Infektionen (Infektion von immunsupprimierten bzw. immunkompetenten Individuen) wurden hierbei zwei Varietäten unterschieden, *C. neoformans* var. *neoformans* mit den Serotypen A und D und *C. neoformans* var. *gattii* mit den Serotypen B und C (10). Mittels molekularbiologischer Methoden wurde noch eine dritte Varietät postuliert, *C. neoformans* var. *grubii*, die mit Serotyp A identisch ist (11,12). *C. neoformans* var. *grubii* scheint hierbei virulenter zu sein als *C. neoformans* var. *neoformans*, da viele Infektionen in immunsupprimierten Menschen, vor allem in den USA, durch Serotyp A-Kryptokokken hervorgerufen wurden und werden (13). *C. neoformans* var. *neoformans* und *C. neoformans* var. *grubii* infizieren immungeschwächte Patienten, vor allem im Rahmen einer HIV-Infektion, während *C. neoformans* var. *gattii* auch immunkompetente Individuen infizieren kann (14). Diese erheblichen Unterschiede in der Virulenz und weitere molekularbiologische Untersuchungen haben dann zu Beginn des 21. Jahrhunderts dazu geführt, dass man zwei getrennte Arten postuliert hat, *C. neoformans* und *C. gattii* (15,16) – *C. neoformans* var. *grubii* konnte aufgrund der molekularbiologischen Untersuchungen hingegen kein Artstatus zuerkannt werden.

Neben dem Menschen hat man Kryptokokkeninfektionen auch bei Wild- und Haustieren nachgewiesen. Eine Besiedelung der Haut bzw. von Schleimhäuten von gesunden Individuen konnte sowohl bei Menschen (17) als auch Tieren (18) beobachtet werden. Beispielsweise fand man im Nasenrachenraum gesunder Hunde Kryptokokken. Auch in einer unauffälligen Ziegenherde, die aufgrund einer Virusinfektion gekeult werden musste, fanden sich bei 6% der Tiere Kryptokokkome in der Lunge. In der nachfolgenden Tabelle (Tab. 2.1) sind Tierarten beschrieben, bei denen Infektionen mit *C. neoformans* bzw. *C. gattii* nachgewiesen werden konnten. Bei Haustieren sind die Symptome je nach Spezies unterschiedlich. Bei Katzen stellt die Kryptokokkose die bedeutendste systemische Mykose dar. Aufgrund ihrer Anatomie sind bei Katzen vor allem der Nasenrachenraum betroffen. Zudem treten vermehrt Infektionen der Haut auf – zentralnervöse Infektionen hingegen sind fast ausschließlich Spätfolgen der Infektion (19). Bei Hunden finden sich neben der Beteiligung des Nasenrachenraums als primärer Infektionsort auch gehäuft Lungeninfektionen, Infektionen des Darmtrakts und eine frühzeitige zentralnervöse Beteiligung (19). Sowohl *C. neoformans* (var. *grubii*) als auch *C. gattii* können bei Kryptokokkosen von Hund und Katze diagnostiziert werden (20). Bei Pferden sind vor allem Infektionen des unteren Respirationstrakts bekannt und auch zentralnervöse Infektionen sind beschrieben. Bei Rindern und Ziegen sind Mastitiden nachgewiesen worden. Auch bei Schafen, Kamelen und Lamas sind

Kryptokokkosen bekannt (19). Epidemiologische Studien haben dabei gezeigt, dass Kryptokokkosen bei Haustieren in Australien und Nordamerika häufiger vorkommen als in Europa.

Tab. 2.1: Veterinärmedizinische Bedeutung der Kryptokokkose

Haustierspezies	Organsystem	Referenz
Hauskatze (*Felis silvestris catus*)[1,2,3]	Nasenrachenraum	(6,21)
	Lunge	(6,22)
	Haut	(6,23,24)
	Augen	(6)
	ZNS	(6,25,26)
Haushund (*Canis lupus familiaris*)[2,3]	Nasenrachenraum	(6,21,27)
	Lunge	(6,27)
	Haut	(6,27)
	Augen	(6)
	Harnweg	(6)
	Darmtrakt	(6)
	ZNS	(6,21,25-28)
Pferd (*Equus cabalus*)[2,3]	Nasenrachenraum	(29,30)
	Lunge	(31,32)
	Darmtrakt	(33)
	Haut	(34)
	Uterus	(35-37)
	ZNS	(38,39)
Rind (*Bos primigenius taurus*)	Euter[1,4,5]	(40-46)
Ziege (*Capra aegagrus hircus)*	Euter[1]	(47,48)
	Lunge[1,3]	(49-51)
Schaf (*Ovis orientalis aries*)	Lunge[1,2]	(8,20)

[1]*C. neoformans* var. *neoformans* [2]*C. neoformans* var. *grubii* [3]*C. gattii* [4]*C. curvatus* [5]*C. laurentii*

Kryptokokken kommen ubiquitär in der Umwelt vor. In ländlichen Regionen spielt für Haustiere vor allem der Kontakt mit kontaminierten Böden eine Rolle. Als wichtiger Vektor in urbanen Regionen hat sich hingegen die Stadttaube erwiesen. In ihren Ausscheidungen finden sich Sporen oder Pilze, die nach Austrocknung aerogen verbreitet und über die Atemluft aufgenommen werden können. Der Pilz gelangt über den Atemstrom in die Alveolen und kann durch Transzytose und Infektion der Epithelien in das Lungengewebe eindringen (52-54), wo er bei immunkompetenten Individuen zu einer latenten persistierenden Infektion führt.

C. neoformans weist eine Reihe von Virulenzfaktoren auf, mit deren Hilfe er im Körper überleben kann. Der Keim ist dazu in der Lage, bei 37°C zu wachsen. Somit kann er in Säugetieren überleben und sich

vermehren (55). Auch in Vögeln überlebt er, kann diese aber nur in Ausnahmefällen infizieren, da Vögel eine höhere Körpertemperatur aufweisen. Es sind Fälle bei Papageienvögeln und Tauben bekannt, hier betrifft die Infektion vor allem kühlere Körperregionen, wie die Haut im Kopfbereich, den Schnabel und die oberen Atemwege (19). Eine Ausnahme ist z.B. der Kiwi, der eine geringere Körpertemperatur aufweist und in dem auch Kryptokokkosen der Lunge und Disseminationen in andere Organe beschrieben sind (19).

Darüber hinaus verfügt *C. neoformans* über eine Kapsel, die vor allem aus Polysacchariden wie Glucuronoxylomannan (GXM) (56-58) besteht. In die Kapsel sind Proteine wie z.B. Mannoproteine eingelagert, die dem Stofftransport dienen. Durch die negative Ladung der Kapsel, kann er den natürlichen Fressfeinden, Amöben (59-63), entkommen. Die Amöben weisen die gleiche Ladung auf, so dass eine Aufnahme erschwert wird.

Den gleichen Mechanismus machen sich die Pilze im infizierten Säugetierwirt zu Nutze (60). Da auch die Makrophagenoberfläche eine negative Ladung aufweist, wird auch hier die Phagozytose verhindert bzw. verzögert (64). Zudem reduziert das Kapselmaterial die Proliferation von T-Zellen (64) und wirkt antichemotaktisch (65-68), es wird also die Rekrutierung von Leukoyzten zum Infektionsort erschwert. Auch hat der Pilz eine einzigartige Möglichkeit entwickelt, nach erfolgter Phagozytose durch Amöbe oder Makrophage der Abtötung zu entgehen. Durch einen Mechanismus, der Expulsion genannt wird, kann der Erreger das Phagosom und die ihn phagozytierende Zelle verlassen, ohne dabei die Zelle zu zerstören (69-72). Es handelt sich bei dieser Expulsion um eine Exozytose. Ein weiterer wichtiger Evasionsmechanismus ist die Bildung einer Melanin-haltigen Schutzschicht, die unterhalb der Kapsel liegt. Diese Melaninschicht ist ebenso wie das vom Pilz gebildete Enzym Superoxiddismutase in der Lage, vom Wirt sezernierte Sauerstoffradikale unschädlich zu machen (73-75). Das Melanin wird durch das Enzym Laccase gebildet (76-79). In der freien Natur kann sich der Pilz durch diese Schutzschicht vor schädlicher UV-Strahlung schützen.

C. neoformans weist im humanen Patienten (und auch in Nagermodellen) einen Neurotropismus auf (80,81), so dass er neben der infizierten Lunge, bei immungeschwächten Individuen auch im Gehirn anzutreffen ist. Untersuchungen der Zerebrospinalflüssigkeit ermöglichen einen eindeutigen Nachweis in der Diagnostik (82). Die Infektion mit *C. neoformans* führt bei den Patienten zu Meningitiden. Zu der Frage, wie der Pilz in das Gehirn eindringt, gibt es mehrere Hypothesen. Einige Autoren gehen von einer hämatogenen Streuung mit einer Transzytose durch Endothelzellen der Bluthirnschranke aus (83), andere von einem *Shuttle* über Immunzellen, z.B. Makrophagen (84,85). Letztendlich können beide Annahmen korrekt sein, wobei es vom *C. neoformans*-Stamm abhängt, welcher Weg eingeschlagen wird. Einige Stämme disseminieren innerhalb weniger Tage ins Gehirn (86), während andere mehr als einen Monat dafür benötigen.

2.2 Die schützende Immunantwort gegen *C. neoformans* und therapeutische Ansätze

Die Immunantwort gegen den Erreger ist ein Zusammenspiel verschiedener Zelltypen, Botenstoffe und Effektormechanismen. Dieses Zusammenspiel wird von T-Helferzellen orchestriert, wobei abhängig von der Differenzierung der T-Helferzellen eine schützende oder eine pathologische Immunantwort induziert wird. Eine schützende Immunantwort ist in erster Linie abhängig von einer durch IL-12 induzierten Immunantwort. Das IL-12 wird hier vor allem von dendritischen Zellen gebildet (87). Die Erkennung von Pilzen durch dendritische Zellen erfolgt z.B. über *C-type lectin* Rezeptoren (CLR), die β-Glukane (88-91) oder Mannane (92), sowie Chitin erkennen können. Diese Rezeptoren sind u.a. in der Lage die Bildung von Zytokinen zu fördern. Makrophagen und dendritische Zellen besitzen zudem weitere Mustererkennungsrezeptoren, die es ihnen erlauben, z.B. die Mannoproteine der Kapselhülle des Erregers zu erkennen (93). Diese Mustererkennungsrezeptoren sind z.B. Mannoserezeptoren. Über diese Mannoserezeptoren können Phagozyten den Pilz erkennen und aufnehmen. Für weitere Mustererkennungsrezeptoren, wie z.B. *Toll-like receptor*(TLR)-2, TLR-4, und Komplementrezeptor CR4 (94) ist beschrieben, dass sie Kapselmaterial (insbesondere Glucuronoxylomannan) erkennen können. Auch die Erkennung von *C. neoformans*-DNA über TLR-9 führt zur Aktivierung von Makrophagen und dendritischen Zellen (95-97).

Eine Interaktion gibt es auch zwischen angeborenem und adaptivem Immunsystem. Das von dendritischen Zellen gebildete IL-12 führt zu einer Ausdifferenzierung naiver T-Helferzellen zu sogenannten Th1- Zellen (98-101). Diese Zellen produzieren nach Aktivierung Interferon gamma (IFN-γ) (101), welches B-Zellen, Natürliche Killerzellen (NK-Zellen), Makrophagen und $CD8^+$ T-Zellen aktiviert. Aktivierte B-Zellen bilden neutralisierende und opsonisierende (102) Immunglobuline der Klasse G, die an der Kapsel des Pilzes haften und Makrophagen befähigen über Fc-Rezeptoren die gebundenen Antikörper zu erkennen und mitsamt Pilz zu phagozytieren (103,104). Des Weiteren kann Komplement an die Polysaccharidkapsel binden (u.a. C3b) und somit opsonisieren. Über die Komplementrezeptoren CR1, CR3 und CR4 kann die Erkennung dieses Komplexes erfolgen (94,104,105).

Makrophagen differenzieren durch das von Th1-Zellen gebildete IFN-γ zu Effektormakrophagen aus. Zudem wird die zytotoxische Antwort durch Aktivierung der NK-Zellen und Ausdifferenzierung von $CD8^+$ T-Zellen zu zytotoxischen T-Zellen (106) verstärkt. – Diese (klassisch aktivierten) Effektormakrophagen (107) sind in der Lage, antifungale Effektorsubstanzen, wie z.B. reaktive Stick- und Sauerstoffintermediate, zu bilden. Mit Hilfe der opsonisierenden Antikörper können Makrophagen über ihre Fcγ-Rezeptoren Pilzzellen erkennen (108) und phagozytieren. NK-Zellen sind über lytischen Granula-Inhalt, wie z.B. Granulysin und NK-Lysin, sowie den Exozytoseweg (Perforin) (109-114) befähigt, Pilzzellen direkt abzutöten. Zytotoxische Zellen können infizierte Zellen erkennen und eliminieren (110,111,115,116). Es ist bekannt, dass *C. neoformans* in der Lage ist, nicht zur Phagozytose befähigte Zelltypen, wie z.B. Epithel- und Endothelzellen zu infizieren (52).

Mit Hilfe von gendefizienten Mäusen konnte gezeigt werden, dass $CD4^+$ T-Zellen (T-Helferzellen) und $CD8^+$ T-Zellen absolut notwendig sind, um eine schützende Immunantwort zu induzieren (117) (Abb. 2.1). Auch sind diese in der Lage, über fungizide Faktoren wie z.B. Granulysin Kryptokokken direkt abzutöten (115,116). Dieser Schutz wird durch sogenannte Th17-Zellen, die IL-17A als Hauptzytokin bilden, unterstützt (86,118).

Das Fehlen einer Th-Antwort ist für die Ausbreitung des Pilzes entscheidend, wie sowohl in Patienten mit AIDS (119-121), als auch in Transplantatempfängern, deren T-Zellen durch immunsuppressive Medikation unterdrückt werden, zu beobachten ist. Beide Patientengruppen sind sehr anfällig für eine Infektion und eine Dissemination des Erregers ins ZNS. Andererseits konnten mit Hilfe von CD4-depletierenden Antikörpern pathologische Effekte im Mausmodell nach einer pulmonalen Infektion mit *C. neoformans* unterdrückt werden (122). Im Rahmen einer pulmonalen Infektion kann sich im empfänglichen Individuum eine Immunpathologie entwickeln, die als Immunpathologie-assoziierte Mykose bezeichnet wird (122). Dieser Befund, dass Th-Zellen auf der einen Seite schützend sind und auf der anderen Seite immunpathologisches Potential haben, wirkt zunächst paradox, aber bei genauerer Betrachtung offenbart sich die Dichotomie des Immunsystems, die auch in diesem Infektionsgeschehen von besonderer Bedeutung ist. *C. neoformans* löst eine persistierende latente Infektion in der Lunge aus (5). Wie in einem Rattenmodell gezeigt werden konnte, welches dem Infektionsgeschehen im immunkompetenten Menschen ähnlich ist, kommt es durch die Infektion der Lunge zu einer verstärkten Th2-Antwort, einhergehend mit einer erhöhten Hyperreagibilität der Atemwege (108,123). Dieser Evasionsmechanismus wird für einige Autoren als Indiz dafür herangezogen, dass eine *C. neoformans*-Infektion ein Risikofaktor für pulmonale Allergien bis hin zum Asthma sein könnte. Es wurde zudem gezeigt, dass Urease, ein bedeutender Virulenzfaktor von *C. neoformans*, an der Induktion einer nicht-schützenden Th2-Antwort in der Lunge beteiligt ist (5,122-124).

Infektionen im Falle einer Immunsuppression rühren dabei neben Neuinfektionen auch von latenten Erregerherden her. So konnte u.a. gezeigt werden, dass Personen aus Afrika, die seit Jahren in Europa lebten, nach einer HIV-Infektion opportunistische Infektionen mit Kryptokokken entwickelten, wobei es sich bei den Erregern um afrikanische *C. neoformans*-Stämme handelte (5,108). Im Falle einer HIV-Infektion kann es im Laufe der Erkrankung zu einer Verschiebung der Qualität der Immunantwort, auch abseits der Reduktion von Th-Zellen im Allgemeinen, kommen.

Wie gezeigt werden konnte, entwickelt sich eine verstärkte Th2-Antwort, diese schwächt die Th1-Antworten ab (5,125,126). Diese Th1-Th2-Verschiebung hat auch deutliche Auswirkungen auf die Kontrolle der pulmonalen Kryptokokkose. Die IL-12-Produktion wird reduziert und die antifungale Kapazität von Lymphozyten und Phagozyten ist beeinträchtigt (5,127-130). Somit bricht der Schutz nicht nur durch die Verringerung der T-Helferzell-Zahlen zusammen, sondern wird noch zusätzlich durch die fehlende Th1-Antwort verringert. Durch die verstärkte Th2-Antwort entwickelt sich eine pulmonale

Immunpathologie-assoziierte Mykose, die einhergeht mit einer Aktivierung der Becherzellen in der Lunge und vermehrter Schleimbildung (5,122,123). Diese Mechanismen sind dabei abhängig von Th2-Zytokinen, die im Rahmen der Infektion von Th2-Zellen sezerniert werden. Die Bedeutung des Th2-Zytokins IL-13 in der Kryptokokkose war bis dato unbekannt. Dieser Frage wurde im Rahmen der vorliegenden Arbeit nachgegangen.

In der Bekämpfung der Kryptokokkose werden diverse Strategien diskutiert. Eine Strategie besteht darin, Vakzinierungen zu entwickeln. Diese neu entwickelten Therapien werden im Tierversuch getestet. So hat man avirulente Mutanten (u.a. kapsellose Stämme (131), eine Mutante, die Pseudohyphen bildet (132)) generiert, die z.B. bei 37°C nicht wachsen können und somit ein gutes Werkzeug zur Ausprägung einer Immunantwort gegen *C. neoformans* sind. Der Vorteil hierbei ist, dass es sich um eine attenuierte Lebendvakzine handelt, die neben der humoralen auch die zelluläre Immunantwort induzieren kann. Auch hitzegetötete Pilze werden zur Vakzinierung eingesetzt, um damit eine zelluläre Immunantwort zu induzieren (133,134). In Lymphknoten mit hitzeinaktivierten Kryptokokken behandelter Ratten konnte eine deutliche Th1-Induktion mit Bildung der Th1-Zytokine IL-12 und TNF-α beobachtet werden (135,136). Des Weiteren wurden Kryptokokken generiert, die IFN-γ produzieren. Diese Pilze sind in der Lage, eine schützende Immunreaktion zu initialisieren, die vor Wildtyp-Stämmen eines homologen virulenten Kryptokokkenstamms schützt (137). Auch Kryptokokken-Kulturfiltrat (Zellkulturüberstand aus einer Pilzkultur), verabreicht mit komplettem Freund'schen Adjuvans, wurde in Vakzinierungsstudien verwendet. Dieses Filtrat führt zu einer starken Überempfindlichkeitsreaktion vom verzögerten Typ (*delayed type hypersensitivity* (DTH)). Die so behandelten Tiere zeigten eine deutliche Reduktion der Keimlast nach Immunisierung im Vergleich zu unbehandelten oder mit abgetöteten Erregern behandelten Kontrolltieren sowie ein längeres Überleben. Die vielversprechendsten Stoffe aus dem Kulturfiltrat stellten bei den Untersuchungen Mannoproteine dar (138,139).

Eine weitere Möglichkeit für Vakzinierungen ist die Verwendung von Konjugatvakzinen. Hierbei wird im Rahmen der Vakzinierungsversuche gegen Kryptokokken Kapselmaterial mit einem stark immunogen wirkenden Agens, wie z.B. Tetanustoxoid, fusioniert, sogenanntes GXM-TT. Die GXM-TT-Immunisierung führt zur Bildung opsonisierender Antikörper, die es Phagozyten erleichtern, Kryptokokken zu phagozytieren und zu elimineren (140). Therapeutisch lassen sich auch monoklonale Antikörper gegen Pilzbestandteile einsetzen (141,142). Für den monoklonalen Antikörper 18B7 konnte z.B. gezeigt werden, dass er spezifisch sowohl das GXM von *C. neoformans* als auch *C. gattii* erkennt und bindet (143), *C. neoformans* opsonisiert, die antifungale Aktivität der Leukozyten erhöht und die Komplementkaskade aktiviert, die zu einer Bindung von C3 an die Kapsel führt.

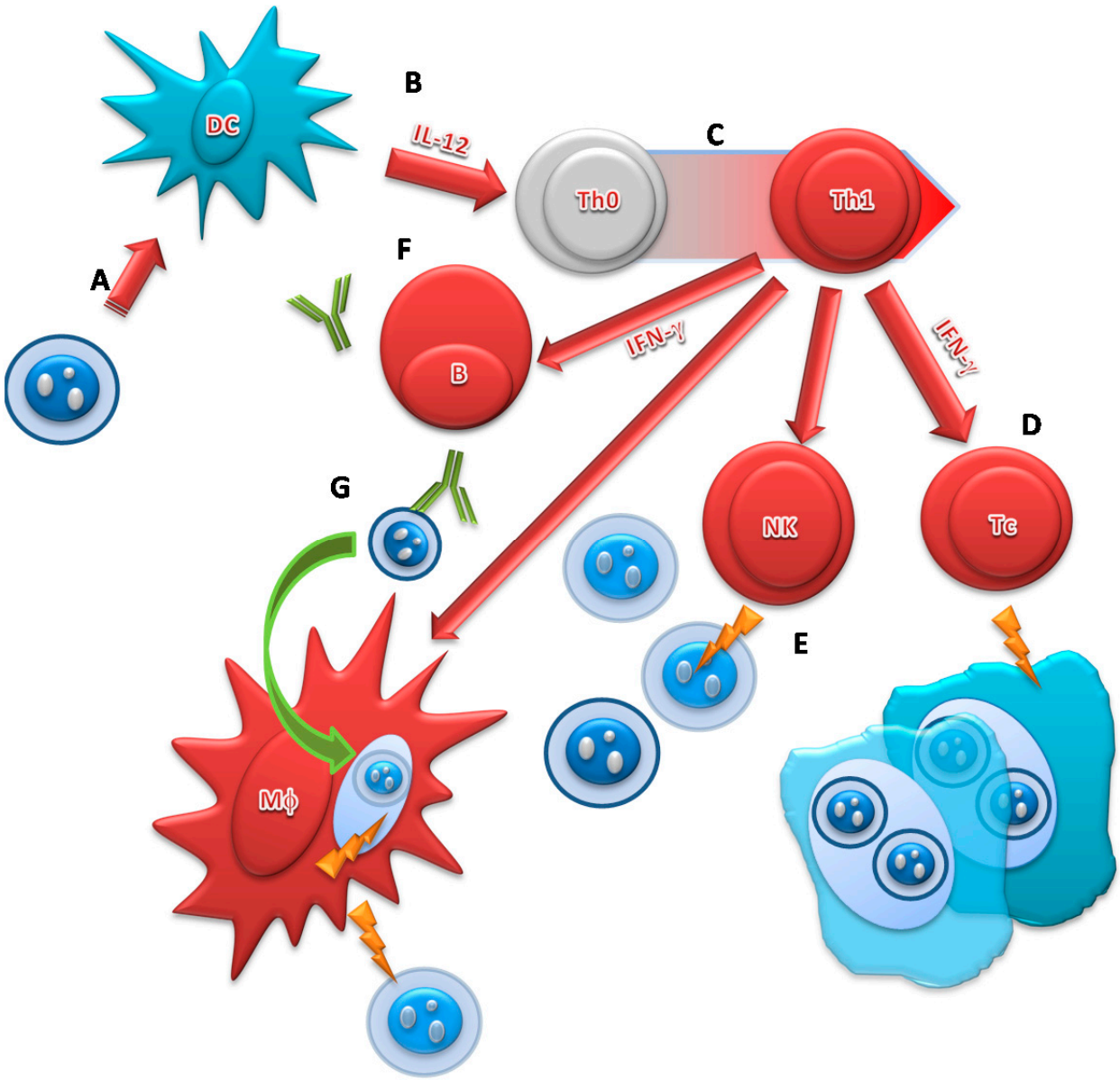

Abb. 2.1: Die schützende Immunantwort gegen C. *neoformans*. Kryptokokken werden durch Mustererkennungsrezeptoren auf dendritischen Zellen (DC) erkannt (A), diese wandern in den drainierenden Lymphknoten und sezernieren dort das Zytokin IL-12 (B). Einwandernde naive T-Helferzellen differenzieren sich durch die IL-12-Stimulation zu Th1-Zellen (C). Th1-Zellen sezernieren das Zytokin IFN-γ, welches verschiedene Immunzellen aktiviert. Durch die Aktivierung von $CD8^+$ T-Zellen (Tc) werden diese zu zytotoxischen T-Zellen, die in der Lage sind, durch Ausschüttung von Perforin und Granzymen, infizierte Zellen zu eliminieren (D). Neben den zytotoxischen T-Zellen werden auch Natürliche Killerzellen (NK) aktiviert. Diese sind in der Lage, durch Ausschüttung lytischer Granula, wie z.B. Granulysin oder NK-Lysin, Kryptokokken abzutöten (E). Die Th1-Zellen stimulieren B-Zellen zur Produktion von opsonisierenden Antikörpern (F). Diese binden an der Kapsel des Pilzes und markieren ihn dadurch für die Phagozytose mittels FcγR (G). Durch IFN-γ werden Makrophagen aktiviert, die Kryptokokken phagozytieren und reaktive Sauerstoff- und Stickstoffstoffintermediate (z.B. NO) sezernieren, die den Pilz schädigen können (H).

Es konnte gezeigt werden, dass der Schutz unter anderem auf der Verstärkung der Phagozytose durch Makrophagen beruht (144).

Eine wirklich erfolgversprechende Therapie gegen Kryptokokken wurde bisher nicht gefunden. Es ist also sinnvoll, nach neuen Therapiemöglichkeiten zu suchen und somit eine Immuntherapie gegen eine pathologisch verlaufende Immunantwort in Betracht zu ziehen.

2.3 Die Th2-Antwort im Schutz gegen extrazelluläre Infektionen

Eine Th2-Antwort bietet Schutz gegenüber extrazellulären Erregern, wie z.B. gegen Würmer. Die von den Th2-Zellen sezernierten Zytokine sind IL-4, IL-5 und IL-13 (145). IL-4 ist dabei besonders wichtig für die Bildung von schützenden Antikörpern gegen extrazelluläre Erreger (in der Maus: Immunglobulin(Ig)G1 und IgE) durch B-Zellen. IgE kann z.B. von Mastzellen und basophilen Granulozyten durch einen hochaffinen IgE-Rezeptor (FcεRI) über seinen Fc-Teil gebunden werden (146). Gebundenes IgE wirkt als Antigenrezeptor und Basophile und Mastzellen können somit antigenspezifisch reagieren. Durch die gleichzeitige Antigenbindung an mehrere IgE-Moleküle kommt es zu einer Aktivierung der Zelle. Unter diesen Umständen sezerniert die Zelle Effektormoleküle, die den Wurm schädigen können. Zudem können IgE-Moleküle, die an den Wurm binden, durch eosinophile Granulozyten erkannt werden, die ebenfalls durch Ausschüttung lytischer Granulainhaltsstoffe den Parasiten schädigen und zu seiner Abtötung bzw. Ausscheidung beitragen können.

Hierbei spielt u.a. auch IL-5 eine Rolle, welches die Rekrutierung von Eosinophilen zum Infektionsort fördert (147). Außerdem können durch die Bildung von IL-13 Becherzellen in den Schleimhäuten (abhängig vom Infektionsort, Darm oder Lunge) zur Mukusproduktion angeregt werden (148,149). Der Schleim fördert dabei die Expulsion der Würmer. In einem solchen Th2-Milieu können Makrophagen entstehen, die keine Effektorfunktionen aufweisen, sondern zur Regeneration von Gewebe befähigt sind (150). Diese Makrophagen werden M2 oder alternativ aktivierte Makrophagen genannt. Im histologischen Schnitt unterscheiden sie sich von den klassisch aktivierten Makrophagen dadurch, dass sie größer sind und das Erscheinungsbild einer Schaumzelle aufweisen (151). In immunhistologischen Untersuchungen kann man alternativ aktivierte Makrophagen durch die Expression des Makrophagen-Mannose-Rezeptors (MMR oder CD206) und der Produktion von Arginase-1 identifizieren (150).

2.4 IL-4 und IL-13 – Liganden des IL-4-Rezeptors

Wie bereits dargelegt, ist IL-4 einer der wichtigsten Faktoren zur Induktion der Th2-Antwort. IL-4 bindet an den IL-4R (IL-4R Typ 1), der aus zwei Untereinheiten besteht, der IL-4Rα-Kette und der γc-Kette, diese teilt er sich mit weiteren Zytokinrezeptoren (z.B. dem IL-2R) (152). IL-4 bindet mit hoher Affinität an die IL-4Rα-Kette. Die IL-4Rα-Kette ist sowohl im IL-4R Typ1 als auch im IL-4R Typ 2 (IL-13R mit der IL-13Rα1-Untereinheit) zu finden (153). Die Signaltransduktion erfolgt über Januskinasen (JAK3 und JAK1 (IL-4R);

Tyk2 und JAK1 (IL-13R)) und den STAT6-Transskriptionsfaktor (Abb. 2.2) (153,154). Der IL-4R ist ubiquitär auf Zellen des Immunsystems und darüber hinaus vorhanden, wobei sich die Expressionsdichte zwischen den Zelltypen unterscheidet. Der IL-13R kommt nicht auf murinen T-Zellen vor, so dass IL-13 im Unterschied zu IL-4 nicht auf diese Zellen wirken kann (155).

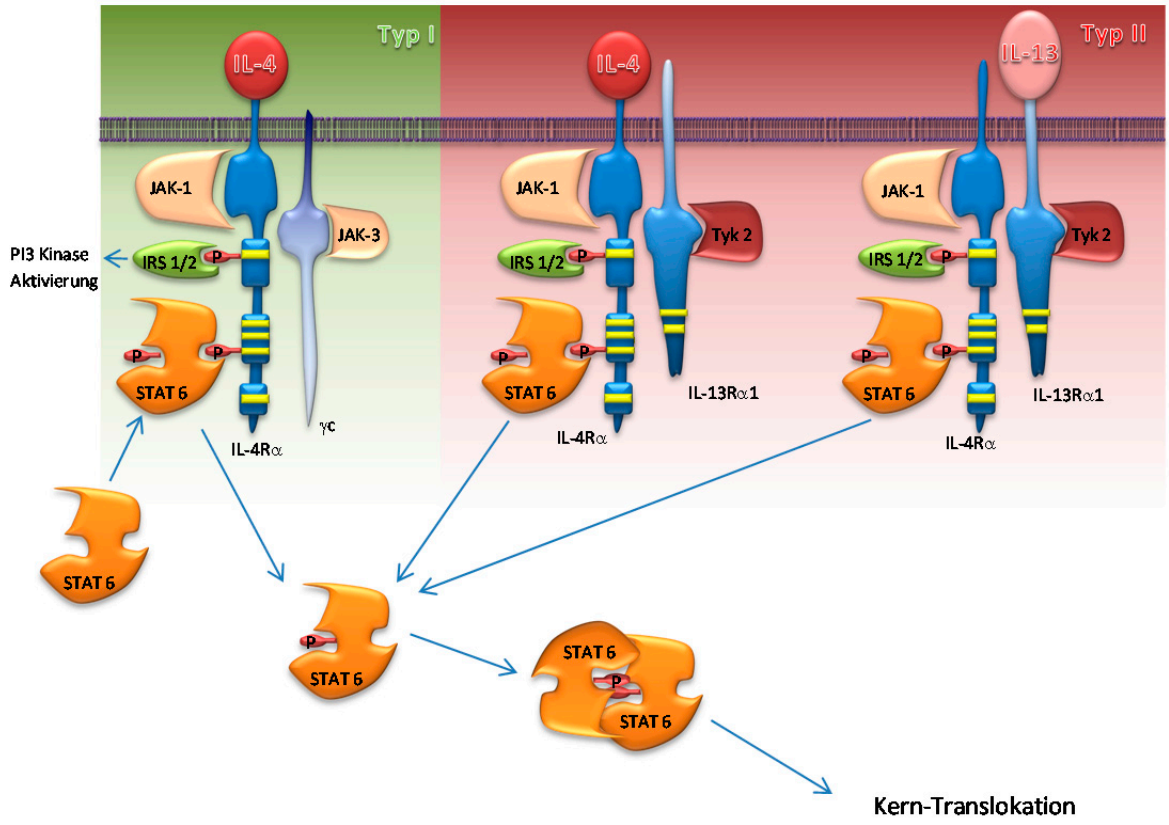

Abb. 2.2: Die IL-4R-Komplexe Typ I und Typ II. Der Typ I-IL-4R_besteht aus der IL-4Rα-Kette und der IL-2γc-Kette. Die Bindung des IL-4 erfolgt hierbei über die IL-4Rα-Untereinheit. Der Typ II-IL-4R besteht aus der IL-4Rα-Kette und der IL-13Rα1-Kette. Die Bindung des IL-13 erfolgt hierbei über die IL-13Rα1-Kette Der Typ II-IL-4R ist auch in der Lage IL-4 über die IL-4Rα-Untereinheit zu binden. Die Signaltransduktion wird über den Insulin-Rezeptor-Substrat-(IRS 1/2) und den JAK-STAT-Signaltransduktionsweg vermittelt. STAT6 wird phosphoryliert (P) und es bildet sich ein STAT6/STAT6-Homodimer aus, das in den Zellkern translozieren kann und dort die IL-4-/IL-13-abhängige Transkriptionen von Genen, wie z.B. Arginase-1 induziert. (modifiziert nach (153,156))

Zu den wichtigsten Effektormechanismen, die durch die Signaltransduktion über den IL-4R induziert werden, zählen die IgE-Produktion (156,157) durch B-Zellen und die alternative Aktivierung von Makrophagen. Es existieren IL-4R-abhängige und -unabhängige Mechanismen der Th2-Induktion, so dass auch in einem Individuum ohne IL-4R z.B. ein Isotypklassenwechsel zu einem Th2-Immunglobulin (Mensch IgG4, in der Maus IgG1) erfolgen kann (158). Der IL-4R ist von Bedeutung in der Abwehr von Würmern, wie z.B. dem Nematoden *Nippostrongylus brasiliensis* oder dem Trematoden *Schistosoma*

mansoni. Die Stimulation des IL-4R auf Makrophagen führt in diesen Modellen zu einer alternativen Aktivierung von Makrophagen über IL-4 und IL-13 (159). Dadurch wird die Th1-Immunantwort gedämpft und immunpathologische Effekte werden reduziert. In Infektionsmodellen mit intrazellulären Erregern, wie z.B. in einem Modell der Malaria mit dem Nagermalaria-Erreger *Plasmodium berghei* konnte gezeigt werden, dass Tiere, denen der IL-4R fehlt, eine erhöhte Resistenz gegen Plasmodien aufweisen (160).

Das murine Infektionsmodell der Leishmaniose weist in Bezug auf den IL-4R ein differenziertes Bild auf. In suszeptiblen Balb/c-Mäusen zeigt sich zu Beginn einer Infektion in Abwesenheit des IL-4R ein resistenter Phänotyp. Überraschenderweise ändert sich das Bild im Verlauf einer Langzeitinfektion, die Pathologie in den IL-4R-defizienten Mäusen nimmt zu. Im Gegensatz dazu sind T-Helferzell-spezifische IL-4R-defiziente Tiere geschützt, einhergehend mit einer geringeren Zahl an IL-10-produziereden Zellen, einer frühzeitigen Induktion einer IL-12-Antwort mit erhöhten IFN-γ-Spiegeln und verstärkter Bildung von Effektormolekülen (161). Dieses differenzierte Bild zeigt, dass IL-4 auf nicht-T-Helferzellen durchaus positive Effekte in der Leishmaniose haben kann, während der Effekt auf T-Helferzellen fatal ist. Die Th2-Immunantwort (d.h. die IL-4-Produktion) in verschiedenen Infektionen unterscheidet sich in ihrer Stärke.

2.5 Die Th2-Antwort bei Allergien und der Kryptokokkose

Die gegenüber extrazellulären Erregern schützende Immunreaktion kann in einem anderen Kontext Pathologien nach sich ziehen (Abb. 2.3). In allergischen Reaktionen des Respirationstrakts und bei Asthma treten ähnliche Reaktionen auf, wie in der Abwehr von Würmern (162). Diese sind allerdings fehlgeleitet und können zu Pathologien führen, da eine Infektion fehlt. Gegen eigentlich apathogene Substanzen (Allergene) wird eine starke lokale Immunreaktion induziert (163). Beispielsweise werden Mastzellen über allergenspezifische Rezeptoren (IgE) zur Ausschüttung von Histamin, Leukotrienen und Prostaglandinen angeregt (164,165). Infolgedessen kommt es zur Reizung der oberen Atemwege (166) und u.a. zur Konjunktivitis (165,167). Im Falle von Asthma kommen Schleimbildung in der Lunge und über muskarinerge Rezeptoren vermittelte Bronchokonstriktion hinzu (168). Für das hier präsentierte Forschungsfeld der Immunpathologie-assoziierten pulmonalen Mykose ist die Bedeutung von FcεRI-tragenden Zellen (z.B. Mastzellen und Basophilen) in der Kryptokokkose noch nicht abschließend geklärt. Im Rahmen der vorliegenden Arbeit sollten wichtige Th2-Zytokine (IL-4 und IL-13) und deren Zytokinrezeptoren (IL-4R Typ1 und Typ2) in der Immunpathologie der pulmonalen Kryptokokkose untersucht werden.

Die Induktion der Th2-Antwort steht gerade im aktuellen wissenschaftlichen Fokus, da neue Zelltypen entdeckt wurden, die an der Bildung von IL-4 beteiligt sind. In pulmonalen Wurminfektionen konnte gezeigt werden, dass wichtige frühe IL-4-Produzenten eosinophile und basophile Granulozyten sind (169-171). Die Entzündungszellen werden T-Zell-abhängig zur Lunge rekrutiert und sezernieren IL-4.

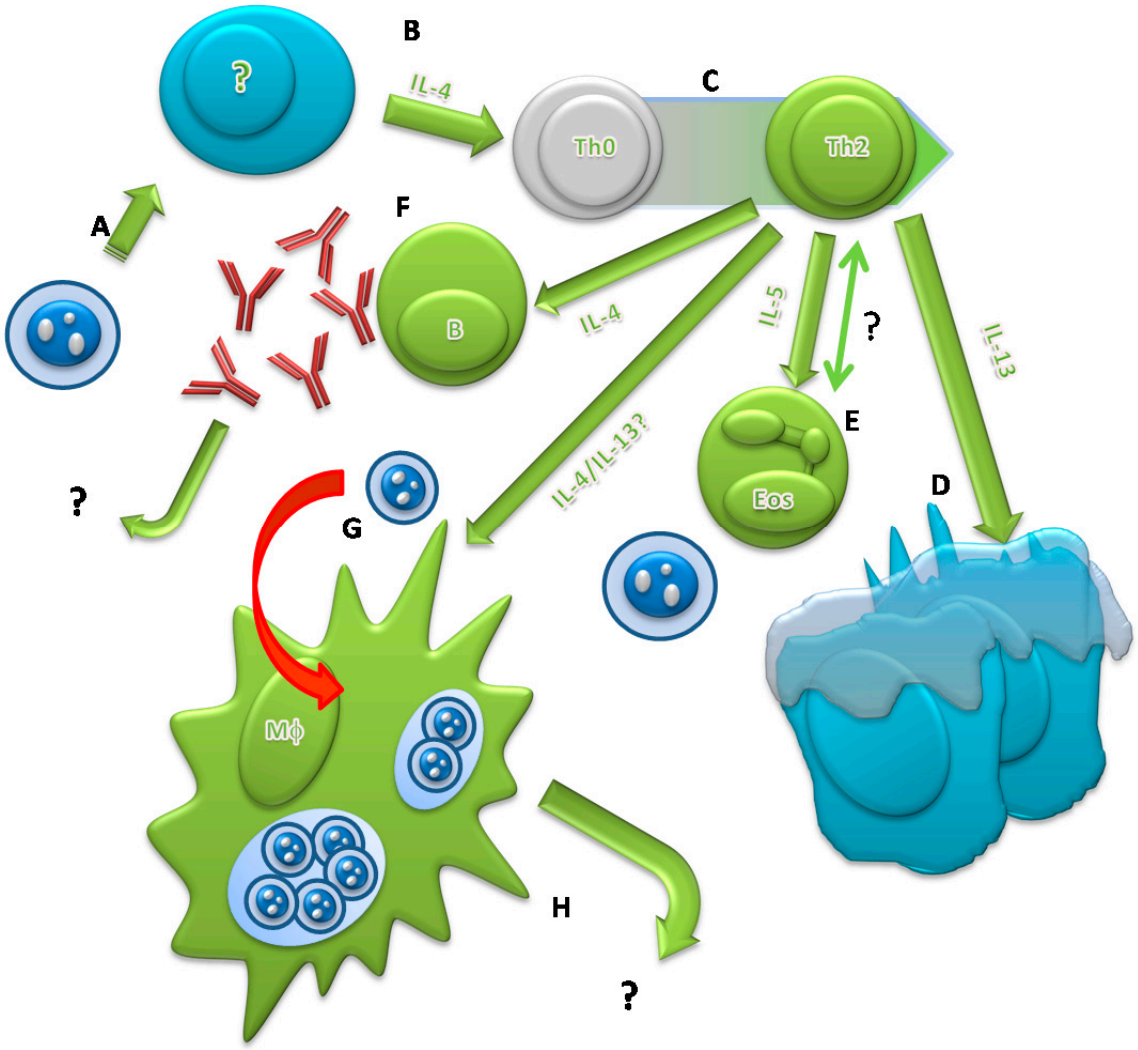

Abb. 2.3: Die pathologische Immunantwort gegen C. *neoformans*. Kryptokokken stimulieren unbekannte Zelltypen, die frühes IL-4 produzieren (A). IL-4 führt zu einer Ausdifferenziereung naiver Th-Zellen zu Th2-Zellen. Th2-Zellen bilden IL-4, IL-5 und IL-13. Becherzellen in der Lunge reagieren auf die Ausschüttung von IL-13 mit einer Schleimbildung, während Eosinophile durch IL-5 zum Infektionsort rekrutiert werden (E). Die Wechselwirkungen zwischen Th2-Zellen und Eosinophilen waren vor Erscheinen der in der vorliegenden Arbeit präsentierten Daten in der Kryptokokkose noch nicht abschließend geklärt. IL-4 führt in B-Zellen zu einem Isotypklassenwechsel hin zu IgG1 und IgE (F). Die Bedeutung von IgE in der Kryptokokkose ist nicht bekannt. Es kann sich um einen diagnostischen Marker, der nur ein Hinweis für den Grad der Immunpathologie ist, handeln oder um einen Pathogenesefaktor, der selber pathologische Effektormechanismen induziert. Makrophagen werden durch IL-4 alternativ aktiviert, die Beteiligung von IL-13 war bis dato noch unbekannt (G). Die Makrophagen nehmen Pilze auf, können sie aber nicht abtöten. Die Möglichkeit als *Shuttle* für eine Dissemination ins ZNS (H).

Insbesondere eosinophile Granulozyten sind wichtig in der Induktion einer Entzündung der Atemwege durch Bildung von Th2-Chemokinen und durch Rekrutierung von Th2-Zellen zum Infektionsort (172). Zudem scheinen Eosinophile in einem Th2-Milieu an der Gewebeschädigung der Lunge, Becherzellhyperplasie und Schleimbildung beteiligt zu sein (173,174).

Es gibt aber auch gegensätzliche Ergebnisse, die aufzeigen, dass in Wurminfektionen nicht eosinophile Granulozyten sondern andere Zellen für die Bildung von IL-4 wichtig sind (170). Aus diesem Grunde resultiert das Interesse für neu entdeckte frühe IL-4-/IL-13-Produzenten, die sogenannten *natural helper cells* (175-177). In diesem Zusammenhang zeigte sich, dass z.B. in der Abwehr von Helminthen oder im pathologischen Geschehen von Asthma Epithelien durch Sezernierung von neu entdeckten Zytokinen, wie z.B. *Thymic stromal lymphopoietin* (TSLP), IL-25 und IL-33 an der Induktion einer Th2-Antwort, beteiligt sind (178).

In einer Infektion mit einem intrazellulären Erreger, wie sie auch die pulmonale Kryptokokkose darstellt, ist eine Th2-Antwort ineffektiv. Da in einem solchen Milieu keine zytotoxischen T-Zellen (179) und Effektormakrophagen (180-182) entstehen, fehlen wichtige Abwehrmechanismen gegen den Erreger. Die Folge ist eine unkontrollierte Erregervermehrung und -ausbreitung (183). In Mausmodellen zur pulmonalen Kryptokokkose lassen sich die beschriebenen Immunpathologien ebenfalls beobachten (81,122,184,185). Mausstämme, die in einer pulmonalen Kryptokokkose eine schützende Th1-Antwort (einhergehend mit der Produktion von IL-12) induzieren, können die Infektion kontrollieren (87). Dagegen können Mausstämme, die eine Th2-Antwort entwickeln, die Infektion nicht kontrolieren, diese zeigen schwerwiegende Pathologien (122,184). In anderen intrazellulären Infektionen ist eine Th2-Antwort ebenfalls schädlich, wie am Beispiel der Leishmaniose des Hundes deutlich wird. Die Th2-Antwort führt dabei durch Immunpathologien zu schweren Komplikationen, wie z.B. einer Glomerulonephritis, die tödlich enden kann (186,187).

2.6 Die Rolle von multi-/polyfunktionalen Th-Zellen in der Immunantwort

Die Art der Immunantwort ist dafür entscheidend, ob ein Erreger effektiv bekämpft werden kann oder ob sich der Erreger unkontrolliert vermehrt (188). Die Bedeutung der T-Helferzellen als Mediatoren einer Th1-, Th17- oder Th2-Antwort ist dabei hoch. Bislang wurde davon ausgegangen, dass die übergreifende Bildung von Leitzytokinen, wie z.B. IFN-γ für die Th1-Antwort und IL-4 für die Th2-Antwort, alleine ausreichend wäre, um Schutz bzw. Immunpathologien zu induzieren. Wie sich aber durch neue Analysemethoden zeigt, ist nicht allein die Quantität der Zytokin-Produzenten von Bedeutung, sondern vielmehr die Qualität der T-Helferzellen. Unter Qualität wird hier verstanden, ob die T-Helferzellen gleichzeitig ein oder mehrere Zytokine produzieren (189). In Vakzinierungsversuchen konnte gezeigt werden, dass Zellen, die gleichzeitig IL-2 und IFN-γ bilden, eher in der Lage sind, zu Gedächtniszellen auszudifferenzieren, als solche, die nur IFN-γ bilden (189,190). Es konnte zudem beobachtet werden, dass Zellen, die gleichzeitig IL-2, IFN-γ und Tumornekrosefaktor alpha (TNF-α) bilden, höhere Mengen an IFN-γ bilden als Einzel- oder Doppelproduzenten (Abb. 2.4) . Ähnliches konnte auch für $CD8^+$ Gedächtniszellen ermittelt werden (191).

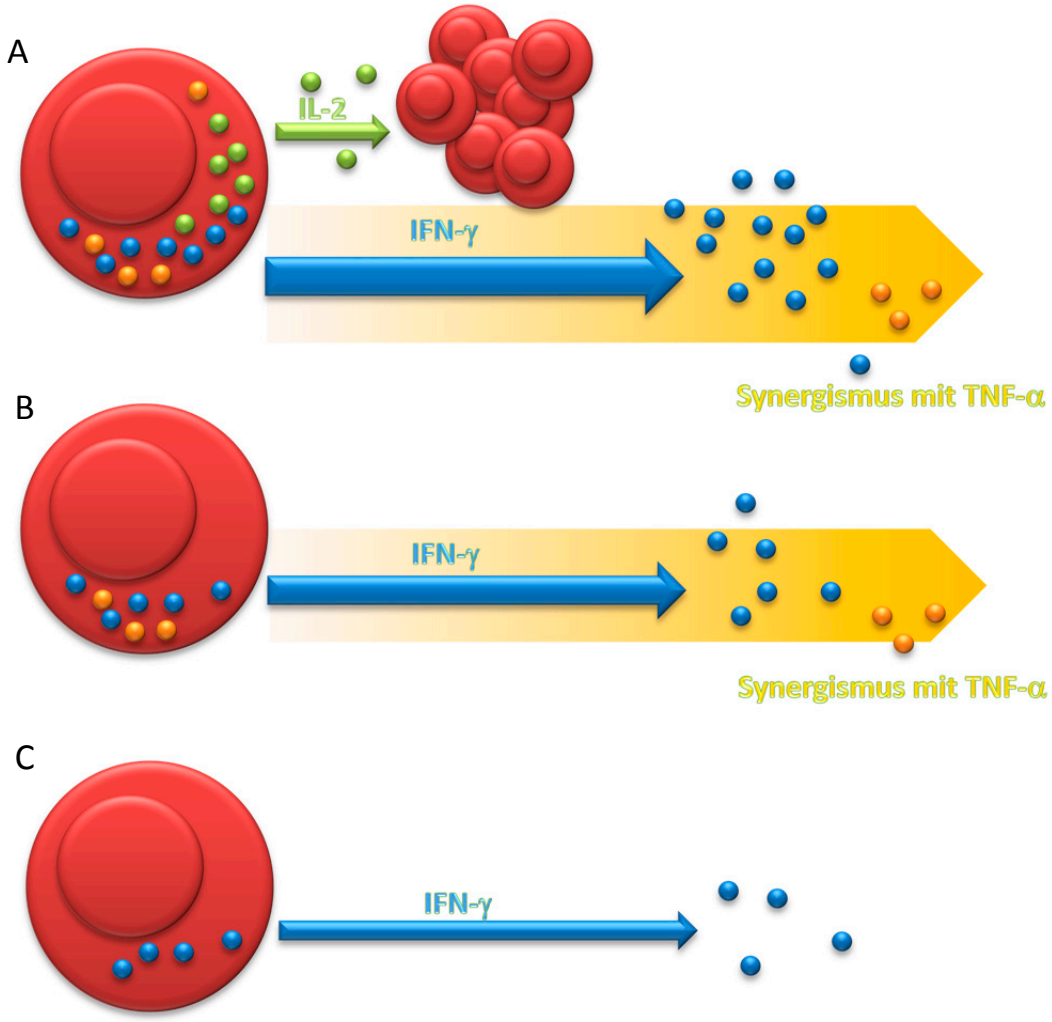

Abb. 2.4: Qualität der Th1 T-Helferfunktionen. (A) Polyfunktionale T-Helferzellen besitzen eine verstärkte Proliferationskapazität durch Sezernierung von IL-2 und der daraus resultierenden höheren Anzahl an Gedächtniszellen, zusätzlich zu einer synergistischen proinflammatorischen Antwort durch die Produktion großer Mengen an IFN-γ und TNF-α. Diese Zelle produziert zudem die größte Menge der jeweiligen Zytokine. (B) Effektorgedächtniszellen mit der Funktion synergistischer Antworten durch Sezernierung von IFN-γ und TNF-α. (C) Die T-Helfer-Effektorzelle sezerniert nur IFN-γ.

Im Falle der Th1-Gedächtniszellen kann man zwischen polyfunktionalen Zellen unterscheiden, die gleichzeitig IFN-γ, IL-2 und TNF-α bilden, Effektorgedächtniszellen, die IFN-γ und TNF-α bilden und ausdifferenzierten Effektorzellen, die nur IFN-γ bilden (192,193). Diese Dreifachproduzenten verfügen über zusätzliche Effektorfunktionen (synergistische Stimulation durch IFN-γ und TNF-α) und über eine verstärkte Proliferation (durch IL-2).

In einem *Leishmania*-Infektionsmodell konnte gezeigt werden, dass Vakzin-induzierte polyfunktionale Th1-Zellen zu einer verstärkten Resistenz gegenüber dem Erreger beitragen. Die Ausprägung der polyfunktionalen Th1-Zellen wird dabei durch das regulatorische Zytokin IL-10 kontrolliert (193). Wird IL-

10 während einer Immunisierung gegen *Leishmania*-Antigene gehemmt, kommt es zu einer verstärkten Ausschüttung von IL-12 durch dendritische Zellen und einem besseren Schutz vor einer anschließenden *Leishmania*-Infektion. Die Qualität der Th-Zellen konnte in diversen Publikationen für Th1-Zellen gezeigt werden (189,194-197), nicht jedoch für Th2-Zellen. Deshalb wurde in der vorliegenden Arbeit die Frage gestellt, inwieweit polyfunktionale Th2-Zellen an der Immunpathologie einer pulmonalen Kryptokokkose beteiligt sind.

2.7 Labormodelle der Kryptokokkose

Um die Interaktionen des Pilzes mit dem Wirt zu untersuchen, wurden eine Reihe von *in vitro*- und *in vivo*-Modellen etabliert. Die Vielzahl von Modellen reicht von Zellkulturen, wie z.B. Makrophagen (198-200) und dendritischen Zellen (201,202) über Invertebraten bis hin zu Wirbeltiermodellen. Mit Hilfe der Zellkulturmodelle kann man direkte Wirkungen des Pilzes auf Zellen ermitteln. Des Weiteren kann man überprüfen inwieweit Zellen in der Lage sind den Pilz z.B. zu phagozytieren oder zu eliminieren.

Da der Pilz ubiquitär in der Umwelt vorkommt, ist es sehr wahrscheinlich, dass er seine Evasionsmechanismen im Wechselspiel mit räuberischen Protozoen und Invertebraten, wie z.B. Amöben und Nematoden – natürlichen Fressfeinden des Pilzes – entwickelt hat (203) und diese nun auch im Wirbeltierwirt nutzt. Aus diesem Grunde werden Untersuchungen in Amöben wie z.B. *Acantamoeba castellanii* (204-207) und Nematoden wie z.B. *Caenorhabditis elegans* (206,208-210) durchgeführt. In diesen einfachen Modellen kann man u.a. Untersuchungen zur Melaninsynthese und Kapselbildung/-wachstum als Virulenzfaktoren und Evasionsmechanismen nach der Phagozytose durchführen (207). *C. neoformans* wirkt auch in diesen Modellen als Pathogen, da er in der Lage ist, die Wirte zu töten. Dabei nutzt der Pilz die gleichen Virulenzfaktoren, die er auch im Säugetier verwendet (203). In Insektenmodellen wiederum kann man Untersuchungen zum angeborenen Immunsystem, insbesondere zur Bedeutung der Phagozytose in der Pilzabwehr, durchführen. In einem Insektenmodell, welches Larven der Großen Wachsmotte (*Galleria mellonella*) verwendet (211-214), können Untersuchungen bei 37°C angestellt werden. Der Pilz wird in diesem Modell in das Haemocoel der Larve injiziert. Somit kann die Motte als Modell für die haematogene Streuung des Pilzes herangezogen werden. Der Pilz führt zum Tode der Larve, obwohl Haemozyten der Larve die Pilze phagozytieren. In diesem Modell werden antifungale Therapien, wie z.B. die Therapie mit Fluconazol in Verbindung mit anderen Medikamenten getestet (215). Auch *Drosophila melanogaster* wird als Modell herangezogen. Diese Fliege ist resistent gegenüber der Infektion mit Pilzen. Der Grund dafür ist ein System von Muster-Erkennungs-Rezeptoren, die Pathogen-assoziierte-Muster erkennen, der sogenannte Toll-Signaltransduktionsweg (216-218). Entsprechende Wege findet man auch im Säugetierwirt, so dass man im Fliegenmodell die Bedeutung der Mustererkennung für die Erregerabwehr studieren kann (219). Die meisten Untersuchungen werden allerdings in Wirbeltieren durchgeführt, allen voran in Mausmodellen (220). Die Maus eignet sich hierbei

besonders gut, da es charakterisierte Stämme gibt und eine Vielzahl an gendefizienten Mutanten, die helfen, wichtige Fragen der Immunantwort gegen *C. neoformans* aufzuklären. Es können hierbei lokale Wechselwirkungen (durch intranasale, intratracheale oder intrazerebrale Applikation (220)), aber auch systemische Infektionen (durch intraperitoneale oder intravenöse Infektion) untersucht werden. Neben der Maus werden auch Ratten für Versuche mit *C. neoformans* verwendet. Die Ratte bietet für gewisse Fragestellungen Vorteile gegenüber der Maus. Beispielsweise kann man bei der Ratte z.B. durch Punktion Zerebrospinalflüssigkeit gewinnen und am lebenden Tier bronchoalveoläre Lavagen durchführen (221-223). Zudem bietet sich dieses Modell an, da Ratten auch in freier Wildbahn eine chronische Kryptokokkose entwickeln können (123). Für Therapieuntersuchungen werden auch Meerschweinchen herangezogen (224,225). So wurde u.a. in Meerschweinchen untersucht, inwieweit die intravenöse Gabe antifungaler Therapeutika die Infektion mit *C. neoformans* verhindern kann (226). Zur Untersuchung der Kryptokokkenmeningitis werden wegen ihrer Größe Kaninchen herangezogen. Da Kaninchen gegen eine Kryptokokkeninfektion resistent sind, werden die Tiere durch Corticosteroide immunsupprimiert, wodurch sie chronische Meningitiden entwickeln (227,228). Zudem wird momentan ein Zebrafischmodell entwickelt, um allgemeine Fragestellungen zu einer Kryptokokkose im Wirbeltierwirt beantworten zu können (203). Das Zebrafischmodell hat einige Vorteile, so kann man durch künstliche Mutagenese von Fischembryonen eine Vielzahl von Mutanten erzeugen, die Pendants in menschlichen Erkrankungen haben. Auch ist die Zahl der möglichen Untersuchungsobjekte hierbei relativ hoch. Daher kann man durch die Verwendung von Zebrafischembryonen die Vorteile der Untersuchungen im Invertebraten in ein Wirbeltier übertragen. Dieses Modell bietet den Vorteil, dass dieses Tier über ein Immunsystem verfügt, wie wir es nur bei Wirbeltieren finden.

3 Publikationen

3.1 Einleitung zum Manuskript „IL-13 Induces Disease-Promoting Type 2 Cytokines, Alternatively Activated Macrophages and Allergic Inflammation during Pulmonary Infection of Mice with *Cryptococcus neoformans*"

Die schützende Immunantwort gegen *C. neoformans* geht mit der Induktion einer Th1-Antwort (unterstützt von einer Th17-Antwort (71,86,229)) und der Bildung von IFN-γ (230,231), IL-12 (87,232-234) und TNF-α (235,236) einher. Eine IL-4-abhängige Th2-Antwort hingegen führt zu einer Verstärkung der Krankheitssymptome (185,231) und zu einem unkontrollierten Wachstum des Erregers. Neben IL-4 gibt es weitere Th2-Zytokine, die eigene Wirkungsspektren besitzen, wie z.B. IL-13 (237), welches u.a. Becherzellen zur Schleimproduktion anregt. Die Wirkung von IL-13 in der pulmonalen Kryptokokkose war bis dato nicht bekannt.

Hauptergebnisse

- **IL-13 ist an der Immunpathologie der pulmonalen Kryptokokkose beteiligt.**
- **Das Überleben von Mäusen in der pulmonalen Kryptokokkose ist in Abwesenheit von IL-13 signifikant erhöht.**
- **Die Kontrolle des Kryptokokkenwachstums wird durch IL-13 gehemmt.**
- **Die Th2-Antwort wird durch IL-13 in der pulmonalen Kryptokokkose verstärkt.**
- **Damit einhergehend kommt es zu einer verstärkten IgE-Bildung.**
- **Zudem wird die Becherzellhyperplasie gefördert und die Schleimbildung in der Lunge angeregt.**
- **Die Hyperreagibilität der Bronchien wird in der pulmonalen Kryptokokkose durch IL-13 verstärkt.**
- **Die Bildung alternativ aktivierter Makrophagen in der Lunge ist in Abwesenheit von IL-13 stark reduziert.**
- **Die Eosinophilen-Rekrutierung wird durch IL-13 gefördert.**

Schlussfolgerungen

IL-13 spielt eine wichtige Rolle in der Immunpathologie der pulmonalen Kryptokokkose. Da IL-13 das Erregerwachstum fördert und pathologische Entzündungsreaktionen der Lunge, wie z.B. die Schleimbildung und Eosinophilie, verstärkt und bei einer IL-13-Depletion, trotz Anwesenheit von IL-4, die Th2-Antwort deutlich reduziert ist, stellt sich IL-13 als guter Kandidat für eine Immuntherapie dar (238).

Tierexperimente

Die Mäuse in der vorliegenden Arbeit wurden im Rahmen des Tierversuchsvorhabens Az. 24-9168.11 TVV15/05 (Landesdirektion Sachsen – Dienststelle Leipzig) verwendet.

3.1.1 Publikation 1: IL-13 fördert die Immunpathologie in der pulmonalen Kryptokokkose

IL-13 Induces Disease-Promoting Type 2 Cytokines, Alternatively Activated Macrophages and Allergic Inflammation during Pulmonary Infection of Mice with *Cryptococcus neoformans*[1]

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In the murine model of *Cryptococcus neoformans* infection Th1 (IL-12/IFN-γ) and Th17 (IL-23/IL-17) responses are associated with protection, whereas an IL-4-dependent Th2 response exacerbates disease. To investigate the role of the Th2 cytokine IL-13 during pulmonary infection with *C. neoformans*, IL-13-overexpressing transgenic (IL-13Tg$^+$), IL-13-deficient (IL-13$^{-/-}$), and wild-type (WT) mice were infected intranasally. Susceptibility to *C. neoformans* infection was found when IL-13 was induced in WT mice or overproduced in IL-13Tg$^+$ mice. Infected IL-13Tg$^+$ mice had a reduced survival time and higher pulmonary fungal load as compared with WT mice. In contrast, infected IL-13$^{-/-}$ mice were resistant and 89% of these mice survived the entire period of the experiment. Ag-specific production of IL-13 by susceptible WT and IL-13Tg$^+$ mice was associated with a significant type 2 cytokine shift but only minor changes in IFN-γ production. Consistent with enhanced type 2 cytokine production, high levels of serum IgE and low ratios of serum IgG2a/IgG1 were detected in susceptible WT and IL-13Tg$^+$ mice. Interestingly, expression of IL-13 by susceptible WT and IL-13Tg$^+$ mice was associated with reduced IL-17 production. IL-13 was found to induce formation of alternatively activated macrophages expressing arginase-1, macrophage mannose receptor (CD206), and YM1. In addition, IL-13 production led to lung eosinophilia, goblet cell metaplasia and elevated mucus production, and enhanced airway hyperreactivity. This indicates that IL-13 contributes to fatal allergic inflammation during *C. neoformans* infection. *The Journal of Immunology,* 2007, 179: 5367–5377.

The opportunistic pathogenic yeast *Cryptococcus neoformans* has become a major health problem for immunocompromised patients, especially AIDS patients, in the last two decades. In particular, the *C. neoformans* variants of the serotype A (*C. neoformans var. grubii*) and serotype D (*C. neoformans var. neoformans*) are of importance for these people (1). Dry feces of birds that contain the organisms can be inhaled and are a source of infection (2, 3). From the respiratory tract *C. neoformans* can disseminate into the body when the immune system lacks specific control mechanisms (4, 5). *C. neoformans* is able to invade the brain, where it causes severe meningoencephalitis (6, 7). The cells and factors of the immune system that help the host to control and eliminate this facultative intracellular yeast are still under investigation in human patients and in particular in murine infection models.

CD4$^+$ T cells that produce proinflammatory cytokines such as IFN-γ (8) are important for the induction of mechanisms which control cryptococcal growth and dissemination (9). Furthermore, the formation of granulomata serves as a major anti-cryptococcal control mechanism (10, 11). Besides CD4$^+$ T cells these granulomata contain macrophages, NK cells, and CD8$^+$ T cells (10). Phagocytic cells can engulf cryptococci (12, 13) and eliminate them by the production of reactive oxygen and in mice by producing nitrogen intermediates such as NO (14). The uptake of cryptococci is supported by complement factors that bind to the yeast surface (15–17) and by opsonizing Abs that are specific for Ags of their capsule (18–20). Effector cells such as NK cells and CD8$^+$ T cells are potent perforin/granulysin producers that destroy *C. neoformans*-infected cells and *C. neoformans* directly (21, 22). Therefore, mice that lack T cells or NK cells are more susceptible to *C. neoformans* infection than wild-type (WT)[5] mice (9, 23–25). The same phenomenon was observed in humans, where HIV-infected patients with advanced AIDS symptoms who have reduced numbers of CD4$^+$ T cells are susceptible to cryptococcosis, whereas immunocompetent people are resistant (26). Several groups have shown that a protective CD4$^+$ T cell immune reaction

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[5] Abbreviations used in this paper: WT, wild type; p.i., postinfection; aaMph, alternatively activated macrophage; caMph, classically activated macrophage; iNOS, inducible NO synthase; Tg, transgenic.

depends on a Th1 response (8, 9, 11, 27). IL-12 has been demonstrated to be essential for induction of such Th1 responses (11, 28–30). Mice which lack the ability to produce IL-12 or other members of the IL-12 family (e.g., IL-23) (31) showed reduced survival times when compared with WT mice (11).

Th2 responses are regulated by IL-4 and IL-13 (32–34). These two closely related cytokines (35, 36) share common functions such as the regulation of macrophages (37) and up-regulation of MHC class II molecules (38). IL-4 and IL-13 mediate their activity via a complex receptor system. The specific high-affinity receptors for IL-4 and IL-13 contain the IL-4Rα-chain (39) in combination with the γ_c chain or the IL-13Rα1/2 chains. Unlike IL-4 that can directly induce the differentiation of naive $CD4^+$ T cells into Th2 cells (type 1 receptor), IL-13 acts on human B cells, macrophages, respiratory epithelial cells and smooth muscle cells but not on T cells (type 2 receptor) (34). IL-4 and IL-13 also display other unique features (40–43). For example, IL-13 is important for the expulsion of gastrointestinal helminths from the gut, but IL-4 is not (33). Furthermore, the induction of allergic inflammation, e.g., asthma is associated with IL-13 and only to a much lower degree with IL-4 (42, 44, 45).

Both, IL-4 and IL-13 were found to induce development of alternatively activated macrophages (aaMph) (46). In experimental studies using the intracellular pathogen *Leishmania major* (47, 48), the switch from classically activated macrophages (caMph) to aaMph could be associated with development of susceptibility. These macrophages do not produce reactive nitrogen intermediates but are characterized by elevated levels of arginase-1, expression of IL-4R, mannose receptor, and YM1 induced by IL-4 or IL-13 (46). In aaMph arginase-1 successfully competes with inducible NO synthase (iNOS) for arginine, the substrate by which iNOS produces NO and other reactive nitrogen intermediates. Following infection of IFN-$\gamma^{-/-}$ mice with *C. neoformans* high numbers of aaMph were found that could be associated with susceptibility in cryptococcosis (49).

IL-4 has been shown to be responsible for susceptibility during murine cryptococcosis (11, 50, 51). However, the role of IL-13 in pathogenesis of *C. neoformans* infection has not been characterized yet. This study elucidates the role of IL-13 in pulmonary (with early local infection of the lung and late systemic infection) cryptococcosis. IL-13-deficient mice (IL-$13^{-/-}$) and IL-13-overexpressing transgenic (Tg) mice (IL-$13Tg^+$) demonstrate profound effects of IL-13 on the pathogenesis of murine cryptococcosis.

Materials and Methods

Mice

Six- to 10-wk-old female WT, IL-$13^{-/-}$ (33) as well as IL-$13Tg^+$ (52) mice on BALB/c background were maintained in an individually ventilated caging system under specific pathogen-free conditions and in accordance with the guidelines approved by the Animal Care and Use Committee of the Regierungspräsidium Leipzig. The murine IL-13 transgene was generated using the human CD2 locus control region to facilitate IL-13 expression from the IL-13 promoter in a T cell-restricted pattern (52). IL-$13Tg^+$ mice used in this study were backcrossed to BALB/c background for fifteen generations. Sterile food and water were given ad libitum. The mice were tested periodically for pathogens in accordance with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations accreditation board. All mice (including IL-$13Tg^+$ mice) were tested negative for pinworms and other endoparasites and ectoparasites.

C. neoformans *and infection of mice*

Encapsulated *C. neoformans*, strain 1841, serotype D was kept as frozen stock in skim milk and was grown in Sabouraud dextrose medium (2% glucose, 1% peptone; Sigma-Aldrich) overnight on a shaker at 30°C. Cells were washed twice in sterile PBS, resuspended in PBS, and counted in a hematocytometer. Inocula were diluted in PBS to a concentration of 2.5×10^4/ml for intranasal administration. Mice were infected by intranasal application of 10-μl volumes per nostril containing 500 CFU in total. For the intranasal infection mice were anesthetized with a 1:1 mixture of 10% ketamine (100 mg/ml; Ceva Tiergesundheit) and 2% xylazine (20 mg/ml; Ceva Tiergesundheit) i.p. administered.

The acapsular *C. neoformans* serotype D strain CAP67 was provided by Dr. B. Fries (Albert-Einstein College of Medicine, Bronx, NY), and cultivated and maintained in the same manner as strain 1841. This acapsular strain was heat-inactivated at 60°C for 1 h and used as a specific stimulus (10^7 cryptococci/ml) for restimulation of splenocytes from *C. neoformans*-infected mice.

Determination of survival rate and organ burden, and generation of lung homogenate supernatants for cytokine analysis

Infected mice were monitored daily for survival and morbidity. Organ burden was determined by removing organs (lung and spleen) under sterile conditions from sacrificed mice. Organs were weighed and dissected. Parts were homogenized in 1 ml of PBS with an Ultra-Turrax (T8; Ika-Werk Instruments). Serial dilutions of the homogenates were plated on Sabouraud dextrose agar plates, and colonies were counted after an incubation period of 72 h at 30°C. For lung cytokine analysis, supernatants of centrifuged lung homogenates were used.

Restimulation of spleen

The spleens were passaged through a 100-μm mesh in PBS to obtain a single-cell suspension. Thereafter erythrocytes were lysed using Gey's solution and cells counted using a hemocytometer. Single cells were resuspended at 5×10^6/ml in Iscove's (containing glutamine) medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were stimulated in medium alone as a control, with 5 μg/ml Con A, or with 1×10^7/ml heat-inactivated acapsulated *C. neoformans* strain CAP67. After 72 h, the supernatant was harvested and analyzed for production of several cytokines and NO.

Histopathological analysis

On day 60 postinfection (p.i.), intranasally *C. neoformans*-infected WT, IL-$13^{-/-}$, and IL-$13Tg^+$ mice, or uninfected mice of the respective genotypes, were perfused intracardially with 0.9% saline under deep CO_2 asphyxia. The lungs of the animals were removed, mounted on thick filter paper with Tissue Tek O.T.C. compound (Miles Scientific), snap-frozen in isopentane (Fluka), precooled on dry ice, and stored at −80°C.

For immunohistochemistry, 10-μm frozen sections were prepared in a serial fashion (15 transversal sections per lung on four consecutive levels). The following anti-mouse Abs were used for staining procedures: iNOS rabbit anti-mouse Abs (Alexis), CD206 (mannose receptor) rat anti-mouse Ab AdB Serotec, YM1 (eosinophil chemotactic factor-L) goat anti-mouse Ab (R&D Systems).

Immunohistochemistry was performed by use of the Vectastain Elite ABC kit (Vector Laboratories) with appropriate biotinylated secondary Abs. The peroxidase reaction product was visualized using 3,3′-diaminobenzidine (Sigma-Aldrich) as chromogen and H_2O_2 as co-substrate. Negative controls, without application of the primary Ab confirmed the specificity of the reactions. Arginase-1 (BD Biosciences) immunostaining was perfomed using DAKO Animal Research Kit Peroxidase, according to the manufacturer's protocol. Histopathological alterations were microscopically evaluated on H&E and immunostained lung sections.

Another part of the lung and the other organs were fixed in neutral-buffered formalin and embedded in paraffin. Sections were stained with H&E to estimate the extent of granulomatous lesion formation in the various organs or with periodic acid Schiff's reagent to study the distribution of cryptococci and mucus production by goblet cells in lung, liver, spleen and kidney. Study of collagen deposition of the organs was done by Elastica van Gieson staining. To study recruitment of granulocytes, the tissue sections were stained with naphthol AS-D-chloracetate.

Digestion of lung tissue and analysis of lung leukocytes

For recruitment studies lungs were perfused through the right ventricle with PBS. Once lungs appeared white, they were removed and one lobe of the lung was cut into small pieces. The dissected lung tissue was digested for 2 h at 37°C using a solution of collagenase A (0.7 mg/ml; Roche) and DNase (30 μg/ml; Sigma-Aldrich) in PBS with 0.1 μM sodium pyruvate (Fluka). The digested lung tissue was gently disrupted by subsequent passage through a 100-μm pore size nylon cell strainer (BD Biosciences) to gain a single-cell solution. Thereafter erythrocytes were lysed using Gey's

solution and cells were counted using a cell counter (Vi-CELL XR; Beckman Coulter). To differentiate between lung cells and cryptococci, the cells were counted twice after an incubation period of 10 min in aqua dest. In aqua dest, the lung cells are lysed but not the cryptococci. To gain the number of lung cells, the number of the second count was subtracted from the first one.

The recruitment of cells was analyzed by flow cytometry (FACSCalibur; BD Biosciences). The cells were stained for leukocytes (leukocyte common Ag CD45, clone HI30; BD Biosciences), Th cells (CD4, clone H129.19; BD Biosciences), CTLs (CD8, clone 53-6.7; BD Biosciences), B cells (CD45R, clone RA3-6B2; Caltag Laboratories), granulocytes (Gr-1, clone RB6-8C5; BD Biosciences), dendritic cells (CD11c, clone HL3; BD Biosciences), and macrophages (Gr-1$^-$/CD11b$^+$, clone M1/70.15; Caltag Laboratories).

Cytokine and Ab analysis

Cytokine concentrations were determined by sandwich ELISA systems with unlabeled capture Abs and labeled detection Abs. To determine the concentration of IL-4, mAb 11B11 was used as the capture Ab and biotin-labeled BVD6-24G2 (BD Pharmingen) was used as the detection Ab followed by incubation with peroxidase-labeled streptavidin. To determine the concentration of IFN-γ, mAb AN18 was coated as the capture Ab and peroxidase-labeled XMG1.2 was used in the detection step. The concentrations of IL-13, IL-5, TNF-α, and IL-17 were detected with the R&D Systems Duoset kits.

The total serum IgE concentration using a murine IgE standard (BD Pharmingen) was determined with R32-72 (BD Pharmingen) as capture Ab and a biotinylated anti-mouse-IgE Ab (R35-118; BD Pharmingen) was used for detection by incubation with peroxidase-labeled streptavidin. Total serum IgG1 and IgG2a were detected with unlabeled polyclonal goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates) as capture Abs, respectively. Biotinylated polyclonal goat anti-mouse IgG1 and IgG2a (Southern Biotechnology Associates) were used for detection by incubation with peroxidase-labeled streptavidin. For quantification of total serum IgG1 and IgG2a we used murine IgG1 and IgG2a standards (Southern Biotechnology Associates).

Measurement of respiratory function of the lung

The respiratory lung function of intranasally infected mice was measured in a plethysmographic chamber (model PLT UNR MS; emka Technologies) for freely moving animals. The pressure inside the chamber was measured by a differential pressure transducer connected to an amplifier (model AC264) and was continuously monitored through software (XA version 1.565). Changes in box pressure represent the difference between the thoracic expansion or contraction and the tidal volume (air removed from or added to the chamber during inspiration or expiration) (53). The box pressure is differentiated to give a pseudoflow signal, which is then analyzed by the software to give a Penh (enhanced pause) index. The Penh value is obtained for each respiration by the following formula: Penh = ((expiratory time − relaxation time)/relaxation time)(PE_{peak}/PI_{peak}), where PE_{peak} is peak expiratory pressure in milliliter per second, and PI_{peak} is peak inspiratory pressure in milliliter per second. Mice were exposed for 3 min to aerosolized 0.9% NaCl alone or supplemented with different methacholine concentrations (5, 10, and 20 μg/ml), produced by a sonicator (model LS 290–990N).

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 *U* test for organ burden and FACS analysis, and the two-tailed Mann-Whitney *U* test for cytokine and isotype levels as well as for the IgG2a to IgG1 ratio and airway hyperreactivity.

Results

Effect of IL-13 on survival and organ burden in pulmonary cryptococcosis

To elucidate the role of IL-13 in the pathogenesis of pulmonary cryptococcosis, we infected WT, IL-13Tg$^+$, and IL-13$^{-/-}$ BALB/c mice intranasally with 500 CFU of the highly virulent *C. neoformans* strain 1841 (11, 31) and observed the animals for a period of 150 days. WT and IL-13Tg$^+$ mice were susceptible with a median survival time of 81 and 72 days p.i., respectively (Fig. 1*A*). The difference in the survival time of susceptible WT and IL-13Tg$^+$ mice was highly significant ($p = 0.0026$). In contrast,

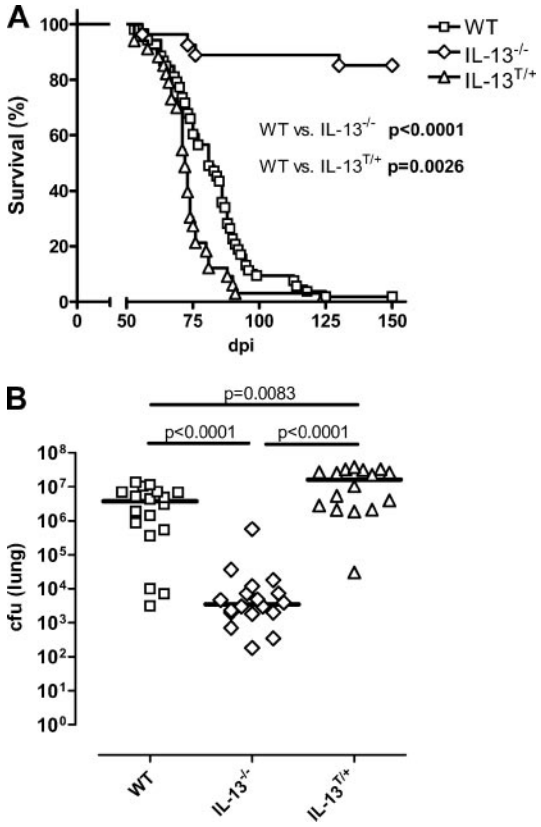

FIGURE 1. Mice are protected against *C. neoformans* in the absence of IL-13. Mice were intranasally inoculated with *C. neoformans (*500 CFU/mouse). Survival time and organ burden were analyzed. *A*, The survival time of WT, IL-13$^{-/-}$, and IL-13Tg$^+$ mice were examined for 150 days p.i. Differences between the genotypes were analyzed by log-rank test and are indicated. Pooled data from four independent experiments are shown (seven mice/group and experiment). *B*, On day 60 p.i., mice were sacrificed and the organ burden of lungs was examined. Pooled data from four independent experiments are shown. Significant differences are shown as indicated.

89% of IL-13$^{-/-}$ mice were resistant to the cryptococcal infection ($p < 0.0001$ comparing WT and IL-13$^{-/-}$ mice). These findings were paralleled by the organ burden of the primary infected organ, the lung (Fig. 1*B*). IL-13Tg$^+$ mice showed a significantly elevated fungal burden in the lung on day 60 p.i. in comparison to WT animals ($p = 0.0083$). In agreement with their elevated survival time, IL-13$^{-/-}$ mice had significantly lower fungal pulmonary burden than WT and IL-13Tg$^+$ mice ($p < 0.0001$) (Fig. 1*B*). Dissemination of *C. neoformans* from the lung to other organs occurred, e.g., to spleen, and similarly as in lungs, higher fungal organ burden was found there in the presence of IL-13 (data not shown).

C. neoformans *infection induces production of IL-13 that drives a pronounced type 2 cytokine and Ab isotype profile*

Elevated levels of IL-4 and IL-13 were found in lungs of susceptible genotypes on day 60 p.i., indicating *C. neoformans*-induced production of type 2 cytokines (Fig. 2*A*). Resistant IL-13$^{-/-}$ mice showed IL-4 levels in the lung that were comparable to levels found in lungs of the susceptible strains, indicating IL-4-independent but IL-13-dependent mechanisms for susceptibility to *C. neoformans* infection (Fig. 2*A*). However, IL-13$^{-/-}$ mice produced only minor levels of IL-4 and IL-5 by ex vivo stimulated splenocytes as compared with WT and especially IL-13Tg$^+$ mice (Fig.

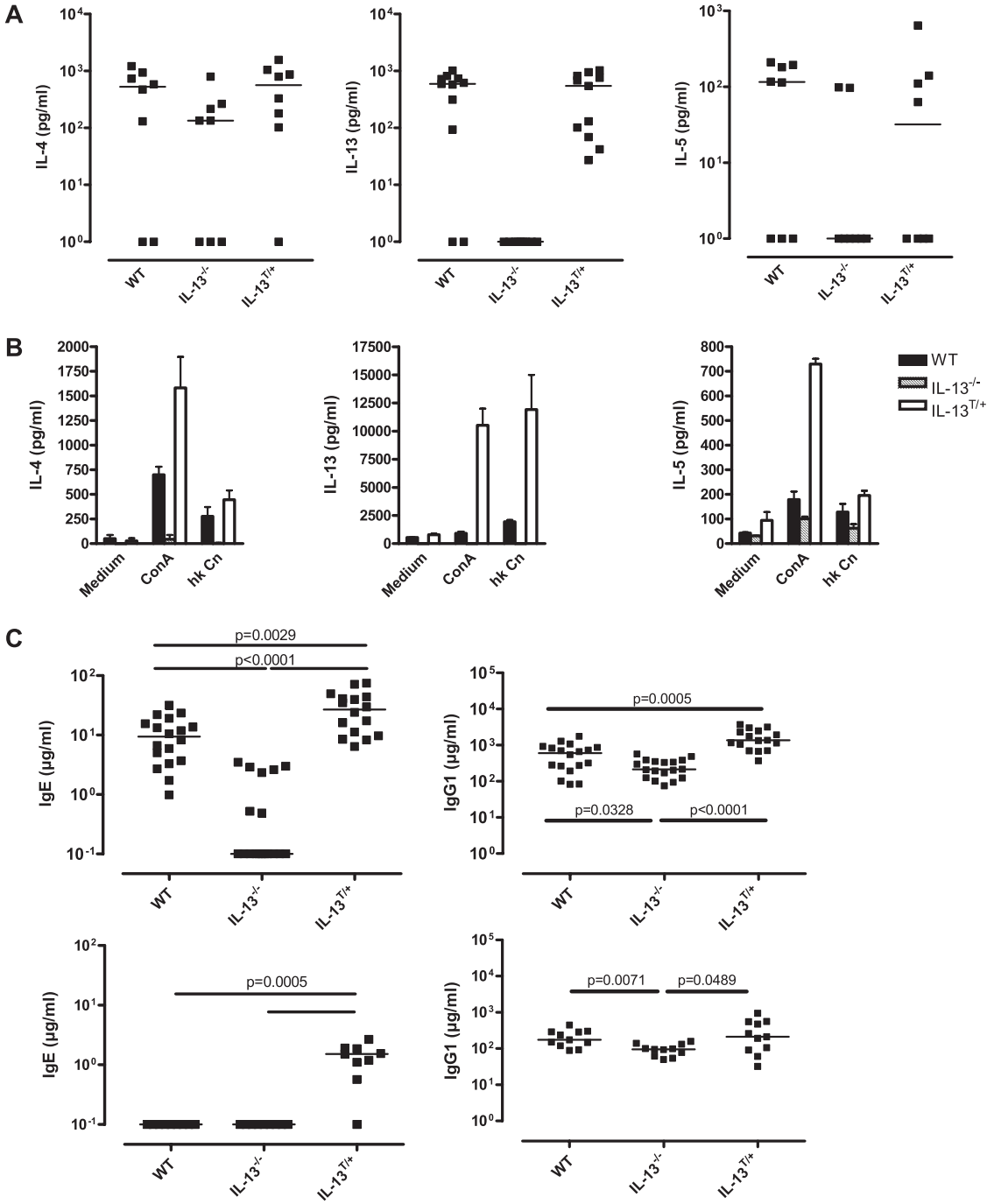

FIGURE 2. In the presence of IL-13 a Th2 phenotype in intranasal cryptococcosis is induced. *A*, WT, IL-13$^{-/-}$, and IL-13Tg^{+} mice were intranasally inoculated with *C. neoformans* (500 CFU/mouse) and sacrificed on day 60 p.i. The production of IL-4, IL-13, and IL-5 by lung cells was analyzed by ELISA. Data from two independent experiments are pooled. *B*, Splenocytes of infected mice were restimulated ex vivo for 72 h and examined for the production of IL-4, IL-13, and IL-5 by ELISA. In comparison splenocytes of naive mice were analyzed (data not shown). Splenocytes of the mice were pooled (5×10^6/ml) and stimulated in triplicates with ConA or heat-killed *C. neoformans* (hk Cn) for 72 h. Results from one representative experiment of two are shown. *C*, Serum of WT, IL-13$^{-/-}$, and IL-13Tg^{+} mice was analyzed for total IgE and IgG1 levels on day 60 p.i. (*top*) and compared with serum of naive mice (bottom). Data from four independent experiments, the same as in Fig. 1*B*, are shown. Significant differences are shown as indicated.

2*B*). The Con A-induced splenocyte response of these susceptible strains was characterized by pronounced production of IL-4, IL-13, and IL-5, indicating a type 2 cytokine bias that is enhanced by IL-13 (Fig. 2*B*). In response to *C. neoformans* Ag, IL-4 production by splenocytes of WT and IL-13Tg^{+} mice was comparable but IL-13 secretion much higher in IL-13Tg^{+} mice. Thus, reduced survival time and higher organ load in IL-13Tg^{+} mice shown in Fig. 1 were found to be associated with elevated production of IL-13 by ex vivo restimulated splenocytes. In splenocytes of naive mice, the production of these cytokines was marginal (data not shown), which demonstrates that the observed type 2 bias in WT and especially IL-13Tg^{+} mice is specifically induced by *C. neoformans* infection.

The cytokine profile of lung and spleen was reflected in the Ab isotype profiles of the examined genotypes. The genotypes with high type 2 cytokine levels developed high levels of IgE on day

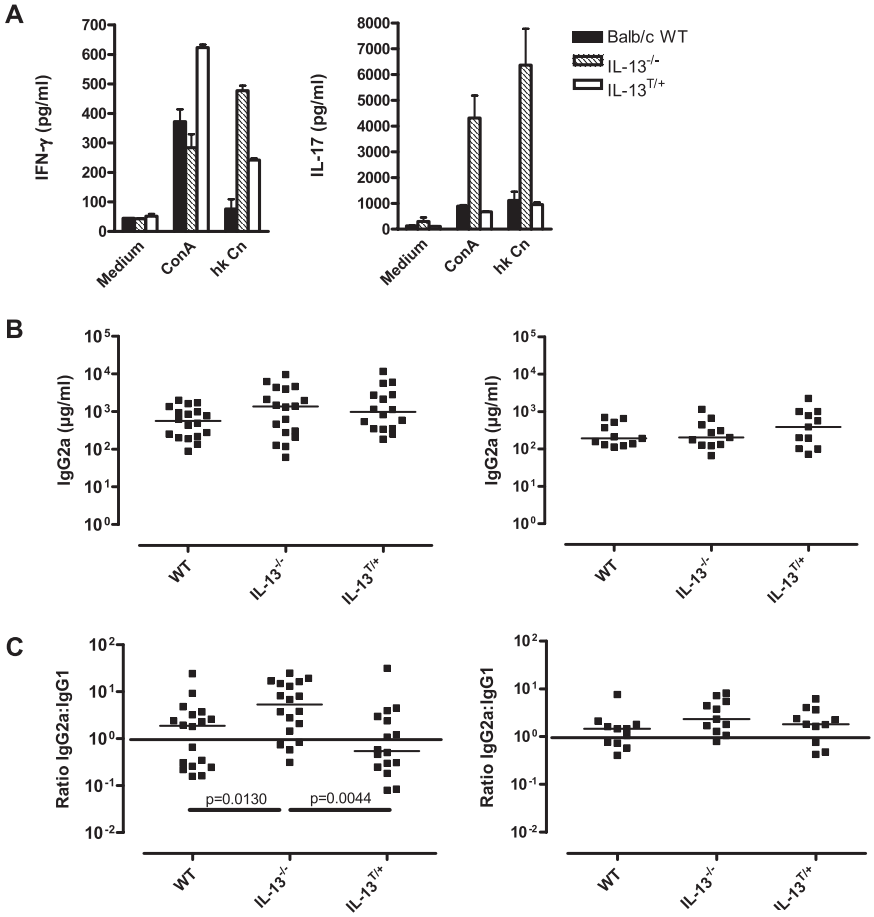

FIGURE 3. Resistant and susceptible mouse strains show only marginal differences in the Th1 response but pronounced differences in the Th17 response. *A*, Splenocytes of *C. neoformans*-infected mice (5×10^6/ml) were pooled and stimulated in triplicates for 72 h. IFN-γ and IL-17 was analyzed by ELISA. In comparison splenocytes of naive mice were analyzed (data not shown). One representative result of two is shown. *B*, Serum of WT, IL-13$^{-/-}$, and IL-13Tg^{+} mice was analyzed for total IgG2a levels on day 60 p.i. (*left*) and compared with serum of naive mice (*right*). Data from four independent experiments are shown. *C*, The ratio of IgG2a to IgG1 levels on day 60 p.i. (*left*) and in naive mice (*right*) was calculated. Data from four independent experiments, the same as in Fig. 1, are shown. Significant differences are as indicated.

60 p.i. in the serum (Fig. 2*C*), whereas only some IL-13$^{-/-}$ mice showed induction of IgE. However, the IgE-producing IL-13$^{-/-}$ mice did not develop higher pulmonary fungal loads than IgE-negative IL-13$^{-/-}$ mice (data not shown). IgE production was induced by *C. neoformans* infection because naive mice did not develop detectable serum IgE (Fig. 2*C*) except for IL-13Tg^{+} mice, which showed low production of IgE. A similar type 2 cytokine-dependent isotype profile is seen in the IgG1 levels of the different genotypes. Resistant IL-13$^{-/-}$ mice develop significantly lower IgG1 levels than susceptible wild-type and IL-13Tg^{+} mice after infection with *C. neoformans* (Fig. 2*C*).

IL-13 has only a minor role in IFN-γ regulation but inhibits IL-17 production

In contrast to the differential production of the type 2 cytokines IL-4 and IL-13, only low levels of IFN-γ were found in lung homogenates. However, pulmonary expression of IFN-γ did not differ between the resistant IL-13$^{-/-}$ mice and the susceptible WT and IL-13Tg^{+} mice (data not shown). In contrast, ex vivo restimulated splenocytes of day 60-infected mice (but not naive mice) secreted IFN-γ (Fig. 3*A*). Resistant and susceptible genotypes showed comparable production of IFN-γ with a slight increase of Th1 cytokines produced by the resistant IL-13$^{-/-}$ mice. The small increase in the Ag-specific secretion of IFN-γ by splenocytes derived from IL-13$^{-/-}$ mice apparently was not associated with elevated serum IgG2a (Fig. 3*B*). The Th1-dependent isotype IgG2a was induced by *C. neoformans* infection for all genotypes in the same manner (Fig. 3*B*). The IgG2a to IgG1 ratio, a marker for type 1 to type 2 responses, was found to be significantly higher for IL-13$^{-/-}$ mice than for the susceptible genotypes, indicating a type 1 bias for infected IL-13$^{-/-}$ mice ($p < 0.013$ WT vs IL-13$^{-/-}$ mice) (Fig. 3*C*). Comparing isotype levels of *C. neoformans*-infected mice with naive mice shows that it is mainly the type 2 cytokine-dependent isotypes IgE and IgG1, which are regulated by IL-13 (Fig. 2*C*).

Recently we showed that an IL-23-dependent Th17 response contributes to protection against *C. neoformans* infection (31). Thus, we were interested in characterizing the production of IL-17A by ex vivo restimulated splenocytes of resistant vs susceptible mouse strains. High amounts of IL-17A were produced by Con A- or Ag-stimulated spleen cells from resistant IL-13$^{-/-}$ mice but only low IL-17A production was found in splenocyte supernatants from susceptible WT and IL-13Tg^{+} mice upon infection with *C. neoformans* (Fig. 3*A*). Again, Ag-specific production of IL-17A by ex vivo restimulated splenocytes was marginal in naive mice (data not shown).

Pulmonary type 2 inflammatory response, lung eosinophilia, goblet cell metaplasia, and elevated mucus production in the presence of IL-13

The inflammatory response was analyzed histologically at day 60 p.i. Lung eosinophilia was detected in WT and with an even higher frequency in IL-13Tg^{+} mice but not at all in IL-13$^{-/-}$ mice (Fig. 4, *A–C*, open arrow). It is evident in the photomicrographs that the extent of the pulmonary inflammatory response was stronger in WT (Fig. 4, *A* and *D*) and IL-13Tg^{+} (Fig. 4, *C* and *F*) mice than in IL-13$^{-/-}$ mice (Fig. 4, *B* and *E*). In WT mice, a nodular inflammatory reaction comprising neutrophils, macrophages, eosinophils, and few lymphocytes was observed. *C. neoformans* was detectable inside of macrophages

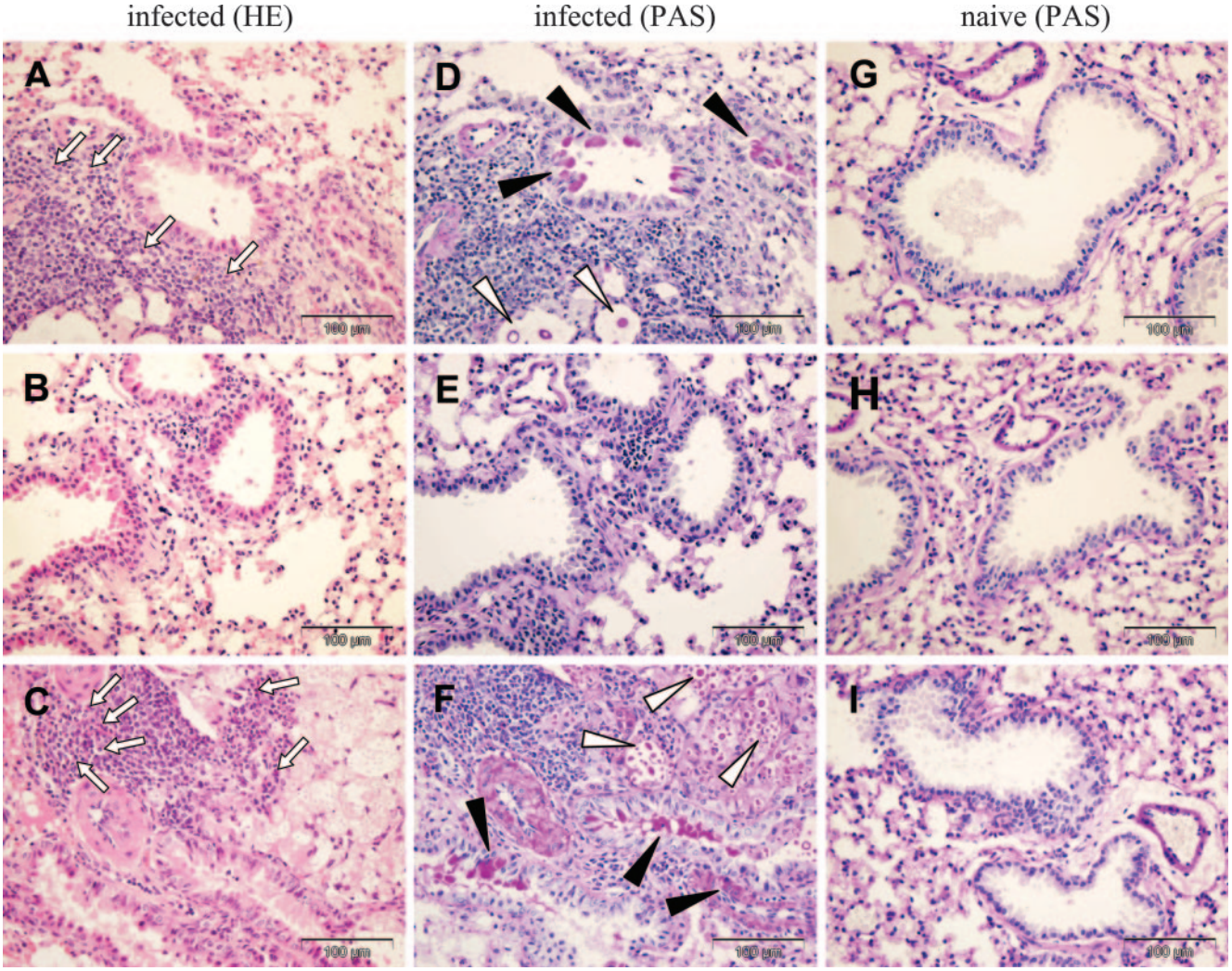

FIGURE 4. Susceptible WT and IL-13Tg$^+$ mice demonstrate pulmonary production of mucus and eosinophilia, and show elevated numbers of cryptococci in the lung. WT (*A* and *D*), IL-13$^{-/-}$ (*B* and *E*), and IL-13Tg$^+$ (*C* and *F*) mice were intranasally inoculated with *C. neoformans* (500 CFU/mouse) and sacrificed on day 60 p.i. Lungs were fixed in formalin and stained with H&E (*A–C*) for detection of eosinophils (arrows) or stained with periodic acid Schiff reagent (*D–F*) for mucus production (filled arrowhead) and cryptococcal capsule (open arrowhead), respectively. The lungs of infected and naive mice from the same genotype were compared (*G–I*; periodic acid Schiff's staining). Note staining of blood vessels in *G–I*. One representative photomicrograph per genotype is shown from a group of 17–18 mice of four independent experiments analyzed. Scale bar, 100 μm.

but also extracellular intra-alveolar proliferation of cryptococci was found in WT mice. In marked contrast cryptococci were found to be widely distributed in the alveoli of IL-13Tg$^+$ mice (Fig. 4, *C* and *F*) associated with frequent massive intra-alveolar lesions. The diffuse distribution of cryptococci in the lung of IL-13Tg$^+$ mice was accompanied by the appearance of several eosinophils, some neutrophils and macrophages with intracytoplasmic yeasts, and few lymphocytes (Fig. 4, *C* and *F*). Only a minimal inflammatory infiltrate was found in lungs of IL-13$^{-/-}$ mice (Fig. 4, *B* and *E*). All naive control mice revealed no inflammatory infiltrate in the lungs (Fig. 4, *G–I*).

Consistent with the histopathological analysis of lung sections, FACS staining of single lung leukocytes of day 60-infected mice revealed a similar pattern (Fig. 5*A*). The proportion of granulocyte, macrophage, and dendritic cell populations was elevated in susceptible WT and IL-13Tg$^+$ mice, whereas IL-13Tg$^+$ mice showed a significantly smaller Th cell population than WT and especially IL-13$^{-/-}$ mice (Fig. 5*A*). The number of total leukocytes was elevated in susceptible WT and IL-13Tg$^+$ mice, whereas a smaller leukocyte population was observed in the lungs of resistant IL-13$^{-/-}$ mice 60 days p.i. (Fig. 5*B*). Elevated absolute numbers of granulocytes, macrophages, and dendritic cells were detected in the presence of IL-13 (Fig. 5*B*). Interestingly, we observed a more moderate inflammatory response with respect to granulocytes and macrophages in the lungs of *C. neoformans*-infected IL-13$^{-/-}$ mice as compared with IL-4$^{-/-}$ mice pointing to distinct activities of IL-13 vs IL-4 in cryptococcosis (data not shown). When comparing lung leukocyte counts from mice infected for 60 days vs naive mice, we found no differences in the numbers of the various leukocyte subpopulations of IL-13$^{-/-}$ mice consistent with the observed minimal inflammatory infiltrate in this genotype at this time point (Fig. 4, *B* and *E*), but a more than 2-fold increase in the infected susceptible genotypes as compared with naive WT and IL-13Tg$^+$ mice (data not shown).

In addition, goblet cell metaplasia was found in WT (Fig. 4*D*) and IL-13Tg$^+$ (Fig. 4*F*) but not in IL-13$^{-/-}$ (Fig. 4*E*) mice. Pulmonary fibrosis was analyzed by Elastica van Gieson staining for collagen but it was neither evident in lungs of the highly susceptible IL-13Tg$^+$ and WT mice nor in the resistant IL-13$^{-/-}$ mice

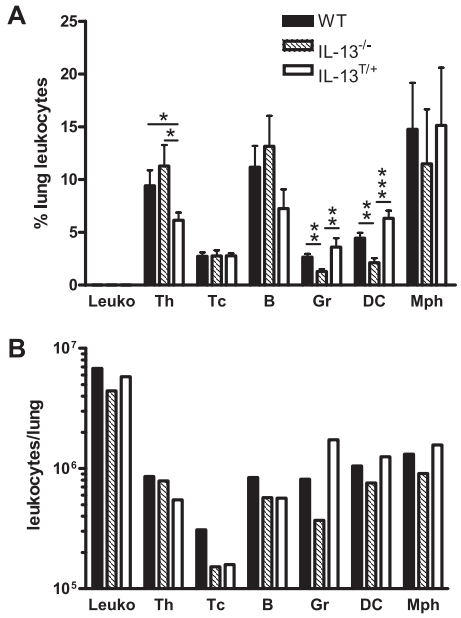

FIGURE 5. An elevated number of granulocytes, macrophages, and dendritic cells in the presence of IL-13 60 days after infection with *C. neoformans*. *A*, relative distribution of leukocytes in WT, IL-13$^{-/-}$, and IL-13Tg^{+} mice. Results from three independent experiments were pooled (three mice/group and experiment). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, The absolute number of lung leukocytes in WT, IL-13$^{-/-}$, and IL-13Tg^{+} mice. One of two similar experiments with the total number of leukocytes as indicated is shown. Cells from three mice/group were pooled, counted (corrected for the number of cryptococci in the samples as described in *Materials and Methods*), and analyzed by FACS analysis. B, B cells; DC: dendritic cells; Gr: granulocytes; Leuko, total leukocytes; Mph: macrophages; Tc, CD8^{+} T cells; Th, CD4^{+} T cells.

(data not shown). Minimal fibrosis was detectable only in the intraalveolar cryptococcosis regions but not in the peribronchial or interstitial lung tissue of IL-13Tg^{+} mice (data not shown).

Development of lung aaMph expressing arginase-1, mannose receptor (CD206), and YM1 in the presence of IL-13

IFN-γ-dependent activation of macrophages for fungicidal activation is essential for resistance against *C. neoformans* (14, 54). In lung macrophages of WT (Fig. 6*A*) and IL-13Tg^{+} (Fig. 6*C*), phagocytosed cryptococci were found. In addition, IL-13Tg^{+} mice showed strong extracellular multiplication of the yeast (Fig. 6*C*). In contrast, macrophages of IL-13$^{-/-}$ mice apparently had cleared most cryptococci at day 60 p.i. (Fig. 6*B*). This indicates insufficient killing mechanisms in the presence of IL-13. Recently, pulmonary macrophages of *Cryptococcus*-infected susceptible IFN-γ$^{-/-}$ mice were shown to be alternatively activated (49). Therefore, lung sections of naive and day 60-infected WT, IL-13$^{-/-}$, and IL-13Tg^{+} mice were stained for markers of caMph vs aaMph. Upon infection of susceptible WT and IL-13Tg^{+} mice with *C. neoformans* high numbers of enlarged lung macrophages expressing arginase-1 (Fig. 6, *D–F*), CD206 (Fig. 6, *G–I*), YM1 (Fig. 6, *J–L*) were detected. In addition, consistent with the observed low-level production of IFN-γ in WT and (IL-13Tg^{+} mice also expression of iNOS was found in these susceptible mouse strains (data not shown). As could be expected, the resistant genotype IL-13$^{-/-}$ with its strikingly reduced pulmonary inflammatory response (see Fig. 4, *B* and *E*) had only few and weakly stained lung macrophages expressing CD206, and almost no cells positive for YM1 or arginase-1 (Fig. 6, *E*, *H*, and *K*). Similarly, the frequency of iNOS expressing cells was minimal in lungs of IL-13$^{-/-}$ mice infected for 60 days (data not shown). However, 2 wk earlier in the course of infection (a time point with higher fungal burden in lungs of IL-13$^{-/-}$ mice as compared with day 60), some expression of iNOS in lungs of IL-13$^{-/-}$ mice was detectable (data not shown) suggesting increasing control of the pathogen by IL-13$^{-/-}$ mice with time and Ag load-driven iNOS expression. In summary, a high number of lung macrophages expressing arginase-1 together with CD206 and YM1 in WT and IL-13Tg^{+} mice indicate development of aaMph in the presence of IL-13.

Pulmonary hyperreactivity in the presence of IL-13

To study lung function in *C. neoformans*-infected mice, we examined naive and infected mice under resting conditions and increasing methacholine concentrations. We found that under resting conditions day 60-infected WT and IL-13$^{-/-}$ mice had a pulmonary resistance similar to naive mice (Fig. 7*A*). However, already in the resting state susceptible IL-13Tg^{+} mice on day 60 p.i. exhibited a significantly increased pulmonary resistance as compared with naive and even to the other infected mice strains (in particular as compared with infected WT mice) (Fig. 7*A*). This result is consistent with the high infection burden found in the lung of the IL-13Tg^{+} mice (Fig. 1*B*). Upon methacholine challenge naive animals of each genotype responded with a small dose-dependent increase in airway resistance (Fig. 7*B*). Infected IL-13$^{-/-}$ mice showed a mild increase in pulmonary resistance upon methacholine challenge, which was significantly lower than the methacholine responses observed in both, infected WT and IL-13Tg^{+} mice (Fig. 7*B*). These findings are consistent with high fungal burden and elevated mucus production in lungs of *C. neoformans*-infected WT and IL-13Tg^{+} mice.

Discussion

Previous experimental work from our group has shown that long-term survival of mice infected with the highly virulent *C. neoformans* strain 1841 can be either achieved by continuous administration of the Th1-inducing IL-12 or by suppression of the Th2-inducing cytokine IL-4 (11). In the present study we show an additional strategy allowing for long-term survival following *C. neoformans* infection: the suppression of IL-13 as another Th2 cytokine.

Indirect evidence for a potential role of IL-13 in cryptococcosis was provided by other earlier studies. Previously, treatment of *C. neoformans*-infected mice with anti-IL-4 mAb resulted in enhanced IFN-γ production and prolonged survival of mice (50). However, despite of the IL-4 mAb treatment IL-4 levels in broncheoalveolar lavage fluid remained elevated. Unchanged IL-4 synthesis upon neutralization of IL-4 might be an indication for IL-4-independent and potentially IL-13-dependent Th2 differentiation (50). Induction of IL-13 during cryptococcosis was reported in a few recent studies (49, 55, 56). However, the functional role of IL-13 during cryptococcosis has not been directly addressed.

We have approached the functional characterization of IL-13 in *C. neoformans* infection by analyzing IL-13 gene-deficient mice and IL-13-overexpressing mice in comparison to WT mice. Our results demonstrate for the first time a role of IL-13 in susceptibility during cryptococcal infection: IL-13 expression leads to greatly reduced resistance as evidenced by reduced survival and higher pulmonary fungal burden, goblet cell metaplasia, mucus production, and elevated airway hyperreactivity. Moreover, our results point to several mechanisms by which IL-13 negatively

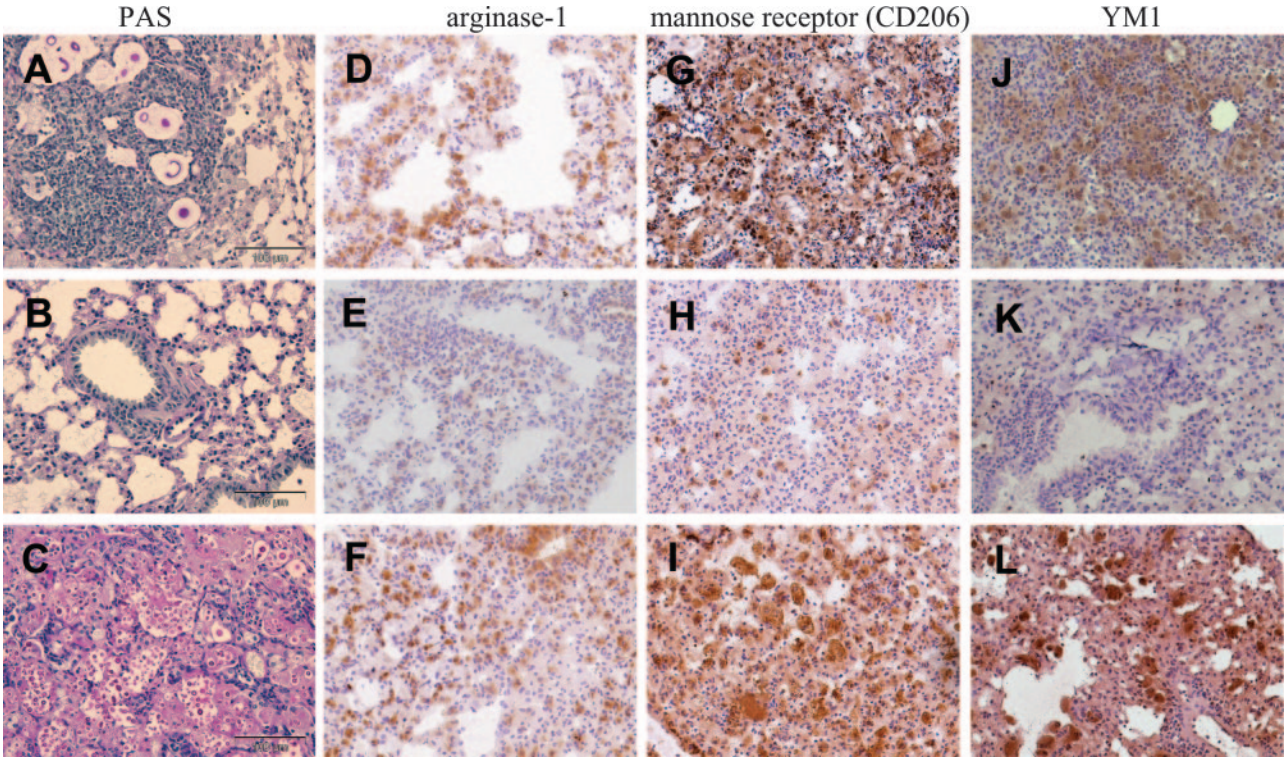

FIGURE 6. The aaMph develop in lungs of susceptible WT and IL-13Tg$^+$ mice after infection with *C. neoformans*. WT (*A*, *D*, *G*, and *J*), IL-13$^{-/-}$ (*B*, *E*, *H*, and *K*), and IL-13Tg$^+$ (*C*, *F*, *I*, and *L*) mice were intranasally inoculated with 500 CFU and sacrificed on day 60 p.i. The distribution of cryptococci in lungs of WT (*A*), IL-13$^{-/-}$ (*B*), and IL-13Tg$^+$ (*C*) mice is shown on day 60 p.i. in periodic acid Schiff (PAS) reagent-stained sections. Note the diffuse extracellular multiplication of *C. neoformans* in lungs of IL-13Tg$^+$ mice (*C*). In addition, lungs were embedded into Tissue Tek, and cryosections were stained with anti-arginase-1 (*D–F*), anti-mannose receptor (CD206) (*G–I*), or anti-YM1 (*J–L*) at original magnification of ×50. In lungs of naive mice of all three groups, no expression of markers typical of aaMph was found (data not shown). Representative organ sections from two independent experiments are shown (five mice/group and experiment).

affects lung function during cryptococcosis. These IL-13-dependent mechanisms include 1) enhanced expression of type 2 cytokine; 2) development of aaMphl; 3) modulation of Th17 responses; and 4) modulation of goblet cell function.

One major mechanism of development of susceptibility to *C. neoformans* appears to be the IL-13-dependent enhancement of type 2 cytokine responses. As shown for leishmanial infection IL-13 is a key factor in the induction of a Th2 response even in the absence of IL-4 expression (57, 58). In our study we could detect elevated levels of IL-4, IL-13, and IL-5 in WT mice and especially in IL-13Tg mice. This type 2 cytokine profile was also evident in the increased serum IgG1 and IgE levels of the susceptible IL-13-expressing genotypes. Mechanistically IL-13-driven enhancement of Th2-dependent IgG1 and IgE must be based on indirect mechanisms because a functional receptor for IL-13 is not expressed on murine T cells (59). Together, type 2 cytokine production, generation of Th2-dependent isotypes and development of lung eosinophilia present in WT and IL-13Tg$^+$ mice can be considered as processes typical of susceptibility to *C. neoformans* infection (55, 60). Type 2 or "allergic" inflammation was found to be enhanced in rats with chronic pulmonary *C. neoformans* infection (56). This recent publication points to a link between chronic exposure to cryptococcal Ags and asthma. Infected rats showed a predisposition to asthma after pulmonary infection with *C. neoformans*. In our infection model we could see a similar phenomenon. Susceptible IL-13-expressing WT mice showed symptoms typical of asthma after exposition to an asthma inducer only when they were previously infected with *C. neoformans* but not in a naive state (Fig. 7). This allergic status is supported by infection-induced elevated IgE levels.

It is noteworthy that the aforementioned markers of IL-13-driven type 2 cytokine production can be closely linked with susceptibility but apparently no direct inverse correlation is possible between IL-13 and protective Th1 development as based on production of IFN-γ or serum IgG2a (see Fig. 3). In agreement with our data, in another pulmonary *C. neoformans* infection model a similar observation was reported. No effect of IL-4 and IL-10 on IFN-γ induction could be found in that study (55). IFN-γ production was found to be similar between resistant and susceptible mouse strains in cryptococcosis. Thus, the cytokines more important for the course of pulmonary cryptococcosis seem to be the type 2 cytokines. It is only in the absence of these cytokines that IFN-γ is able to act in a protective manner. IL-13 has been found to down-regulate IL-12Rβ2 expression (61). We have analyzed lung mRNA expression of IL-12Rβ1 and IL-12Rβ2 (as well as IL-12p35 and p40) in the absence and presence of IL-13 expression at 60 day p.i., but found no obvious correlation with resistance or susceptibility to *C. neoformans* (data not shown). This is consistent with minor differences in IFN-γ production and serum IgG2a (see Fig. 3) and similar levels of lung IFN-γ in supernatants of lung homogenates (data not shown) of resistant IL-13$^{-/-}$ mice and susceptible WT and IL-13Tg$^+$ mice at day 60 p.i. Moreover, expression of iNOS by pulmonary macrophages was found to be independent of their susceptible or resistant phenotype at day 60 p.i. (data not shown). In summary, these observations support the hypothesis that the presence of type 2 but not type 1 cytokines critically regulates immunity to pulmonary *C. neoformans* infection.

In contrast, our data point to an association of Th17 responses and protection in cryptococcosis. Significant T cell-derived IL-17

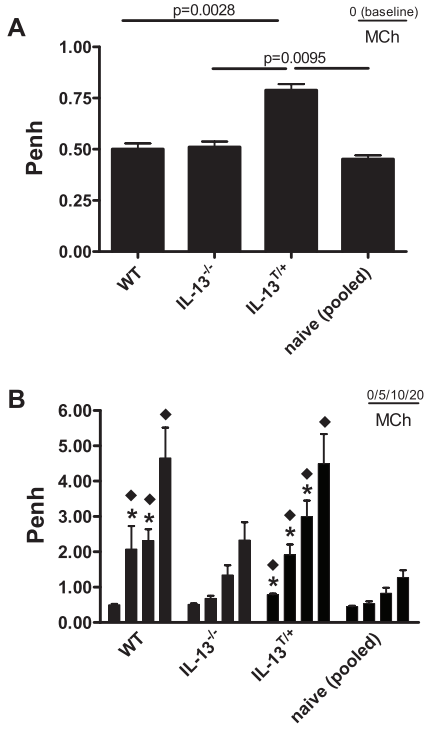

FIGURE 7. Elevated pulmonary hyperreactivity is seen in susceptible *C. neoformans*-infected mice. *A*, The baseline of the pulmonary resistance of *C. neoformans* infected WT, IL-13$^{-/-}$, and IL-13Tg^{+} mice was measured in comparison to naive mice that were pooled, independent of their genotype. Results from two independent experiments were pooled (n = 5–8 mice). *B*, The mice as shown in *A* were exposed to increasing levels of methacholine (MCh), and the pulmonary resistance was measured. *, $p < 0.05$ compared with IL-13$^{-/-}$ mice with indicated methacholine levels. ◆, $p < 0.05$ compared with naive mice at indicated methacholine levels.

was detected in resistant mice as compared with susceptible IL-13 expressing mice. Thus, IL-13 expression appears to down-regulate Th17 responses. A possible link between IL-13 and IL-17 was described in a model of autoimmune inflammation of the brain (i.e., experimental allergic encephalomyelitis) (62). Mice supplemented with IL-25 produced elevated levels of IL-13 that suppressed the production of Th17 cells resulting in complete suppression of Th17-induced experimental allergic encephalomyelitis disease. Recently, we could show in an i.p. model of *C. neoformans* strain 1841 infection that the protective function of IL-23 is associated with IL-17 production (62). In the present pulmonary model the elevated levels of Ag-specific IL-17 produced by splenocyte cultures (Fig. 3) and lung cell cultures (data not shown) of resistant strains point to a role of Th17 responses in local pulmonary cryptococcal infection. By infecting IL-17A$^{-/-}$ mice intranasally with *C. neoformans*, we obtained evidence for a significant role of IL-17 for survival (U. Müller and G. Alber, unpublished observations). The mechanism of IL-17-dependent protection is still under investigation. Further support for a protective activity of IL-17 was provided very recently. In an allergic asthma model it could be shown that IL-17 treatment reduces pulmonary IL-5 and IL-13 production and the recruitment of eosinophils (63). Both these parameters are consistent with data from our study (e.g., elevated splenocyte IL-17 production and diminished lung eosinophilia in the absence of IL-13).

Activation of macrophages harboring cryptococci is a central effector mechanism in immunity to *C. neoformans* (30, 64, 65). Whereas in mice T cells and B cells only express IL-4R type 1 and therefore can only respond to IL-4, macrophages express IL-4R type 2, which can respond to IL-4 and also IL-13 (59). Therefore, both IL-4 and IL-13 have the potential to induce development of aaMph. In the present study we could detect markers specific for aaMph such as arginase-1, mannose receptor (CD206), and YM1 in macrophages of susceptible IL-13-producing strains but not in IL-13$^{-/-}$ mice. This demonstrates that IL-13 is able to induce the development of aaMph in *C. neoformans* infection and links high fungal lung burden (see Fig. 1) with insufficiently fungicidal pulmonary macrophages. As shown earlier susceptible mice such as IFN-$\gamma^{-/-}$ mice (which were found to produce IL-13) also show evidence of alternatively activated macrophages in pulmonary cryptococcosis as judged by arginase-1 vs iNOS expression and expression of eosinophilic crystalline protein YM1 (49). Development of aaMph appears to be a common feature of and maybe a marker for susceptibility to cryptococcosis. Very recently it could be shown that chitin, which is an important component of the cell wall of *C. neoformans* (66), is able to induce the accumulation of IL-4-producing innate immune cells, e.g., eosinophils and basophils, in tissue and mediates the activation of alternatively macrophages (67). Interestingly, in our study development of aaMph was not associated with significant fibrosis in contrast to studies looking at e.g., *Schistosoma mansoni* (68). The lack of a fibrotic response in our study is different from *C. neoformans*-infected (serotype D) IFN-$\gamma^{-/-}$ mice on a C57BL/6 background where some lung fibrosis was found by Masson's trichrome staining of lung sections (49).

In summary, IL-13 plays an immunopathological role that adds to the fatal action of IL-4, which has been observed in studies looking at *C. neoformans* infection and other pathogens (11, 50, 51, 58, 61). IL-13 but not IL-4 can activate goblet cells to produce mucus that is beneficial in the gut to expulse helminths (69) but can be deleterious in experimentally induced asthma (44). By analyzing *Cryptococcus*-infected IL-13Tg mice together with WT and IL-13$^{-/-}$ mice, we could identify IL-13-specific actions. In several parameters (e.g., lung fungal burden, survival period, lung eosinophilia, inflammatory response of the lung) we found a gradual increase of these parameters in IL-13Tg^{+} mice as compared with WT mice, suggesting a quantitative correlation between IL-13 production and disease severity. In addition, using IL-13Tg^{+} mice and comparing these mice with IL-13$^{-/-}$ mice was important to us because it has been shown that the IL-13$^{-/-}$ mice used in this study may be partially defective in IL-4 gene expression due to disrupting a potential regulatory element of the IL-4 gene by the IL-13 gene targeting. Therefore, we determined IL-4 production in lung and spleen cells of IL-13$^{-/-}$ mice and compared it with WT mice. Fig. 2*A* shows similar levels of IL-4 produced in lungs of infected WT and IL-13$^{-/-}$ mice. In contrast, IL-4 levels secreted by splenocytes of IL-13$^{-/-}$ mice were found to be lower than in WT mice. Also, serum IgE levels were reduced in IL-13$^{-/-}$ mice as compared with WT mice. Therefore, comparing WT and IL-13Tg^{+} mice enabled us to draw conclusions on a specific role of IL-13 independent of IL-4. Based on this comparison, we were able to show a correlation between higher pulmonary fungal load (see Fig. 1) and elevated Ag-specific IL-13 production (see Fig. 2*B*), whereas IL-4 production was similar in WT and IL-13Tg^{+} mice.

In our present study for the first time to our knowledge the individual contributions of the Th2 cytokine IL-13 for fatal allergic inflammation and development of aaMph during *C. neoformans* infection have been characterized. Future therapeutic strategies have to consider IL-13 or target the IL-13-specific receptor component IL-13Rα1 or the receptor component common for IL-13 and IL-4, IL-4Rα. Neutralizing IL-13 or blocking its receptor should be a promising therapy for long-lasting

protection during *C. neoformans* infection in human patients as well.

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Disclosures

The authors have no financial conflict of interest.

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3.2 Einleitung zum Manuskript „Eosinophils Contribute to IL-4 Production and Shape the T-Helper Cytokine Profile and Inflammatory Response in Pulmonary Cryptococcosis"

Die Beteiligung von IL-4 und IL-13 an der Immunpathologie der pulmonalen Kryptokokkose konnte durch die eigenen Studien (87,238) und Arbeiten anderer Arbeitsgruppen (185,231) geklärt werden. Allerdings stellte sich die Frage nach den Produzenten der Th2-Zytokine. Hierbei war IL-4 von besonderem Interesse, da bekannt ist, dass es die Th2-Induktion fördert. Aus diesem Grunde sollte untersucht werden, welche Zelltypen an der IL-4-Bildung während einer Kryptokokkose beteiligt sind und zu welchem Zeitpunkt die Bildung stattfindet.

Hauptergebnisse

- **Th-Zellen und Eosinophile sind die Hauptproduzenten des IL-4 in der Lunge von *C. neoformans*-infizierten Mäusen (in anderen Zelltypen konnte keine IL-4-Bildung nachgewiesen werden).**
- **Sechs Wochen nach Infektion konnte frühestens eine IL-4-Bildung nachgewiesen werden.**
- **Dazu passt die späte IgE-Bildung, die erst mit Beginn der IL-4-Produktion einsetzt. Zu diesem Zeitpunkt beginnt auch die Dissemination des Erregers in periphere Organe (Daten nicht gezeigt)).**
- **Die Zahl an Th-Zellen und Eosinophilen steigt im Lungenparenchym parallel an.**
- **Die Depletion von Eosinophilen und die daraus resultierende Abnahme der IL-4-Spiegel zeigen, dass dieses IL-4 nicht durch andere zelluläre Quellen kompensiert wird.**
- **Daraus ergibt sich, dass Eosinophile eine wichtige Bedeutung in der Ausprägung des Zytokinprofils und der daraus resultierenden Th-Antwort haben.**

Schlussfolgerungen

Eosinophile und Th-Zellen sind die IL-4-Quellen in der pulmonalen Kryptokokkose. Es erfolgt eine gemeinsame Rekrutierung in die Lunge, die zeitabhängig während der Infektion erfolgt. Eosinophile scheinen die Th2-Antwort in der Infektion zu modulieren (Zytokin-Bildung und Rekrutierung von Leukozyten), da die Depletion von Eosinophilen zu einer Verringerung der IL-4-Bildung führt, die nicht durch Th- oder andere Zellen kompensiert wird.

Die Fragen zur frühen Quelle von IL-4 zur Induktion der Th2-Antwort und der Quellen der IL-13-Bildung müssen in weiterführenden Untersuchungen beantwortet werden (239).

Tierexperimente

Die Mäuse in der vorliegenden Arbeit wurden im Rahmen des Tierversuchsvorhabens Az. 24-9168.11 TVV16/09 (Landesdirektion Sachsen – Dienststelle Leipzig) verwendet.

3.2.1 Publikation 2: Eosinophile sind wichtige IL-4-Produzenten und fördern die Th2-Antwort in der Kryptokokkose

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Immunopathology and Infectious Diseases

Eosinophils Contribute to IL-4 Production and Shape the T-Helper Cytokine Profile and Inflammatory Response in Pulmonary Cryptococcosis

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Susceptibility to infection with *Cryptococcus neoformans* is tightly determined by production of IL-4. In this study, we investigated the time course of IL-4 production and its innate cellular source in mice infected intranasally with *C. neoformans*. We show that pulmonary IL-4 production starts surprisingly late after 6 weeks of infection. Interestingly, in the lungs of infected mice, pulmonary T helper (Th) cells and eosinophils produce significant amounts of IL-4. In eosinophil-deficient ΔdblGATA mice, IL-33 receptor–expressing Th2s are significantly reduced, albeit not absent, whereas protective Th1 and Th17 responses are enhanced. In addition, recruitment of pulmonary inflammatory cells during infection with *C. neoformans* is reduced in the absence of eosinophils. These data expand previous findings emphasizing an exclusively destructive effector function by eosinophilic granulocytes. Moreover, in ΔdblGATA mice, fungal control is slightly enhanced in the lung; however, dissemination of *Cryptococcus* is not prevented. Therefore, eosinophils play an immunoregulatory role that contributes to Th2-dependent susceptibility in allergic inflammation during bronchopulmonary mycosis. ***(Am J Pathol 2011, 179:733–744; DOI: 10.1016/j.ajpath.2011.04.025)***

Cryptococcus neoformans is a facultative intracellular pathogen that is acquired by inhalation of spores and/or desiccated yeasts and leads to latent pulmonary infection in immunocompetent humans.[1] The development of cryptococcal meningitis occurs mainly in immunocompromised HIV-1–infected patients, most likely by reactivation of latent pulmonary *C. neoformans* infection.[2] It is estimated that 504,000 HIV-1–infected patients die every year from cryptococcal meningitis in sub-Saharan Africa,[3] which surprisingly exceeds the annual death rate of tuberculosis-associated HIV cases. Resistance against *C. neoformans* primarily involves monocytic effector mechanisms.[4–6] In this context, T helper (Th) cells are central regulatory players with profound effects. Whereas IL-12–dependent Th1 responses are protective, with an additional contribution by IL-23–dependent Th17 responses,[7–9] Th2 cells producing IL-4, IL-13, and IL-5 are detrimental.[10,11] Studies[12–14] that used i.v. inoculation examined the traversal of the blood-brain barrier by *C. neoformans* and led to the conclusion that transmigration can occur with intracellular and extracellular fungi. In case of bronchopulmonary infection, dissemination seems to rely more on Th2 cytokines. This allergic Th2-driven inflammation represents the immunopathological pathway promoting disease by allowing cryptococci to grow inside the lung and finally enabling dissemination to the brain, ultimately causing fatal meningoencephalitis.[15] This sequela is accompanied by development of IL-4/IL-13–dependent alternatively activated macrophages, suggesting that those cells may be involved in dissemination. Alternatively activated macrophages are found only in susceptible mice[15] and show significantly reduced control of intracellular growth.[5] In addition, IL-13–dependent mucus production by goblet cells, IL-4–dependent IgE production, IL-5–dependent eosinophilia, and functional pulmonary impairment can be found; these features

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are also typically described in asthma.[16–18] Studies[10,11,19] of pulmonary and cerebral cryptococcosis in IL-4–, IL-13–, IL-4 receptor α–, and IL-4/IL-13–deficient mice or mice treated with anti-IL-5 convincingly provide a basis for future immunotherapies by targeting one or several of these Th2-associated molecules. However, it is unclear when IL-4 production starts after pulmonary infection. In addition, potential innate immune cell(s) producing IL-4 and thereby promoting Th2 initiation and/or Th2 maintenance remain to be identified. Therefore, in this study, we aimed to define the following: i) the onset and time course of IL-4 production, ii) the IL-4–producing innate cell type(s) supporting Th2 development, and iii) the immunological and phenotypic consequences of innate IL-4 production in pulmonary cryptococcosis. Our results indicate that eosinophilic granulocytes are a significant source of IL-4, with distinct regulatory consequences in murine cryptococcosis.

Materials and Methods

Mice

Female wild-type (WT) mice (Janvier, Le Genest Saint Isle, France), 4get mice[20] (provided by André Gessner, Clinical Microbiology and Immunology, Erlangen, Germany), and ΔdblGATA mice[21] (provided by Achim Hoerauf, Institute of Medical Microbiology, Immunology und Parasitology, Bonn, Germany), aged 6 to 10 weeks, on a BALB/c background were maintained in an individually ventilated caging system 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. The mice were tested periodically for pathogens, in accordance with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations accreditation board. All mice had negative test results for pinworms and other endoparasites and ectoparasites.

C. neoformans *and Infection*

Encapsulated *C. neoformans*, strain 1841, serotype D, was kept as a frozen stock in skim milk and was grown in Sabouraud dextrose medium (2% glucose and 1% peptone; Sigma, Deisenhofen, Germany) overnight on a shaker at 30°C. Cells were washed twice in sterile PBS, resuspended in PBS, and counted in a hematocytometer. Inocula were diluted in PBS to a concentration of 2.5×10^4/mL for intranasal (i.n.) infection. Mice were infected by i.n. application of 20-μL volumes containing 500 colony-forming units (CFUs). Before infection, mice were anesthetized i.p. with a 1:1 mixture of 10% ketamine (100 mg/mL; Ceva Tiergesundheit, Düsseldorf, Germany) and 2% xylazine (20 mg/mL; Ceva Tiergesundheit).

Leukocyte Preparation for Flow Cytometry and CFU Enumeration

Infected mice were monitored daily for survival and morbidity. After sterile removal of the brain from sacrificed mice, half was processed for histological examination and the remaining half was processed for determination of organ burden (CFU). After homogenization in 1-mL PBS with an Ultra-Turrax (T8; IKA-Werke, Staufen, Germany), serial dilutions of the homogenates were plated on Sabouraud dextrose agar plates and colonies were counted after an incubation period of 48 hours at 30°C. After sterile removal, lungs from sacrificed mice were minced and digested for 30 minutes at 37°C in RPMI 1640 medium supplemented with collagenase (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), 100 μmol/L sodium pyruvate, and DNase IV (Sigma-Aldrich, Steinheim, Germany). After passage through a 100-μm nylon mesh (BD Biosciences, Heidelberg, Germany), filtrate was resuspended in 1-mL RPMI 1640 medium (PAA Laboratories, Pasching, Austria); and 50 μL was taken for CFU enumeration. Serial dilutions were plated on Sabouraud dextrose agar plates, and colonies were counted after an incubation period of 48 hours at 30°C. Remaining filtrate was resuspended in 70% Percoll (GH Healthcare Biosciences AB, Uppsala, Sweden) and layered under 26% Percoll. Leukocytes were recovered from interphase, washed with Iscove's modified Dulbecco's medium (PAA Laboratories), and counted in trypan blue (Fluka Chemie AG, Buchs, Switzerland). For surface staining, 1×10^5 to 2×10^5 cells were used; and for intracellular cytokine staining, 1×10^6 cells were acquired.

Flow Cytometry

Purified cells were adjusted to 5×10^6/mL in Iscove's modified Dulbecco's medium and stimulated either 6 hours with ionomycin (1 μg/mL; Sigma-Aldrich) and phorbol 12-myristate 13-acetate (PMA) (40 ng/mL; Alexis Corporation, Lausen, Switzerland) or 22 hours with specific antigen. For the accumulation of cytokines, brefeldin A (5 μg/mL; Sigma-Aldrich), was added for the last 4 hours. The acapsular *C. neoformans* serotype D strain CAP67 (provided by Dr. Bettina Fries, Albert Einstein College of Medicine, Bronx, NY) was used as a specific stimulus (1×10^7 cryptococci/mL, termed C.n. antigen) for restimulation of pulmonary leukocytes from *C. neoformans*–infected mice. The CAP67 strain has better restimulatory capacities than the highly virulent strain 1841. It was cultured and maintained in the same manner as strain 1841; before use, it was heat inactivated at 60°C for 1 hour.[22]

First, Near-IR Dead Cell Stain (Invitrogen, Darmstadt, Germany) was used to ensure discrimination and exclusion of dead cells during analysis. Second, cells were fixed with 2% paraformaldehyde (Serva, Heidelberg, Germany) for 20 minutes on ice. When intracellular staining was performed, permeabilization was included by using fluorescence-activated cell sorting buffer (ie, PBS containing 3% heat-inactivated fetal calf serum and 0.1% sodium azide) containing 0.5% saponin (w/v; Serva).

Cells were incubated for 15 minutes on ice with FcR block (2×10^6 μg cells/L; purified from 2.4G2 hybridoma supernatant) and rat serum (Sigma-Aldrich) to avoid unspecific staining. Antibodies (Abs) and FcR block for intracellular staining were diluted in fluorescence-activated cell sorting buffer containing 0.5% saponin (w/v; Serva). For specific stainings, the following Abs were used: anti-CD4-PerCP-Cy5.5 (RM4-5; eBioscience, Frankfurt, Germany); anti-interferon (IFN)-γ–fluorescein isothiocyanate (XMG1.2; eBioscience); anti-IL-4–allophycocyanine (APC) (11B11; Biolegend, Fell, Germany); anti-IL-17-PE-Cy7 (eBio17B7; eBioscience); anti-Siglec-F (E50-2440; BD Biosciences) biotinylated, following standard procedures; anti-Siglec-F-PE (E50-2440; BD Biosciences); anti-F4/80-PE-Cy7 (BM8; eBioscience); anti-CD11c–APC (N418; eBioscience); anti-CD154-PE (MR1; Miltenyi Biotec, Bergisch Gladbach, Germany); and anti-T1/ST2–fluorescein isothiocyanate (MD Biosciences, Zürich, Switzerland). Appropriate isotype Abs were all from eBioscience, except for anti-IL-4 from Biolegend. Cells labeled with biotinylated Abs were further stained with streptavidin-PerCp (eBioscience). Cells were acquired on a BD FACS Calibur using CellQuest software version 3.0.1 and BD FACS CANTO II using DIVA version 6.1.1 and FlowJo version 7.6.1 (Treestar Inc., Ashland, OR) software for analysis.

IL-4 Secretion Assay

An IL-4 secretion assay (Miltenyi Biotec) was performed according to manufacturer's instructions. Percoll (GH Healthcare) purified pulmonary leukocytes were stained with anti-CD4–fluorescein isothiocyanate (RM4-5; eBioscience) and, afterward, with anti-fluorescein isothiocyanate MicroBeads (Miltenyi Biotec). Cells were then applied to an MS column (Miltenyi Biotec) to separate $CD4^+$ and $CD4^-$ cells. Both fractions were stimulated for 2 hours with ionomycin (1 μg/mL; Sigma-Aldrich) and PMA (40 ng/mL; Alexis Corporation). After stimulation, cells were applied to an IL-4 secretion assay (Miltenyi Biotec) and were additionally stained with anti-CD11c–APC (N418; eBioscience), anti-CD3-biotin (145-2C11; eBioscience), and biotinylated anti-Siglec-F (E50-2440; BD Biosciences). Cells labeled with biotinylated Abs were further stained with streptavidin-PerCp (eBioscience).

Cytokine ELISA

Cytokine concentrations were determined by sandwich enzyme-linked immunosorbent assay (ELISA) systems with unlabeled capture Abs and labeled detection Abs. To determine the concentration of IL-4, monoclonal Ab (mAb) 11B11 was used as the capture Ab and biotin-labeled BVD6-24G2 (BD Biosciences) was used as the detection Ab, followed by incubation with peroxidase-labeled streptavidin (Southern Biotechnology Associates, Birmingham, AL). IFN-γ was captured by mAb AN-18 and detected by peroxidase-labeled mAb XMG1.2. The concentration of IL-17 was detected with the R&D Systems Duoset kit (R&D Systems GmbH, Wiesbaden, Germany).

Histopathological Analysis

Lung samples were processed for histological analysis, as previously described.[11]

IHC

Lung samples were processed for histological analysis, as previously described.[15] In brief, the accessory lobe of the lung was sterilely removed, mounted on thick filter paper with Tissue Tek optimal cutting temperature compound (Miles Scientific, Naperville, IL), snap frozen in isopentane (Fluka, Neu-Ulm, Germany) precooled on dry ice, and stored at -80°C. For immunohistochemistry (IHC), 10-μm frozen sections were prepared in a serial fashion (30 transversal sections on six consecutive levels per lung). Glucuronoxylomannan immunostaining was performed using mAb 18B7 (provided by Dr. Arturo Casadevall, Albert Einstein College of Medicine, New York, NY). The mAb 18B7 was biotinylated (Sigma-Aldrich) before use, and lung slides were incubated in a secondary step with ExtrAvidin peroxidase (Sigma-Aldrich). The peroxidase reaction product was visualized using 3,3′-diaminobenzidine (Sigma-Aldrich) as the chromogene and H_2O_2 as the cosubstrate.

Serum Immunoglobin Measurement

Total serum IgG1, IgG2a, and IgE levels were analyzed, as described earlier.[11] For determination of *C. neoformans*–specific serum IgE, the following capture ELISA was developed. ELISA plates (Nunc GmbH & Co KG, Langenselbold, Germany) were coated with 5 μg/mL rat–anti-mouse IgE mAb (R35-72; BD Biosciences) in carbonate buffer (pH 9.5) overnight at 4°C. To prevent unspecific binding, plates were washed and blocked with 5% (w/v) skim milk (Sigma-Aldrich) in PBS for 3 hours at room temperature. After washing, serum samples were diluted 1:10 in blocking buffer containing 0.1% (v/v) Tween 20 (Karl Roth AG, Karlsruhe, Germany) and added in duplicate for 2 hours at room temperature. Plates were washed again and biotinylated (Sigma-Aldrich). *C. neoformans* 1841D homogenate (5 μg/mL) was incubated for 2 hours at room temperature. This step was followed by incubation with peroxidase-labeled streptavidin (Southern Biotechnology Associates), diluted 1:4000 in Tween 20 containing blocking buffer, for 45 minutes at room temperature for detection. Plates were washed, and the TMB Microwell peroxidase system (KPL, Gaithersburg, MD) was used as a substrate for the final colorimetric reaction. The reaction was discontinued after 2 hours by adding 1 mol/L H_3PO_4, and ODs were read using a Spectra-max 340 ELISA reader (Molecular Devices GmbH, Ismaning, Germany) at 450 nm, with background subtraction at 630 nm. Wells incubated without serum samples but with all other reagents were used as plate blank. To control the specificity of this ELISA, we added a monoclonal IgE isotype control (C38-2; BD Biosciences) specific for the hapten trinitrophenyl that we also used as a standard for the total IgE quantification after coating with IgE mAb (R35-72; BD Biosciences) and blocking. After an-

other incubation with blocking buffer, plates were either incubated with anti-mouse IgE–horseradish peroxidase (23G3; Southern Biotechnology Associates) or biotinylated *C. neoformans* homogenate. Plates incubated with biotinylated homogenate were additionally incubated with peroxidase-labeled streptavidin (Southern Biotechnology Associates). Development was performed with the TMB Microwell peroxidase system (KPL). This confirmed that even high concentrations of anti-trinitrophenyl IgE (eg, 20 μg/mL) do not bind to biotinylated *C. neoformans* homogenate nonspecifically. Specificity was further confirmed by using serum samples from naïve BALB/c WT instead of anti-trinitrophenyl IgE. The median OD_{450} was 0.015.

Statistical Analysis

The one-tailed Mann-Whitney *U*-test was performed to determine the significance of differences in kinetic analyses of 4get mice and between WT and ΔdblGATA mice. Data are presented as the mean ± SEM. The level of confidence for significance was $P < 0.05$.

Results

Pulmonary Th2 Development Occurs after 6 Weeks of Infection and Coincides with IL-4–Producing Eosinophils

Susceptibility in cryptococcosis is tightly linked with IL-4 production.[7,11] BALB/c WT mice infected i.n. with only 500 CFUs of *C. neoformans* strain 1841 show dissemination from lung to brain beginning at approximately 6 weeks after infection (data not shown), leading to death 10 weeks after infection.[7,11] This pulmonary cryptococcosis model is a long-term model compared with other published murine models.[8,23–27]

To monitor IL-4 production over time in this long-term model, IL-4 reporter mice, termed 4get mice, were infected, and enhanced green fluorescent protein (eGFP) expression, which is known to correlate with IL-4 transcription, was assessed.[20] There were constitutively $eGFP^+$ pulmonary leukocytes in naïve mice (Figures 1A and 2, A and B), as published by others.[28,29] On infection of 4get mice, the frequency of $eGFP^+$ leukocytes increased almost 10-fold, at 42 days postinfection (dpi), and stayed on this elevated level up to 70 dpi (Figure 1A). Because eGFP expression may indicate only IL-4 transcription,[20] we wanted to assess IL-4 protein production. Indeed, we found that IL-4 production in response to antigen-specific stimulation of pulmonary leukocytes with cryptococcal antigen starts to become detectable at a similarly late time point as found for eGFP expression. IL-4 was not detectable at 35 dpi (data not shown) or earlier; instead, it started to become detectable 6 weeks after infection and increased up to 70 dpi (Figure 1B). Late IL-4 expression, monitored by eGFP expression or restimulation of pulmonary leukocytes, was further confirmed by intracellular staining of IL-4 in Th cells and revealed similar results (data not shown). Consistent with the time course of IL-4 production, total and specific IgE started to increase after 42 days of infection (Figure 1, C and D). Although the sandwich ELISA for total IgE detected a median concentration of approximately 13.65 μg/mL, starting after 42 days of infection, the ELISA for specific IgE resulted in minor signals of only up to 0.099 OD_{450}, with a substrate development time of 2 hours. Thus, similar to parasite models,[30] only a minor portion of the total IgE appears to be specific for cryptococcal antigens (Figure 1D).

$CD4^+$ Th cells, and innate immune cells, have been described as cellular sources of IL-4.[29] To define the cell types producing IL-4 in pulmonary cryptococcosis, we characterized $eGFP^+$ cells in the lungs of infected 4get mice. At 70 dpi, we found elevated numbers of $eGFP^+$

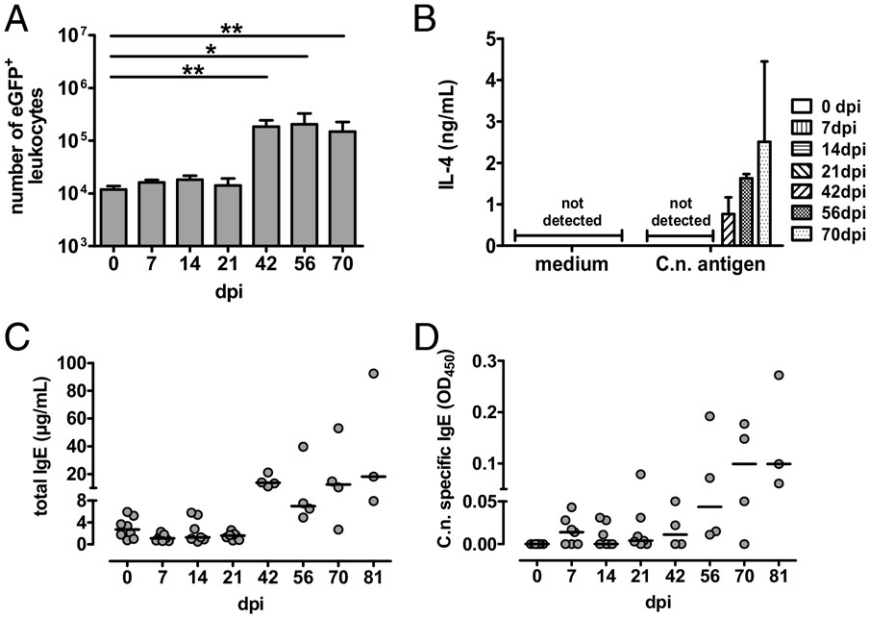

Figure 1. Coincidental accumulation of pulmonary leukocytes competent for IL-4 expression (indicated by eGFP), onset of IL-4 secretion, and increase of total and specific IgE. 4get mice were infected i.n. with *C. neoformans* 1841D. **A:** On the indicated dpi, leukocytes were isolated from lungs (n = 3 to 5 per time point), counted, and analyzed for eGFP-expression by flow cytometry. **B:** Pulmonary leukocytes were restimulated for 22 hours with C.n. antigen. IL-4 was measured by ELISA in culture supernatant. **C** and **D:** Serum samples from the same mice were examined for total IgE and *Cryptococcus*-specific IgE according to the *Materials and Methods* section. Data from two independent experiments were pooled and are expressed as the mean ± SEM. Statistical analysis was performed using the Mann-Whitney *U*-test. $^*P < 0.05$ and $^{**}P < 0.01$ comparison with naive 4get mice.

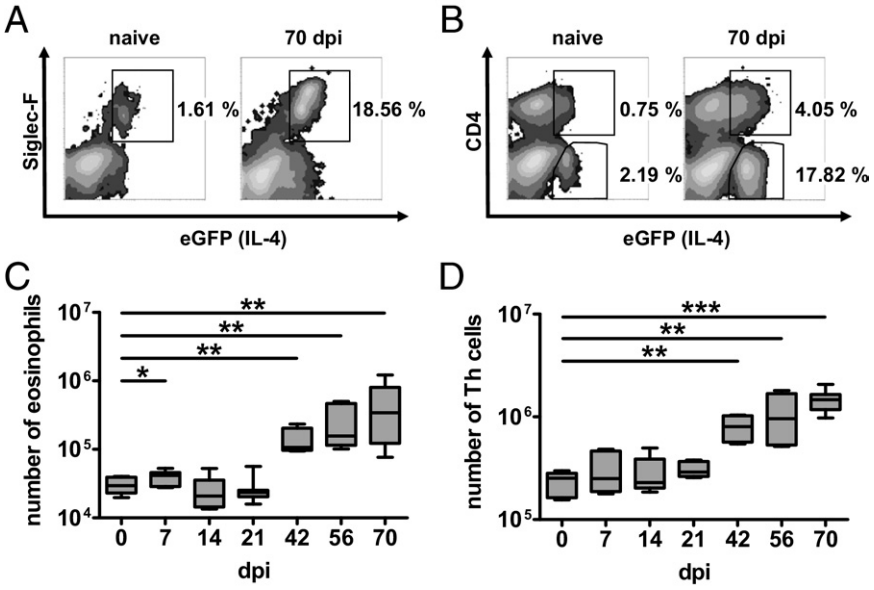

Figure 2. Th cells and eosinophils exclusively constitute eGFP$^+$ cells during pulmonary cryptococcosis. Flow cytometry was performed on pulmonary leukocytes from i.n. infected 4get mice on the indicated dpi (n = 3 to 5 per time point). **A:** CD4$^-$eGFP$^+$ cells identified by Siglec-F expression as eosinophils in representative 4get mice are shown. **B:** eGFP expression in Th cells in the same 4get mice shown in **A**. **C:** Kinetic analysis of enumerated eosinophils. **D:** Th cells at the indicated dpi are shown. Kinetic data were pooled from two independent experiments. The mean ± SEM is shown for kinetic analyses. Statistics were performed with the Mann-Whitney U-test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with naive 4get mice.

pulmonary leukocytes in both CD4$^-$ and CD4$^+$ populations (Figure 2, A and B). Interestingly, the CD4$^-$eGFP$^+$ population was identified to be Siglec-F$^+$, pointing to eosinophils (Figure 2A).[31] This prompted us to characterize the time course of recruitment of Th cells and eosinophilic granulocytes. The data shown in Figure 2, C and D, demonstrate that there is a similarly late time course of recruitment to the lung for both Th cells and eosinophils.

To directly define the cellular source(s) of pulmonary IL-4 production in cryptococcosis, we applied IL-4 secretion assays on magnetic cell sorting (MACS)-separated (Miltenyi) lung CD4$^+$ and CD4$^-$ cells to avoid cross feeding between different cells (Figure 3). Th2s represent a cell type already known to be responsible for efficient IL-4 production in cryptococcosis.[7,11] In addition to the Th cells as IL-4 sources (Figure 3C), we show that eosinophilic granulocytes (Figure 3A; further gated on Siglec-F$^+$/CD11c^{dim}) produce significant amounts of IL-4 on *Cryptococcus* infection (Figure 3B). Interestingly, a major portion of these eosinophils (ie, 17.49%) produced IL-4 constitutively after pulmonary infection with *C. neoformans* (Figure 3B, top; mean fluorescence intensity of isotype control (not shown) versus medium, 133.59 versus 330.43). This could be further enhanced (41.63% of all eosinophils) by *ex vivo* stimulation with a combination of PMA and ionomycin (mean fluorescence intensity, 784.66; Figure 3B, bottom). Together, these data demonstrate that, during pulmonary cryptococcosis, Th2 cells and eosinophils contribute to late IL-4 production at a point when IgE production is significantly increased and *C. neoformans* disseminates from the lung to the brain.

In the Absence of Eosinophils, Th2 Responses Are Reduced and Th1/Th17 Responses Are Enhanced

IL-4 is not essential for Th2 differentiation but plays a nonredundant role in the maintenance of Th2 responses.[32] We were interested in whether eosinophils

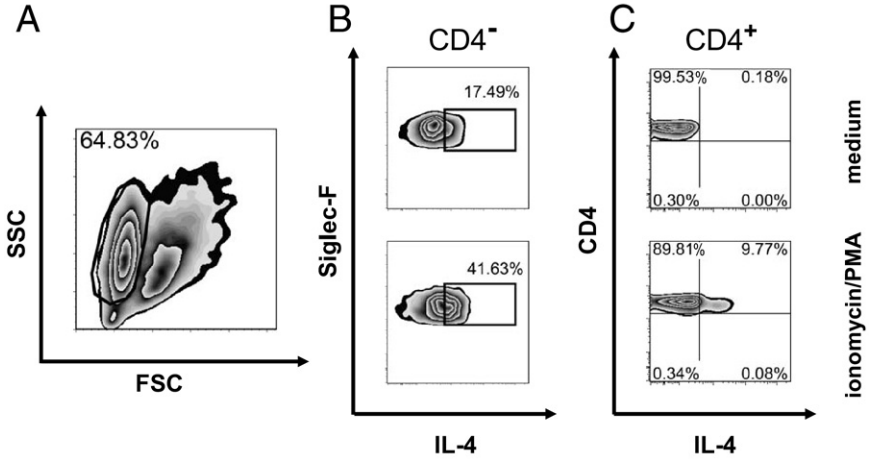

Figure 3. Both Th cells and eosinophils are the main producers of IL-4 in the lungs of infected mice. Isolated pulmonary leukocytes from i.n. infected BALB/c WT mice were positively enriched for CD4$^+$ Th cells by MACS. **A:** The remaining CD4$^-$ cells include eosinophils [gated on a side scatter (SSC)^{high}/forward scatter (FSC)^{low} plot]. Both CD4$^-$ cells (**B**) and CD4$^+$ Th cells (**C**) were either stimulated with ionomycin and PMA (**bottom**) or left untreated (**top**) before performing an IL-4 secretion assay. One of two independent experiments is shown.

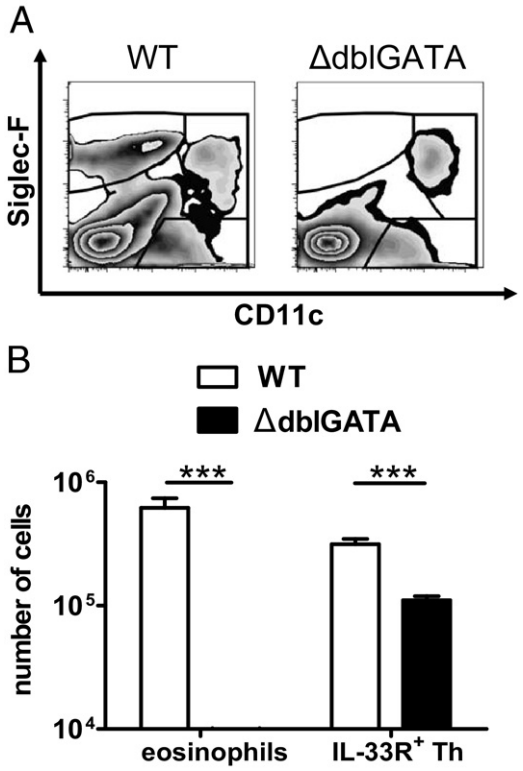

Figure 4. Mice devoid of eosinophils harbor fewer Th cells, indicated by IL-33R expression. Flow cytometry was performed on pulmonary leukocytes from i.n. infected BALB/c WT and ΔdblGATA mice at 60 dpi. **A:** The absence of eosinophils is confirmed by plots because no $CD11c^{dim}$/$Siglec\text{-}F^{high}$ can be detected in the ΔdblGATA mice (**right**). A representative WT mouse is shown (**left**). **B:** Enumeration of total $IL\text{-}33R^+$ $CD4^+$ Th cells is shown, together with eosinophils. One of three independent experiments is shown (n = 6 to 7 per genotype). Values are given as the mean ± SEM. Statistical analysis was performed by using the Mann-Whitney U-test. $^{***}P < 0.001$.

can contribute to Th2 responses in cryptococcosis. Thus, we infected WT and eosinophil-deficient ΔdblGATA mice[21] i.n. with *C. neoformans*. The i.n. infection of WT mice led to accumulation of $Siglec\text{-}F^+$/$CD11c^{dim}$ eosinophils in the lung, which was not the case for ΔdblGATA mice, as expected (Figure 4A). We then assessed the frequency of pulmonary Th2 cells in infected WT versus ΔdblGATA mice. In the absence of eosinophils, pulmonary Th2 cells, characterized by the expression of IL-33 receptor (IL-33R), are greatly reduced, but not completely lacking, in ΔdblGATA mice (Figure 4B). Earlier data demonstrated that the IL-33R, also termed T1/ST2, is specifically expressed on differentiated but not on naïve Th cells.[33]

Th cells are central regulators of anticryptococcal immune responses.[25,34,35] Although Th2 responses are detrimental,[11,15] Th1 and Th17 responses are protective.[7,9,25,35,36] To gain a deeper insight into the Th cytokine profile in the absence of eosinophils, we analyzed IL-4, IFN-γ, and IL-17A production by pulmonary Th cells on infection of WT and eosinophil-deficient ΔdblGATA mice. Analysis of IL-4, IFN-γ, and IL-17A in the supernatants of pulmonary leukocytes stimulated with cryptococcal antigen revealed reduced IL-4 and enhanced IFN-γ and IL-17 production by restimulated pulmonary leukocytes of ΔdblGATA versus WT mice (Figure 5, A–C). IL-4 production is substantially reduced, but not completely lacking, in pulmonary $CD4^+$ T cells from ΔdblGATA mice. This indicates a shift from Th2 to Th1/Th17 responses in the absence of eosinophils.

By direct intracellular staining of IL-4 in $CD4^+$ T cells (Figure 6), we corroborate the data shown in Figure 5, A–C, clearly demonstrating a pronounced Th2 response in *Cryptococcus*-infected WT mice. We were also able to demonstrate that only $CD154^+$ Th cells (ie, antigen-specific Th cells[37,38]) from infected mice responded with IL-4 production on stimulation with cryptococcal antigen (data not shown). Pulmonary Th cells from ΔdblGATA mice have similar proportions of Th1 cells, while they generate higher proportions of Th17 cells (Figure 6, antigen panel). A similar relative frequency of IFN-γ^+ Th1 cells (Figure 6) but elevated IFN-γ levels in supernatants of antigen specifically restimulated pulmonary leukocytes (Figure 5C), suggests higher IFN-γ production on a per-cell basis in Th1 cells from ΔdblGATA mice (Figure 6, C.n. antigen; IFN-γ mean fluorescence intensity, 529.30 for WT and 713.26 for ΔdblGATA). The hypothesis of a greater IFN-γ potency of ΔdblGATA Th cells on a single-cell basis is further supported by the results shown in Figure 5D ($CD4^+$ ionomycin/PMA; mean concentration of WT versus ΔdblGATA, 0.078 versus 0.835 pg/mL) because purified pulmonary Th cells were restimulated at an equal cell concentration when using this approach. Analysis of IL-4 from the same Th cells revealed comparable potency in IL-4 secretion (Figure 5E; $CD4^+$ ionomycin/PMA). Therefore, the reduced amounts of IL-4 shown in Figure 5A may result from fewer Th cells in the lungs of eosinophil-deficient mice (Figure 7B). More important, we detected a substantial amount of IL-4 in the Th-depleted fraction after ionomycin/PMA stimulation (Figure 5E; $CD4^-$ ionomycin/PMA; mean concentration of WT versus ΔdblGATA, 294 versus 0.056 pg/mL). Because ΔdblGATA mice are devoid of eosinophils (Figure 4A) and no other potential source(s) of IL-4 could be identified in this infection model, the detected IL-4 appears to depend on eosinophils in WT mice and reaches approximately one third of the Th-derived IL-4 (Figure 5E; $CD4^+$ ionomycin/PMA; mean concentration of WT versus ΔdblGATA, 890 versus 832 pg/mL). Consistent with lower IL-4 production in ΔdblGATA mice (Figure 5A), we observed substantially reduced IgE and elevated IgG2a (a marker for a Th1 response) serum levels in the absence of eosinophils (data not shown). Therefore, the presence of eosinophils contributes to enhanced fatal Th2 and reduced protective Th1 and Th17 responses.

Elevated Recruitment of Leukocytes in the Presence of Eosinophils

To study the pulmonary inflammatory response in the presence and absence of eosinophils, infected WT versus ΔdblGATA mice were analyzed at 60 dpi when significant eosinophils were present in the lungs of *C. neoformans*–infected 4get mice (Figure 2C). The pulmonary inflammatory response of infected 4get mice closely resembles WT mice (data not shown). Interestingly, more lung leukocytes were found in WT compared with

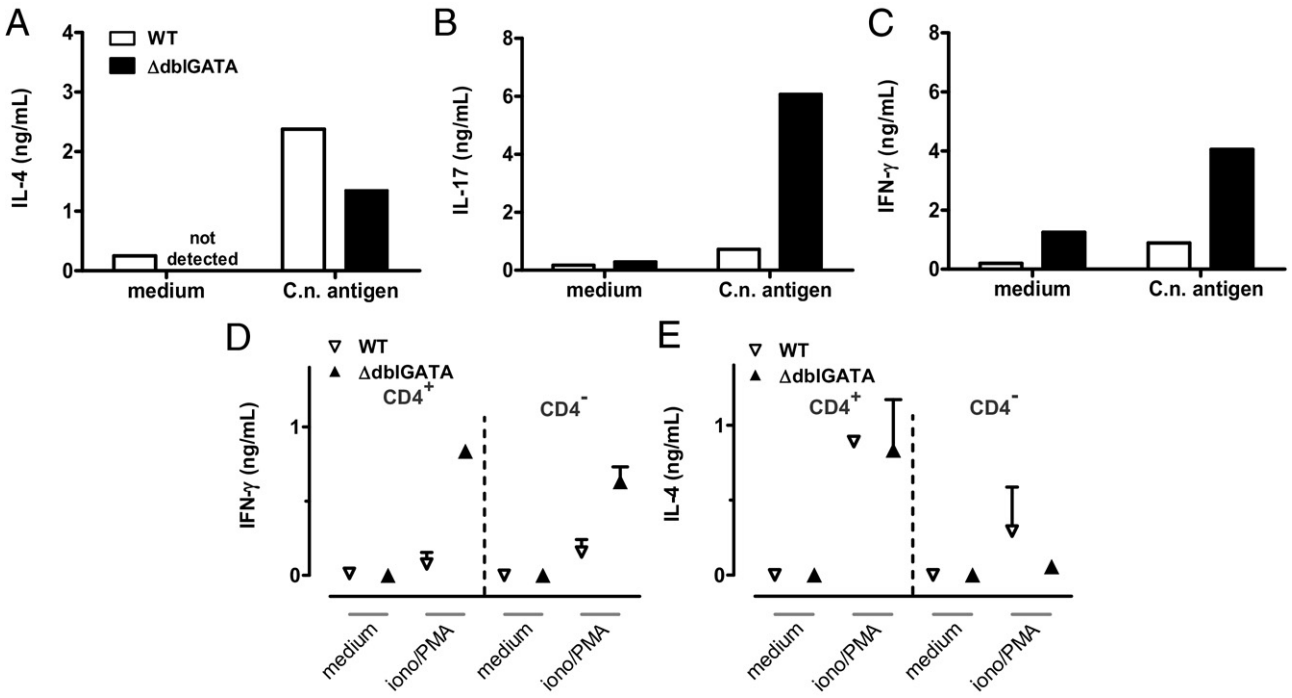

Figure 5. The absence of eosinophils promotes pronounced secretion of Th1- and Th17-associated cytokines and reduces the Th2 response. Pulmonary leukocytes were isolated from i.n. infected BALB/c WT and ΔdblGATA mice at 60 dpi. Cells were pooled per group and stimulated with C.n. antigen for 22 hours. **A** through **C:** IL-4, IL-17, and IFN-γ were measured by ELISA in culture supernatant, according to the *Materials and Methods* section. One representative of three independent experiments is shown (n = 6 to 7 per genotype). In addition, IFN-γ (**D**) and IL-4 (**E**) in the supernatant from MACS-enriched pulmonary $CD4^+$ Th cells and remaining cells either stimulated with ionomycin/PMA or left untreated for 2 hours from BALB/c WT and ΔdblGATA mice at 60 dpi are shown. Data were pooled from two independent experiments (n = 3 pooled mice per genotype and experiment).

ΔdblGATA mice (Figure 7A). An elevated frequency of total leukocytes in the presence of eosinophils corresponded with elevated numbers of Th cells in the lung (Figure 7B). We wanted to characterize the composition of other pulmonary leukocytes important in cryptococcosis. Macrophages are central effector cells that are able to direct the outcome of *C. neoformans* infection.[4,5,39,40] Alveolar and interstitial macrophages, and pulmonary dendritic cells were reduced in the absence of eosinophils (Figure 7, C–E). In summary, the data demonstrate a significant role of eosinophils in the recruitment of inflammatory cells on pulmonary infection with *C. neoformans*.

In the Absence of Eosinophils, Fungal Replication Is Reduced, but This Does Not Prevent Dissemination of C. neoformans *to the Brain*

Histopathological analysis of lungs from infected WT and ΔdblGATA mice revealed fewer and smaller foci of cryp-

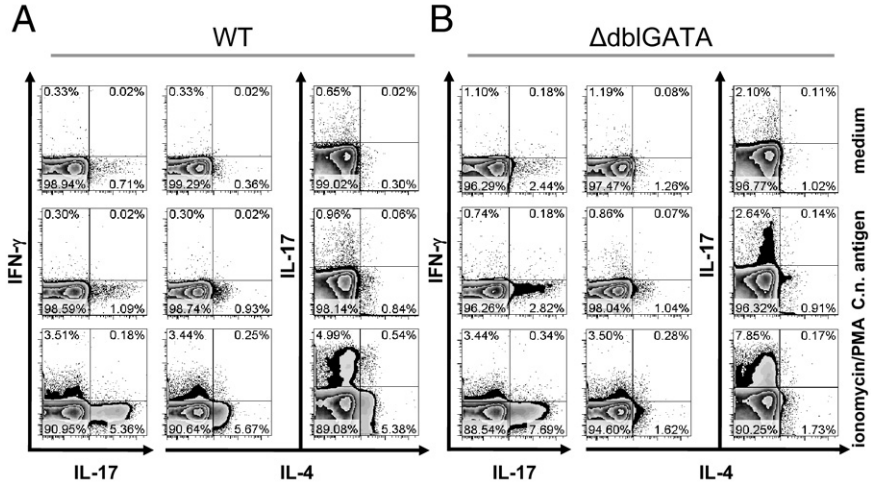

Figure 6. The Th cytokine profile is dependent on eosinophils on infection. Pulmonary leukocytes were isolated from i.n. infected BALB/c WT (**A**) and ΔdblGATA (**B**) mice at 60 dpi. Cells were pooled per group and allowed to rest for 22 hours as the negative control (**top**), stimulated with C.n. antigen for 22 hours (**middle**), or stimulated with ionomycin/PMA for 6 hours (**bottom**). Intracellular cytokine staining was performed according to the *Materials and Methods* section, and plots are gated on living $CD4^+$ Th cells. Appropriate isotype controls for staining Abs were used (data not shown), confirming the specific staining. One representative of three independent experiments is shown (n = 6 to 7 per genotype).

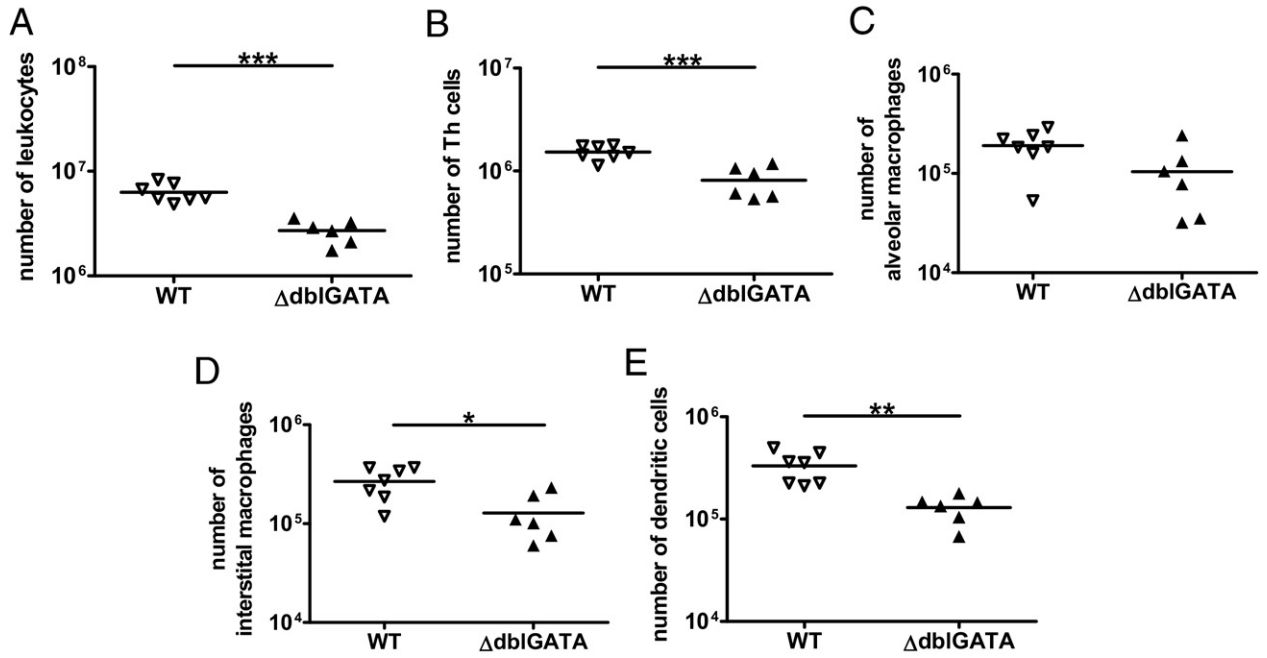

Figure 7. Recruitment of leukocytes to lung parenchyma after cryptococcal infection in the absence and presence of eosinophils. Pulmonary leukocytes were isolated from i.n. infected BALB/c WT and ΔdblGATA mice at 60 dpi. The numbers of total leukocytes (**A**), $CD4^+$ Th cells (**B**), alveolar macrophages (**C**), interstitial macrophages (**D**), and dendritic cells (**E**) are shown. One representative of three independent experiments is shown as the mean ± SEM ($n = 6$ to 7 per genotype). Statistical analysis was performed by using the Mann-Whitney *U*-test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with BALB/c WT and ΔdblGATA mice.

tococci in the absence of eosinophils (Figure 8, A and B). Infected WT mice developed large aggregates of fungi in their lungs (Figure 8, C–F), which was also corroborated by detection of the cryptococcal capsular component glucuronoxylomannan (Figure 8, G and H). When we analyzed the total number of viable cryptococci contained in the lung, we observed substantially fewer (approximately 84-fold reduction of median fungal load) in ΔdblGATA mice compared with WT mice; however, this difference did not reach statistical significance (Figure 9A, $P = 0.0734$). In line with this finding, dissemination of *Cryptococcus* to the brain was not prevented in ΔdblGATA mice (Figure 9B, $P = 0.1375$). This indicates that the absence of eosinophils has a limited impact on protective pulmonary immunity against *C. neoformans* and does not suffice to prevent fungal dissemination.

Discussion

In this study, we report a regulatory role of eosinophilic granulocytes in cryptococcosis. Eosinophils have been mentioned before in murine models of cryptococcosis,[10,23,41–43] observed in human cryptococcosis,[44–46] and described with an emphasis on tissue damage.[41] Although *in vitro* eosinophils have phagocytosed *C. neoformans*[23] and presented cryptococcal antigens,[47] *in vivo*, no evidence for uptake of *C. neoformans* by eosinophils has been found by others[4] and in this study (data not shown). Herein, we highlight an immunoregulatory role of eosinophils that contribute to IL-4–dependent immunopathological features during murine pulmonary *C. neoformans* infection. We provide evidence for previously unrecognized features of eosinophils during bronchopulmonary infection. The protective immune response against *C. neoformans* relies on Th1-biased cellular immunity.[7,48] However, even in the presence of IFN-γ, IL-4 production has been detrimental in pulmonary cryptococcosis.[11] An exquisite role of IL-4 signaling strength has been demonstrated in our pulmonary cryptococcosis model, with a gene dosage effect of the IL-4 receptor α alleles.[18] Thus, in this report, we focus on nonprotective IL-4 production by Th cells and innate immune cells. In brief, the IL-4 competence of Th cells and eosinophils was determined by eGFP expression during 10 weeks of infection. Both cell populations show a concomitant late increase in lung parenchyma. The quantitative data obtained for IL-4 derived from Th cells and eosinophils indicate that Th2 cells are a major cellular source, followed by eosinophils as an innate cellular source of IL-4 (Figure 5E). Moreover, in cryptococcosis, eosinophils promote Th2 responses but are not essential for Th2 differentiation because we found a considerable residual frequency of $IL\text{-}33R^+$ Th2 cells in eosinophil-deficient mice (Figure 4B). Although the absence of eosinophils favors the development of a more Th1/Th17 pronounced response by modulating the Th cell cytokine secretory capacity (Figures 5, D and E, and 6), this cannot prevent dissemination of fungi, as shown by the brain cryptococcal burden (Figure 9B). Dissemination of cryptococci to the brain is only abrogated when IL-4, IL-13, or IL-4/IL-13 signaling is completely abolished.[15]

The Th2 promoting property of eosinophils has also been shown recently in a murine asthma model induced by an *Aspergillus fumigatus* extract.[49] Similar to pulmonary cryptococcosis, eosinophil-deficient ΔdblGATA mice showed reduced levels of pulmonary Th2-related cyto-

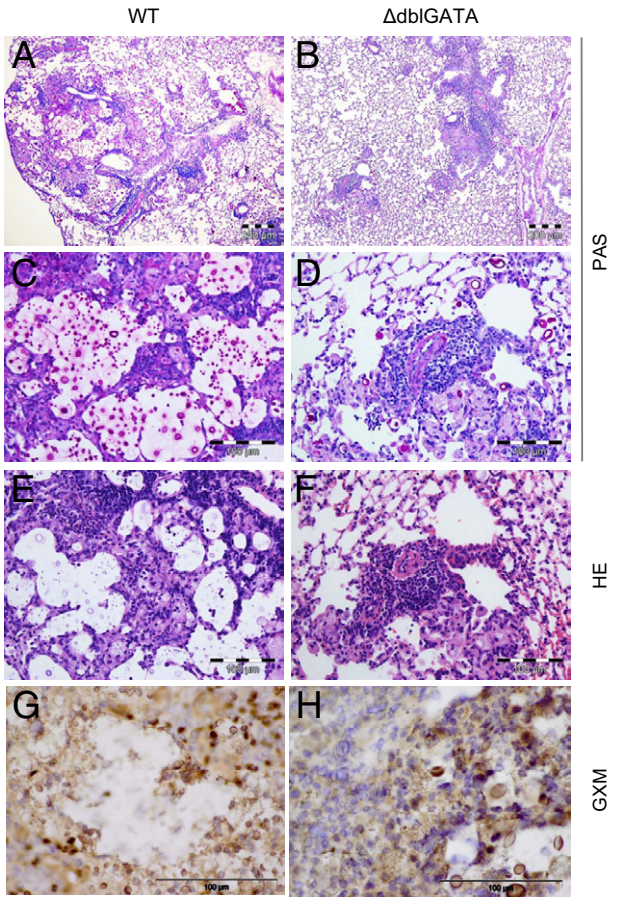

Figure 8. Lung sections from infected WT and ΔdblGATA mice indicate better fungal control in the absence of eosinophils at 60 dpi. **A** through **D:** PAS staining. Scale bars: 200 μm (**A** and **B**); 100 μm (**C** and **D**). **E** and **F:** H&E staining. Scale bar = 100 μm. **G** and **H:** IHC was performed on sections from the same mice, and glucuronoxylomannan (GXM)–containing foci are brown. Scale bar = 100 μm. ΔdblGATA mice show reduced numbers of cryptococci in the lungs (**A**–**F**) and formation of smaller foci of accumulating cryptococci and GXM compared with WT mice (**G** and **H**). There was pronounced influx of inflammatory cells in WT compared with ΔdblGATA mice.

kines and mononuclear cell recruitment.[49] The contribution of other innate immune cells (eg, basophils) potentially involved in fatal Th2 initiation in cryptococcosis remains to be tested, because basophils have been shown recently in models of parasitic disease and a protease allergen model to play an essential role in Th2 differentiation.[50–52]

In addition to well-described developmental requirements for Th1 and Th17 responses,[53,54] mechanisms of Th2 initiation are less unraveled and controversy on Th2-inducing molecules and cells continues.[32,33,54–58] Since the introduction of the Th1/Th2 paradigm, IL-4 has been tightly associated with Th2 responses[59–61]; there is clear evidence that IL-4 is indispensable for Th2 maintenance.[62] For Th2 initiation, current studies point to non-hematopoietic cells that appear to be able to support innate immune cells by secretion of chemokines[63] and novel cytokines, such as IL-33,[33] IL-25,[32,58] and thymic stromal lymphopoietin (TSLP).[64] In pulmonary cryptococcosis, airway epithelial cells and eosinophils would be candidates for cross talk between resident tissue cells and leukocytes.[65] Interestingly, we found expression of IL-33R on eosinophils in this study of pulmonary cryptococcosis (data not shown). Thus, eosinophils could be cellular targets of IL-33 produced by epithelial cells[66] and, thereby, could contribute to Th2 initiation. The definitive roles of IL-33, its cellular sources, and targets in anticryptococcal immunity remain to be defined.

Eosinophils were recognized for a long time as effector cells acting by degranulation in helminth/parasitic infections with *Trichinella spiralis*[67,68] or *Schistosoma mansoni*.[69] Eosinophils demonstrate protective mechanisms that rely on degranulation in bacterial infections[70] and release mitochondrial DNA in a unique way that clumps bacteria together.[71] In addition, antiviral effects have been reported.[72] Investigations of the role of eosinophils in fungal infection with *Candida albicans*,[73–75] *Alternaria alternata*,[76] or *C. neoformans*[10,23,41,42] were made, with a focus on their effector function. Similarly, a study[10] using anti-IL-5 treatment showed an association of eosinophil frequency with susceptibility during cryptococcosis. During the past decade, several reports[77,78] extended the function of eosinophils beyond the sole defense against nonphagotizable pathogens. Eosinophils produced various chemokines and cytokines modulating immune responses in different models.[29,49,79,80] Lee and colleagues[81] recently introduced the term LIAR (regulators of local immunity and/or remodeling/repair) for eosinophils; this term summarizes more recently discovered regulatory properties of eosinophilic granulocytes. Our data from a chronic fungal infection support the regulators of local immunity and/or remodeling/repair concept of eosinophil function. Other eosinophil-dependent factors, in addition to IL-4, that are involved in regulation of

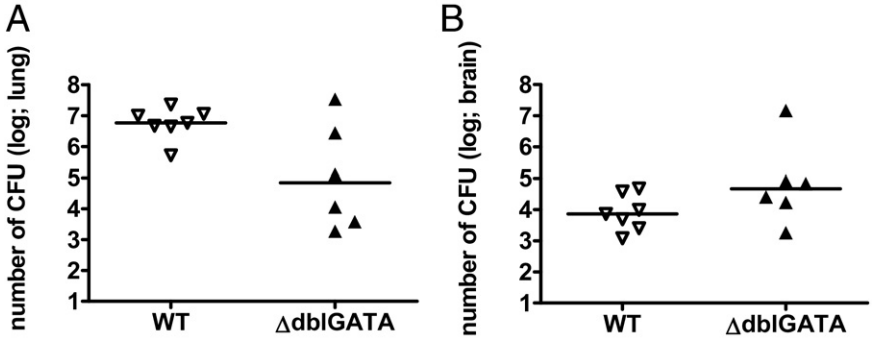

Figure 9. Organ burden in the presence and absence of eosinophils. Lung (**A**) and brain (**B**) fungal burden of WT and ΔdblGATA mice at 60 dpi was evaluated according to the *Materials and Methods* section. One representative of three independent experiments is shown as the median (n = 6 to 7 per genotype). Statistical analysis was performed by using the Mann-Whitney U-test, indicating $P = 0.0734$ for lung burden and $P = 0.1375$ for brain burden.

the Th cytokine profile and leukocyte recruitment require further investigation to enlighten the pathophysiological role that eosinophils play in cryptococcosis.

In conclusion, IL-4 production by both eosinophils and antigen-specific Th2 cells is a relatively late event in pulmonary cryptococcosis. A late and as of yet unidentified process appears to promote the onset of IL-4 production that dominates the production of otherwise protective cytokines IL-17 and IFN-γ. This suggests a cytokine hierarchy, with IL-4 on top of IFN-γ/IL-17 underlining the exquisite role of IL-4 in cryptococcosis. Therefore, it is intriguing to develop therapies antagonizing IL-4 or its receptor. Certainly, the late onset of IL-4 production by Th cells and eosinophils (shown herein) and functional studies in IL-4– or IL-4 receptor α–deficient mice, reported earlier by us,[18] make IL-4 or its receptor attractive drug targets in allergic bronchopulmonary mycosis and possibly in asthma.

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3.3 Einleitung zum Manuskript „A Gene-Dosage Effect for Interleukin-4 Receptor α-Chain Expression Has an Impact on Th2-Mediated Allergic Inflammation during Bronchopulmonry Mycosis"

Die wichtigen Befunde zu IL-13 und IL-4 belegen, wie bedeutend die Rolle dieser beiden Zytokine in der Immunpathologie der pulmonalen Kryptokokkose ist. Nun galt es zu klären, inwieweit die Typ 1- und Typ 2-IL-4R (153,237), welche die Signaltransduktion von IL-4 und IL-13 vermitteln (153), an der Pathogenese beteiligt sind. Die beiden Rezeptoren besitzen eine gemeinsame Kette, die IL-4Rα-Kette. Durch Depletion der IL-4Rα-Kette in der Maus war es deshalb möglich, die Signaltransduktion der IL-4R zu unterbinden, um so eine funktionale IL-4-/IL-13-doppeldefiziente Maus zu erhalten. Durch Kreuzung dieser Maus mit einer Wildtyp-Maus konnten IL-4Rα-heterozygote Tiere erhalten werden. Durch Vergleich der verschiedenen Genotypen wurde untersucht, ob die Zahl der IL-4Rα-Allele Einfluss auf die Immunpathologie in der pulmonalen Kryptokokkose hat.

Hauptergebnisse

- **Im Gegensatz zu Wurminfektionen, bei der große Mengen IL-4 gebildet werden, wird in der pulmonalen Kryptokokkose die IL-4R-Expression gegenüber naiven Tieren nicht erhöht. In der Kryptokokkose werden nur geringe Mengen IL-4 gebildet, die nicht ausreichen, um die Expression des Rezeptors zu erhöhen (240).**
- **Die nach Anzahl der IL-4Rα-Allele abgestufte Expression von Wildtyp-Tieren ($^{+/+}$ biallel), über heterozygote F1-Tiere ($^{+/-}$ monoallel) zu defizienten Tieren ($^{-/-}$) spiegelt sich in der Organlast, der IgE-Bildung und dem Überleben wider. Je höher die Zahl der Allele, umso empfänglicher ist das Tier für immunpathologische Effekte der pulmonalen Kryptokokkose.**
- **Die Anzahl der Allele und die damit einhergehende IL-4Rα-Expression korreliert auch mit der Hyperreagibilität der Atemwege und der alternativen Aktivierung von Makrophagen.**
- **Eine hohe IL-4Rα-Expression fördert die Bildung von IL-5 und reduziert die Bildung von IL-17.**

Schlussfolgerungen

Die IL-4Rα-Expression moduliert die Immunpathologie in der pulmonalen Kryptokokkose, wobei die Expression von der Anzahl der IL-4Rα-Allele abhängt. Da während der Infektion die Expression des IL-4R nicht steigt, korreliert die Stärke der IL-4-/IL-13-Antwort direkt mit der Anzahl der IL-4Rα-Allele und der Ausprägung von immunpathologischen Effekten. Da schon eine teilweise Depletion der IL-4Rα-Kette deutliche Effekte auf die Ausprägung des Krankheitsbilds hat, ist die IL-4Rα-Kette eine ideale Zielstruktur für die Therapie gegen eine pulmonale Kryptokokkose (241).

Tierexperimente

Die Mäuse in der vorliegenden Arbeit wurden im Rahmen des Tierversuchsvorhabens Az. 24-9168.-36/04 (Landesdirektion Sachsen – Dienststelle Leipzig) verwendet.

3.3.1 Publikation 3: Der IL-4R als Schlüsselmolekül in der Immunpathologie der pulmonalen Kryptokokkose

MAJOR ARTICLE

A Gene-Dosage Effect for Interleukin-4 Receptor α-Chain Expression Has an Impact on Th2-Mediated Allergic Inflammation during Bronchopulmonary Mycosis

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Interleukin (IL)–4 and IL-13 are key factors in the pathogenesis of bronchopulmonary mycosis induced in mice by infection with *Cryptococcus neoformans*. Both cytokines use the IL-4 receptor α-chain (IL-4Rα). In this study, we investigated the role played by IL-4Rα expression in susceptibility to pulmonary *C. neoformans* infection. IL-4Rα$^{-/-}$ mice were extremely resistant. To characterize the effect of IL-4Rα expression level on disease outcome, we generated IL-4Rα$^{+/-}$ first-generation (F1) mice. IL-4Rα$^{+/-}$ mice showed intermediate levels of IL-4Rα expression, in contrast to higher levels in wild-type mice and no expression in IL-4Rα$^{-/-}$ mice, indicating biallelic expression of the gene for IL-4Rα *(Il4ra)*. Concomitant with intermediate IL-4Rα expression, F1 mice showed intermediate susceptibility associated with altered Th2/Th17 cytokine production, decreased immunoglobulin E levels, and reduced allergic inflammation. This indicates a gene-dosage effect of IL-4Rα expression on susceptibility to bronchopulmonary mycosis. These data provide the basis for novel therapies antagonizing IL-4Rα in Th2-related pulmonary infection and possibly also in asthma.

The interleukin (IL)–4 receptor α-chain (IL-4Rα) is ubiquitously expressed and is part of the receptor for the Th2 cytokines IL-4 and IL-13 [1], which have been shown to be involved in the fatal outcome of experimental murine cryptococcosis [2–6]. The regulation of Th2 responses is controlled by early IL-4 production, which induces the differentiation of naive T helper cells to Th2 cells [7]. Th2 cells, along with basophils, eosinophils, mast cells, NKT, and γδ T cells, are the main producers of IL-4 and IL-13. IL-4 acts as an important factor affecting B cells, causing isotype switching that leads to the production of IgG1 or IgE, antibody classes that are important for antiparasitic defense mechanisms.

However, these mechanisms are ineffective or even disease promoting against a facultative intracellular pathogen such as *Cryptococcus neoformans*. IL-13, on the other hand, can act on smooth muscle cells and goblet cells to cause bronchoconstriction and mucus production, respectively [8–10]. It is of interest that *C. neoformans* infection can contribute to asthma development, as shown in a rat model [11]. The IL-4– and IL-13–dependent mechanisms lead to the pathogenesis of pulmonary cryptococcosis, especially by induction of alternatively activated macrophages (aaMphs) [3, 5, 12]. Thus, the IL-4 receptor is likely a key regulator in the pathogenicity of cryptococcosis, but this has not yet been proved experimentally. Downstream of the IL-4

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receptor, the signal transduction cascade is controlled by signal transducer and activator of transcription (STAT) 6 [13, 14].

In the present study, we sought to determine whether antagonism of Th2 development during pulmonary cryptococcosis by IL-4Rα ablation (i.e., using IL-4Rα$^{-/-}$ mice) has an additive or synergistic effect compared with antagonism of individual ligands (i.e., using IL-4$^{-/-}$ or IL-13$^{-/-}$ mice). Moreover, we wished to clarify whether the gene for IL-4Rα *(Il4ra)* is expressed biallelically and whether 1 allele containing functional IL-4Rα (i.e., using IL-4Rα$^{+/-}$ mice) is sufficient to induce a full or only a gradual Th2 response in cryptococcosis. Our findings unambiguously demonstrate that *Il4ra* is indeed expressed biallelically and that its expression level is critical for susceptibility to experimentally induced bronchopulmonary mycosis. Depending on the IL-4Rα expression level, there is a gradual appearance of Th2-dependent mechanisms, such as IgE production, allergic inflammation with eosinophilia, goblet cell metaplasia, mucus hyperproduction, and alternative macrophage activation after pulmonary *C. neoformans* infection.

METHODS

Mice. Female mice (6–10 weeks old) included 3 groups: wild-type (WT; IL-4Rα$^{+/+}$), IL-4Rα$^{-/-}$ mice on a BALB/c background [15], and the first generation of WT by IL-4Rα$^{-/-}$ intercrosses (IL-4Rα$^{+/-}$). They were maintained in an individually ventilated caging system under specific pathogen–free conditions and in accordance with the guidelines approved by the Animal Care and Usage Committee of the Regierungspräsidium Leipzig. Sterile food and water were given ad libitum. The mice were tested periodically for pathogens, in accordance with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations accreditation board.

C. neoformans *and infection of mice.* Encapsulated *C. neoformans* (strain 1841, serotype D) was kept as frozen stock in skim milk and was grown in Sabouraud dextrose medium (2% glucose and 1% peptone; Sigma) overnight on a shaker at 30°C. The mice were infected intranasally, and the acapsular *C. neoformans* serotype D strain CAP67 was used as an in vitro stimulus, both as described elsewhere [5].

Determination of survival rate and organ burden. Infected mice were monitored daily for survival and morbidity. Organ burden was determined as described elsewhere [5].

Restimulation of spleen. Splenocytes were stimulated as described elsewhere [5].

Histopathological analysis. On day 70 after infection, *C. neoformans*–infected WT, IL-4Rα$^{-/-}$, and IL-4Rα$^{+/-}$ mice as well as uninfected mice of the same genotypes were perfused intracardially with 0.9% saline while under deep CO_2 asphyxia. The lungs of the animals were removed, mounted on thick filter paper with Tissue-Tek OTC compound (Miles Scientific), snap-frozen in isopentane (Fluka) precooled on dry ice, and stored at −80°C.

For immunohistochemical analysis, 10-μm frozen sections were prepared in a serial fashion (15 transversal sections per lung on 4 consecutive levels). The YM1 (ECF-L) goat anti-mouse antibody was used to detect aaMphs (R&D Systems). Arginase-1 (BD Biosciences) immunostaining was performed using the Dako ARK peroxidase kit, in accordance with the manufacturer's protocol. Immunohistochemical analysis was performed as described elsewhere [16]. Negative controls, without application of the primary antibody, confirmed the specificity of the reactions.

Other parts of the lungs and of the other organs were fixed in neutral-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E) to estimate the extent of granulomatous lesion formation in the various organs or with periodic acid–Schiff reagent to study the distribution of cryptococci and mucus production by goblet cells in lungs, liver, spleen, and kidneys. Analyses of collagen deposition in the organs were done by elastica–van Gieson staining. To study the recruitment of granulocytes, the tissue sections were stained with naphthol AS-D-chloracetate esterase. Histopathological alterations were microscopically evaluated on H&E–stained and immunostained lung sections.

Digestion of lung tissue and analysis of lung leukocytes and blood cells. For IL-4Rα expression experiments, lungs were perfused through the right ventricle with PBS. The lungs were treated as described elsewhere [5]. IL-4Rα expression in cells was analyzed by flow cytometry (FACSCalibur; BD). The cells were stained for IL-4R (CD124; clone mIL4R-M1; BD; phycoerythrin conjugate for staining of lung cells; biotinylated M1 antibody in combination with streptavidin-allophycocyanin [BD] for blood leukocytes), T helper cells (CD4; clone H129.19; BD), cytotoxic T cells (CD8; clone 53–6.7; BD), B cells (CD45R; clone RA3-6B2; Caltag), NK cells (DX-5; clone DX5; BD), granulocytes (Gr-1; clone RB6-8C5; BD), dendritic cells (CD11c; clone HL3; BD), and macrophages (CD11c^{-}/Gr-1^{-}/CD11b^{+}; clone M1/70.15; Caltag).

Cytokine and antibody analysis. Cytokine concentrations were determined by sandwich ELISA systems using unlabeled capture antibodies and labeled detection antibodies, followed by incubation with peroxidase-labeled streptavidin if not otherwise indicated, as described elsewhere [5]. The total serum IgE, IgG1, and IgG2a concentrations in naive and infected mice were measured as detailed elsewhere [5].

Measurement of respiratory lung function. The respiratory lung function of intranasally infected mice was measured in a plethysmographic chamber (model PLT UNR MS; Emka Technologies) for freely moving animals. The pressure inside the chamber was measured by a differential pressure transducer connected to an amplifier (model AMP-B01) and continuously

monitored with IOX software (version 22.17.19). Airway hyperreactivity was examined as described elsewhere [17].

Statistical analysis. The statistical significance of differences between experimental groups of animals was determined using the log-rank test for survival analysis, the 1-tailed Mann-Whitney *U* test for organ burden and fluorescence-activated cell sorter (FACS) analysis, and the 2-tailed Mann-Whitney *U* test for cytokine, isotype levels, and airway hyperreactivity. Differences for which $P < .05$ were considered significant.

RESULTS

Susceptibility to pulmonary* C. neoformans *infection determined by IL-4Rα expression level. We and others have shown that each of the Th2 cytokines IL-4 and IL-13 is associated with susceptibility to *C. neoformans* infection [2–6]. To analyze the effect of simultaneous abrogation of IL-4 and IL-13 on *C. neoformans* infection, we used IL-4Rα$^{-/-}$ mice, which were deficient in the common receptor chain of the IL-4 and IL-13 receptor [18]. The survival of *C. neoformans*–infected IL-4– or IL-13–deficient mice was compared with that of IL-4Rα–deficient mice. In these experiments, the IL-4Rα–deficient (IL-4Rα$^{-/-}$) mice were more resistant than the ligand-deficient mice, which were significantly more resistant than the WT (IL-4Rα$^{+/+}$) mice (proportion of surviving mice in total from 3 independent experiments: for IL-4Rα$^{-/-}$ mice, 26/26; for IL-4$^{-/-}$ mice, 24/25; for IL-13$^{-/-}$ mice, 24/28). Although 100% of the IL-4Rα$^{+/+}$ mice died of the infection, the IL-4Rα$^{-/-}$ mice were completely resistant during an observation period of >200 days after infection (up to 275 days after infection) (figure 1*A*). Examination of the organ burden in lung and brain at day 70 after infection showed significantly reduced numbers of cryptococci in the lungs of the IL-4Rα$^{-/-}$ mice (figure 1*B*). In the absence of IL-4Rα expression, dissemination of *C. neoformans* to the brain is prevented (figure 1*C*). Long-term examination for >200 days after infection (up to 275 days after infection) revealed that stable control of the fungal burden in the lung is achieved in IL-4Rα$^{-/-}$ mice, but not sterile elimination (figure 1*B*).

In light of the contrasting phenotypes observed in infected IL-4Rα$^{+/+}$ versus IL-4Rα$^{-/-}$ mice, we wanted to learn whether an intermediate level of expression of IL-4Rα would have an effect on the resistance or susceptibility to pulmonary *C. neoformans* infection. Therefore, IL-4Rα heterozygous (IL-4Rα$^{+/-}$ mice) were generated. To determine the relative expression of IL-4Rα on leukocytes of naive and *C. neoformans*–infected IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ mice, we determined the median fluorescence intensity by flow cytometry, using a monoclonal antibody specific for IL-4Rα. Expression of IL-4Rα was characterized for leukocytes in lungs, blood (table 1), brain, lymph nodes, and spleen (data not shown). Interestingly, IL-4Rα expression in all examined tissues of naive IL-4Rα$^{+/-}$ mice showed an intermediate level between the high levels found in IL-4Rα$^{+/+}$ mice and

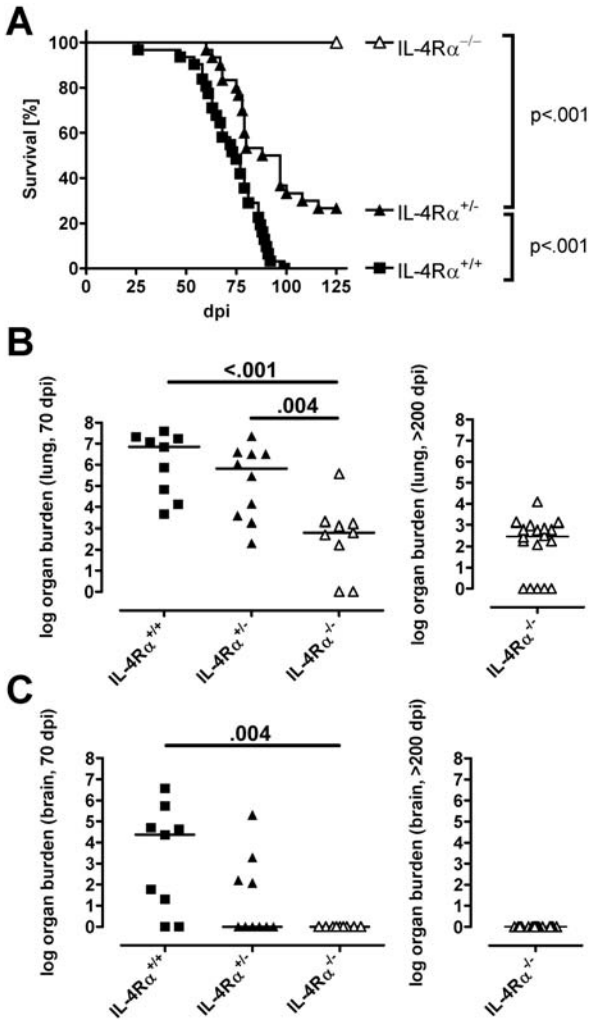

Figure 1. Resistance of homozygous and prolonged survival of heterozygous interleukin (IL)–4 receptor α-chain (IL-4Rα)–deficient mice relative to susceptible wild-type (WT) mice during pulmonary *Cryptococcus neoformans* infection. IL-4Rα$^{+/+}$, IL-4Rα$^{+/-}$, and IL-4Rα$^{-/-}$ mice were intranasally infected with 500 cfu of *C. neoformans (A).* The survival time of mice was recorded for 150 days after infection. Although no IL-4Rα$^{-/-}$ mice died of the infection, IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ mice had median survival times of 75 and 92.5 days, respectively; 26.7% of IL-4Rα$^{+/-}$ mice survived for >150 days after infection. In one experiment the mice were examined for 272 days after infection, and 1 of 12 survived the whole period. The survival graph represents data from 3 independent experiments; the log-rank test was used for statistical analysis. Organ burdens in lungs *(B)* and brain *(C)* were analyzed on day 70 after infection and, for IL-4Rα$^{-/-}$ mice, on days 202, 246, 272, and 275 after infection as well (pooled as >200 days after infection). IL-4Rα$^{-/-}$ mice had significantly lower organ burdens in lungs and brain than did IL-4Rα$^{+/+}$ mice. IL-4Rα$^{+/-}$ mice tended to have lower organ burdens than the WT mice. IL-4Rα$^{-/-}$ mice did not completely eliminate the cryptococci but had low organ burdens even beyond 200 days after infection. There were no differences in the lung burdens of the IL-4Rα$^{-/-}$ mice between 70 and >200 days after infection. Data from 3 independent experiments were pooled. Significance was calculated with the 1-tailed Mann-Whitney *U* test. dpi, days post infection.

Table 1. Interleukin (IL)–4 receptor α-chain (IL-4Rα) expression in blood leukocytes and lung cells of naive and *Cryptococcus neoformans*–infected mice 70 days after infection.

Cell type	Median fluorescence intensity		
	IL-4Rα$^{+/+}$ mice	IL-4Rα$^{+/-}$ mice	IL-4Rα$^{-/-}$ mice
Naive PBLs			
T helper cells	53.61	32.93[a]	11.05[a,b]
Cytotoxic T cells	56.20	41.28[a]	14.67[a,b]
B cells	57.38	27.14	16.35
Macrophages	47.92	27.79	20.28[a]
Granulocytes	33.50	24.07[a]	21.46[a]
Dendritic cells	29.25	16.70	16.38[a]
NK cells	31.10	24.95	15.94
Lung cells			
Naive			
All lung cells	5.75	4.16	2.88
CD3$^+$ lung cells	6.58	3.57	1.86
CD11b$^+$ lung cells	2.94	2.56	2.32
70 days after infection			
All lung cells	4.54	3.52[a]	2.68[a]
CD3$^+$ lung cells	4.28	3.20[a]	2.24[a]
CD11b$^+$ lung cells	3.19	2.51[a]	2.81

NOTE. Heterozygous IL-4Rα–deficient mice have an intermediate level of IL-4Rα expression in blood and lung leukocytes. Although the fluorescence intensity of IL-4Rα in IL-4Rα$^{-/-}$ mice marks the minimum median fluorescence intensity and IL-4Rα$^{+/+}$ leukocytes show the maximum value of IL-4Rα expression, intermediate expression levels of IL-4Rα were found in IL-4Rα$^{+/-}$ leukocytes. For peripheral blood leukocytes (PBLs), each value represents blood analyses for 5–9 naive mice from 4 independent experiments. For lung cells, data are shown from 1 of 2 similar experiments with lung cells from naive mice and pooled data from 2 independent experiments 70 days after infection.

[a] $P < .05$ for the comparison with IL-4Rα$^{+/+}$ mice (1-tailed Mann-Whitney *U* test).

[b] $P < .05$ for the comparison with IL-4Rα$^{+/-}$ mice (1-tailed Mann-Whitney *U* test).

the absent expression in IL-4Rα$^{-/-}$ mice. This argues for biallelic expression of *Il4ra*. The levels of IL-4Rα expression were especially high in lymphocytes compared with innate immune cells (table 1). No difference could be found between the IL-4Rα expression levels of IL-4Rα$^{+/-}$ and IL-4Rα$^{-/-}$ phagocytes and dendritic cells. This may be related to the detection limit of the FACS method (owing to the lower IL-4Rα expression in innate immune cells) or to monoallelic expression of IL-4Rα in phagocytes and dendritic cells in contrast to lymphocytes (table 1). Importantly, intranasal infection of mice with the highly virulent *C. neoformans* strain 1841 did not modulate IL-4Rα expression in lung cells (table 1) or blood leukocytes (data not shown).

To define the susceptibility of IL-4Rα$^{+/-}$ mice expressing intermediate levels of IL-4Rα against *C. neoformans*, we infected IL-4Rα$^{+/-}$ mice intranasally, together with IL-4Rα$^{+/+}$ and IL-4Rα$^{-/-}$ mice. Strikingly, *C. neoformans*–infected IL-4Rα$^{+/-}$ mice exhibited a significantly prolonged median survival time compared with IL-4Rα$^{+/+}$ mice (92.5 vs. 75 days after infection) (figure 1*A*). In accordance with the lower susceptibility found in infected IL-4Rα$^{+/-}$ mice, the lung and brain fungal burden of the IL-4Rα$^{+/-}$ mice was reduced (figure 1*B*). This finding indicates a gene-dosage effect of IL-4Rα expression on resistance to pulmonary infection with *C. neoformans*.

Degree of fatal Th2 induction induced by* C. neoformans *infection determined by IL-4Rα expression level. To define the mechanism(s) responsible for the partial resistance of the heterozygous IL-4Rα$^{+/-}$ mice, immune parameters were investigated. It has been shown elsewhere that development of protective Th1 versus pathological Th2 responses is critical for resistance rather than susceptibility to *C. neoformans* infection [19–22]. Interestingly, the absence of a Th2 response appears to be even more important than the magnitude of the Th1 response for the course of cryptococcosis [3, 5]. The Th2-dependent isotypes IgE and IgG1 were chosen as in vivo markers of Th2 development. Although serum IgE was significantly reduced after *C. neoformans* infection of IL-4Rα$^{+/-}$ mice compared with IL-4Rα$^{+/+}$ mice, the former group showed comparably elevated levels of IgG1 (table 2). Both IgE and IgG1 levels were significantly reduced in IL-4Rα$^{-/-}$ mice; IgE was not even detectable in infected IL-4Rα$^{-/-}$ mice. Naive mice of the IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ genotypes showed comparably low IgE levels (table 2). Thus, induction of IgE is strictly IL-4Rα dependent (table 2) and correlates with the IL-4Rα expression level in leukocytes, as shown above (table 1). Furthermore, with IgE as an important marker of fatal Th2 development during cryptococcosis, the data showed that IL-4Rα expression levels critically determine the degree of Th2-mediated susceptibility. For the Th1-related isotype IgG2a, no differences were found between the 3 genotypes. Compared with naive mice, all 3 groups showed comparably

Table 2. Serum IgE and IgG1 levels in naive and *Cryptococcus neoformans*–infected mice 70 days after infection.

Immunoglobulin	Serum concentration, μg/mL		
	IL-4Rα$^{+/+}$ mice	IL-4Rα$^{+/-}$ mice	IL-4Rα$^{-/-}$ mice
IgE			
Naive	0.97	1.42	0.00
70 days after infection	24.50	4.02[a]	0.00
IgG1			
Naive	58.04	24.07	22.75
70 days after infection	701.08	1173.94	66.83[a,b]

NOTE. Intermediate interleukin (IL)–4 receptor α-chain (IL-4Rα) expression leads to reduced IgE but comparable IgG1 levels after infection with *C. neoformans*. In infected mice, the serum levels of IgE differ significantly between these genotypes, whereas naive IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ mice show comparable levels of IgE and IgG1. Values shown are medians from 3–4 experiments with 3 mice per experiment.

[a] $P < .01$ for the comparison with IL-4Rα$^{+/+}$ mice (2-tailed Mann-Whitney *U* test).

[b] $P < .001$ for the comparison with IL-4Rα$^{+/-}$ mice (2-tailed Mann-Whitney *U* test).

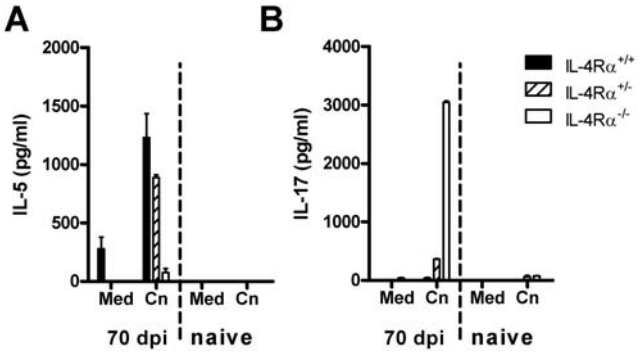

Figure 2. Dependence of antigen-specific production of Th2 *(A)* and Th17 *(B)* cytokines on the level of expression of interleukin (IL)–4 receptor α-chain (IL-4Rα). Restimulated splenocytes from naive and infected mice 70 days after infection produced *Cryptococcus neoformans*–specific IL-5 and IL-17. Although the IL-5 level increased with IL-4Rα expression, IL-17 production decreased. Data from 1 of 2 independent experiments are shown. Cn, heat-killed cryptococci (MOI of 10); dpi, days post infection; med, medium.

elevated serum IgG2a levels on day 70 after *C. neoformans* infection (data not shown).

To assess Th2 responses more directly in IL-4Rα$^{+/+}$, IL-4Rα$^{+/-}$, and IL-4Rα$^{-/-}$ mice infected intranasally with *C. neoformans,* we analyzed ex vivo cytokine production by splenocytes. Because IL-4 was consumed by cultured WT (IL-4Rα$^{+/+}$) splenocytes [23] but to a lesser degree by IL-4Rα$^{+/-}$ splenocytes and not at all by IL-4Rα$^{-/-}$ splenocytes, it was difficult to interpret antigen-specific production of IL-4. Therefore, we studied IL-5, another Th2 cytokine that has been characterized as being associated with the immunopathology of cryptococcosis [3, 5]. Antigen-specific stimulation of splenocytes with heat-killed cryptococci led to high levels of IL-5 production by splenocytes derived from susceptible IL-4Rα$^{+/+}$ mice, whereas splenocytes from partially resistant IL-4Rα$^{+/-}$ mice and totally resistant IL-4Rα$^{-/-}$ mice showed gradually reduced IL-5 production (figure 2*A*). In addition, splenocytic IL-17 production, recently shown by us to be associated with protection in *C. neoformans* infection [24], was found to be gradually elevated in partially resistant IL-4Rα$^{+/-}$ mice and totally resistant IL-4Rα$^{-/-}$ mice, compared with susceptible IL-4Rα$^{+/+}$ mice (figure 2*B*).

Nature of the pulmonary inflammatory response to* C. neoformans *infection determined by IL-4Rα expression level, which affects lung function. The pulmonary inflammatory response to *C. neoformans* infection crucially depends on the nature of the T cell response, that is, a protective Th1/Th17 versus a nonprotective Th2 response [6, 19, 24–26]. Th2 responses are associated with the appearance of eosinophils, the modulation of goblet cells, and alternative macrophage activation. In accordance with lower IL-5 production (figure 2*A*), fewer eosinophils were detected in the lungs of partially resistant IL-4Rα$^{+/-}$ mice compared with IL-4Rα$^{+/+}$ mice with pronounced cryptococcosis (figure 3*A* and 3*B*). The goblet cell metaplasia and mucus hyperproduction found in susceptible *Cryptococcus*-infected IL-4Rα$^{+/+}$ mice was strongly reduced in IL-4Rα$^{+/-}$ mice (figure 3*D* and 3*E*). No eosinophils and no alteration of goblet cells could be found in the highly resistant IL-4Rα$^{-/-}$ mice (figure 3*C* and 3*F*). Therefore, the allergic inflammatory response of the lungs reflects the IL-4Rα expression levels responsible for susceptibility against *C. neoformans* infection (figure 1 and table 1).

Alternative activation of lung macrophages is associated with a fatal course of cryptococcosis [3, 5, 12]. The immunostaining of chitinase-like YM1 and arginase-1, both markers of aaMphs, revealed only marginal expression of YM1 and arginase-1 in the lungs of infected IL-4Rα$^{-/-}$ mice but strongly enhanced expression of both in IL-4Rα$^{+/+}$ mice (figure 3*G,* 3*I,* 3*J,* and 3*L*). The number of aaMphs was found to be reduced in the heterozygous mice compared with IL-4Rα$^{+/+}$ mice (figure 3*G,* 3*H,* 3*J,* and 3*K*), again pointing to an intermediate phenotype of IL-4Rα$^{+/-}$ mice. YM1^{+} cells appeared voluminous and stained strongly positive for macrophage markers, such as F4/80 and CD11b (data not shown). The lungs of IL-4Rα$^{+/+}$ mice showed massive focal accumulations of YM1^{+} macrophages. In contrast, the distribution of YM1^{+} macrophages in IL-4Rα$^{+/-}$ mice was more diffuse, and these cells appeared smaller and more compact. These results provide evidence for IL-4Rα–dependent development of aaMphs in *C. neoformans* infection.

To see whether the IL-4Rα–dependent pulmonary inflammatory response had a functional consequence, we studied airway hyperreactivity in the 3 mouse groups. After receipt of high doses of the asthma-inducing agent methacholine, *C. neoformans*–infected IL-4Rα$^{+/+}$ mice displayed significantly elevated airway hyperreactivity compared with IL-4R α$^{-/-}$mice (figure 3*M*). IL-4Rα$^{+/-}$ mice showed a phenotype between those of WT and IL-4Rα$^{-/-}$ mice, an important hint for a critical effect of the IL-4Rα expression level on lung function.

DISCUSSION

Susceptibility to *C. neoformans* infection is critically correlated with Th2 development [2–4, 6]. In the present study, we chose to target IL-4Rα to interfere simultaneously with the fatal action of IL-4 and IL-13. Interestingly, IL-4Rα$^{-/-}$ mice were found to acquire a particularly high degree of protective immunity associated with complete control of brain infection and long-term survival. As expected, targeting the shared receptor IL-4Rα was more effective than targeting the individual ligands (especially IL-13) in protecting against *C. neoformans* infection [5]. Interestingly, IL-4Rα$^{-/-}$ mice were unable to eliminate *C. neoformans* in the lungs during a prolonged period (up to 275 days after infection) but were very well able to control the pulmonary fungal load at a stable level between 70 and 275 days after infection. This shows the impressive efficacy of antagonizing Th2 activity to prevent reactivation of *C. neoformans* infection from the

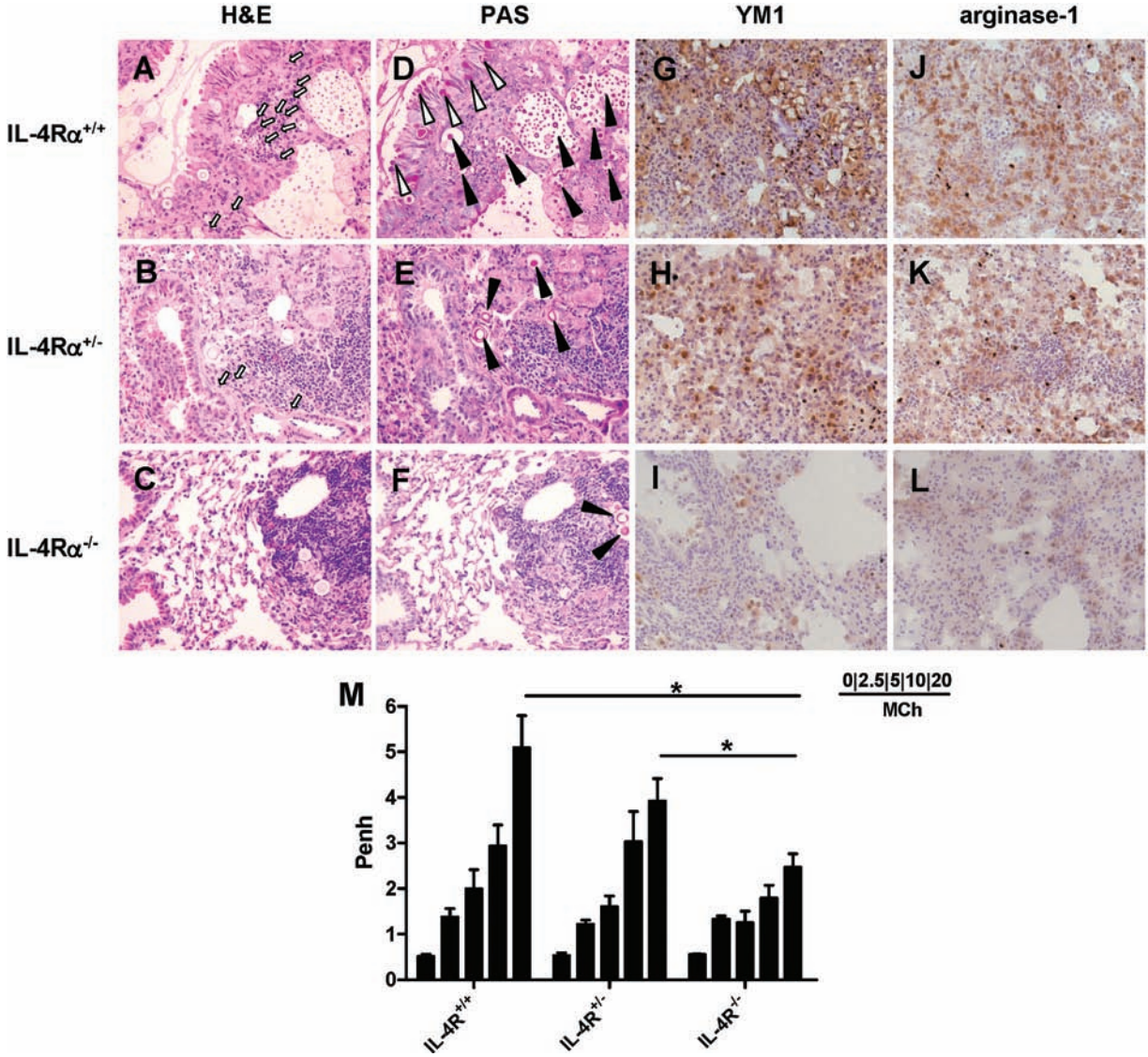

Figure 3. Reduced pulmonary eosinophilia, reduced nos. of alternatively activated macrophages (aaMphs), goblet cell metaplasia, and mucus production associated with decreased airway hyperreactivity resulting from lower expression of interleukin (IL)–4 receptor α-chain (IL-4Rα). The degree of susceptibility correlates with the nos. of eosinophils *(white arrows),* goblet cell metaplasia and mucus production *(white arrowheads),* and organ burden *(black arrowheads). A–C,* Eosinophils. Large nos. of eosinophils could be detected in the lungs of *Cryptococcus neoformans*–infected IL-4R$\alpha^{+/+}$ mice on day 70 after infection, whereas, in IL-4R$\alpha^{-/-}$ mice, no eosinophils could be detected. IL-4R$\alpha^{+/-}$ mice represent an intermediate phenotype; all eosinophils in the high-power field are marked *(arrows),* in contrast to the IL-4R$\alpha^{+/+}$ mice, for which only some of the eosinophils are marked by arrows. H&E, hematoxylin-eosin. *D–F,* Lung burden. The organ burden in the lungs correlated with the degree of susceptibility in the examined genotypes. Although IL-4R$\alpha^{+/+}$ mice showed large amounts of *C. neoformans*, the IL-4R$\alpha^{-/-}$ mice displayed only minimal nos. of fungal pathogens on day 70 after infection. The IL-4R$\alpha^{+/-}$ mice represented an intermediate phenotype, with reduced nos. of cryptococci. In addition, the IL-4R$\alpha^{+/+}$ mice displayed profound goblet cell metaplasia with mucus production, whereas IL-4R$\alpha^{+/-}$ mice showed only reduced and focally occurring metaplasia. In IL-4R$\alpha^{-/-}$ mice, no goblet cell metaplasia and mucus hyperproduction could be detected. *G–L,* aaMphs. Focal dense accumulations of large, rounded aaMphs, positive for YM1 *(G–I)* and arginase-1 *(J–L),* were detected in IL-4R$\alpha^{+/+}$ mice. YM1- and arginase-expressing aaMphs could also be found in IL-4R$\alpha^{+/-}$ mice, albeit with a more diffuse and loose distribution, but IL-4R$\alpha^{-/-}$ mice exhibited no significant accumulations of this cell type. Micrographs representative of 2 independent experiments are shown. *M,* Airway hyperreactivity. The airway hyperreactivity of the infected genotypes was compared between the indicated groups on day 70 after infection. Data from 2 independent experiments were pooled. MCh, methacholine (in μg/mL); Penh, enhanced pause. *$P < .05$.

lungs. Moreover, our data indicate, for the first time, a gene-dosage effect of IL-4Rα in anti-infective immunity.

The data from *C. neoformans*–infected IL-4R$\alpha^{-/-}$ mice do not allow us to draw conclusions on the individual contribution of IL-4 versus IL-13. Certainly, the survival rates of IL-4$^{-/-}$ mice (96%) and IL-13$^{-/-}$ mice (86% and [5]) suggest that the action of IL-4 is somewhat more important than that of IL-13 for susceptibility during cryptococcosis. However, as we have shown

recently, goblet cell metaplasia and mucus production during pulmonary cryptococcosis are IL-13 dependent [5]; this may even contribute to enhanced airway hyperreactivity (figure 3*M* and [5]). Owing to the reduced goblet cell metaplasia and mucus production found in infected IL-4R$\alpha^{+/-}$ mice (figure 3*D*), IL-13 definitely plays a role in susceptibility different from that played by IL-4.

The strikingly contrasting phenotypes of WT versus IL-4R$\alpha^{-/-}$ mice prompted us to determine whether an intermediate level of expression of IL-4Rα would also result in intermediate susceptibility. Indeed, our study describing IL-4Rα expression level–dependent immunity to *C. neoformans* infection is, to the best of our knowledge, the first example of a gene-dosage effect of IL-4Rα in antimicrobial immunity. Infection with *C. neoformans* appears to be different from a number of other infections studied, for which the level of IL-4Rα expression has not been found to be critical for IL-4/IL-13 responsiveness.

In *Leishmania* and *Schistosoma* infection models, heterozygous IL-4Rα mice were found to have phenotypic and immunological responses similar to those in WT mice [27, 28]. It is intriguing to speculate that, in these models, higher IL-4 levels are generated, making it impossible to distinguish between IL-4R$\alpha^{+/+}$ and IL-4R$\alpha^{+/-}$ mice. Presumably, both the level of IL-4 production and the level of IL-4Rα expression together regulate the intensity of Th2-driven disease. Small changes in the level of IL-4Rα expression may be particularly limiting with low-level IL-4 production (as may be the case for *C. neoformans* infection).

As shown earlier, IL-4Rα up-regulation is dependent on the concentration of IL-4 [29]. With higher IL-4 levels produced, low or intermediate levels of IL-4Rα may be up-regulated and less limiting. It is noteworthy that we could not find modulation or even up-regulation of IL-4Rα due to *C. neoformans* infection (table 1), arguing for a low-level IL-4 system in our model. In vitro experiments need to be designed to model quantitatively the IL-4/IL-13/IL-4Rα–dependent pulmonary allergic immune response driven by *C. neoformans*.

The data from this bronchopulmonary mycosis model may have relevant implications for future therapeutic strategies against asthma and atopy. IL-4Rα polymorphism has been shown elsewhere to affect asthma development and prevalence [30–37]. It has been demonstrated that strong IL-4R–dependent signaling in newborns' monocytes and Th lymphocytes could contribute to Th1/Th2 imbalance [38]. These authors concluded that IL-4R overexpression in newborns' monocytes and lymphocytes could be an early risk marker of allergy development. In line with these findings, significantly reduced expression of IL-4Rα associated with reduced IL-4–induced signaling was found in neonatal B cells [39].

In another study, combined extended haplotypes involving IL-4, IL-13, IL-4Rα, and STAT6 were analyzed to assess the combined effect of single-nucleotide polymorphisms in the IL-4/IL-13 signaling pathway [35]. When polymorphisms in all 4 major pathway genes were combined in a stepwise procedure, the risk of high serum IgE levels increased 10.8-fold and the risk of the development of asthma increased 16.8-fold, compared with the maximum effect of any single polymorphism. Interestingly, in *Leishmania*-infected BALB/c STAT6$^{+/-}$ mice, Burgis and Gessner [40] found evidence of distinct STAT6 dosage requirements for different IL-4 functions. Their finding underscores the fact that the IL-4/IL-13 pathway is tightly regulated at different levels of the cascade. Earlier it was shown that different IL-4Rα allotypes exist in inbred mouse strains associated with different levels of IL-4–neutralizing activity [41]. Different degrees of IL-4 responsiveness may be involved in the specific phenotypes of inbred mouse strains in IL-4–dependent infections.

For therapeutic strategies against Th2-related diseases, such as asthma and atopy, our findings, together with other epidemiological data, reveal IL-4Rα to be an attractive target. It is obvious that a complete blockade would not be feasible and, as we have shown, is not necessary. Even a partial blockade could be effective for ameliorating IL-4/IL-13–driven diseases.

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3.4 Einleitung zum Manuskript „IL-4/IL-13-Dependent Alternative Activation of Macrophages but Not Microglial Cells Is Associated with Uncontrolled Cerebral Cryptococcosis"

Das entscheidende Krankheitsbild in der Kryptokokkose bei humanen immungeschwächten Patienten ist die Kryptokokken-Meningitis (4,119). Aus diesem Grunde stellt sich nach Aufklärung der Effekte von IL-4, IL-13 und dem IL-4R die Frage, wie sich diese Faktoren auf das Geschehen in der zerebralen Kryptokokkose, insbesondere Makrophagen und Mikrogliazellen und die Entzündung, auswirken.

Hauptergebnisse

- **Im Gegensatz zu Wildtypmäusen findet sich in IL-4R-defizienten Mäuse für IL-13, IL-4 oder IL-4R nur eine schwache Dissemination von Kryptokokken in das Gehirn, einhergehend mit fehlenden oder schwachen Gehirnläsionen. Tiere, die diese Faktoren besitzen, entwickeln starke Läsionen im Gehirn mit deutlicher Dissemination der Erreger in das ZNS.**
- **Die Zahl an Makrophagen im Gehirn steigt während der pulmonalen Infektion mit *C. neoformans* nur in den Gruppen an, die IL-13, IL-4 und IL-4R exprimieren können. Die schaumzelligen Makrophagen weisen in diesen Tieren eine alternative Aktivierung auf. In den schaumzelligen Makrophagen sind Kryptokokken nachweisbar. In den resistenten Mausstämmen steigt die Zahl an Makrophagen, die keine Zeichen einer alternativen Aktivierung aufweisen, nur geringfügig an.**
- **Die Zahl der Mikrogliazellen ändert sich in allen Gruppen gleichmäßig. Sie werden aktviert, weisen aber in keiner Gruppe eine alternative Aktivierung auf. In Mikrogliazellen sind keine Kryptokokken nachweisbar.**

Schlussfolgerungen

IL-4, IL-13 und IL-4Rα fördern die Dissemination des Erregers aus der Lunge in das Gehirn, wo pseudozystische Läsionen entstehen. Da zwar Makrophagen, aber nicht Mikrogliazellen alternativ aktiviert werden, kann man davon ausgehen, dass die Aktivierung bereits außerhalb des ZNS stattfindet. Da nur in Makrophagen Kryptokokken nachweisbar sind, lässt sich vermuten, dass die alternativ aktivierten Zellen Kryptokokken außerhalb des ZNS (z.B. in der Lunge) aufgenommen und dann in das Gehirn transportiert haben. Die vorliegenden Ergebnisse sind ein Beweis für die immunpathologischen Auswirkungen von alternativ aktivierten Makrophagen in einer Infektion auf das Gehirn (242).

Tierexperimente

Die Mäuse in der vorliegenden Arbeit wurden im Rahmen des Tierversuchsvorhabens Az. 24-9168.-36/04 (Landesdirektion Sachsen – Dienststelle Leipzig) verwendet.

3.4.1 Publikation 4: IL-4, IL-13 und der IL-4R fördern die Immunpathologie der zerebralen Kryptokokkose

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Immunopathology and Infectious Diseases

IL-4/IL-13-Dependent Alternative Activation of Macrophages but Not Microglial Cells Is Associated with Uncontrolled Cerebral Cryptococcosis

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Both interleukin (IL)-4- and IL-13-dependent Th2-mediated immune mechanisms exacerbate murine *Cryptococcus neoformans*-induced bronchopulmonary disease. To study the roles of IL-4 and IL-13 in cerebral cryptococcosis, IL-4 receptor α-deficient (IL-4Rα$^{-/-}$), IL-4-deficient (IL-4$^{-/-}$), IL-13-deficient (IL-13$^{-/-}$), IL-13 transgenic (IL-13$^{T/+}$), and wild-type mice were infected intranasally. IL-13$^{T/+}$ mice displayed a higher fungal brain burden than wild-type mice, whereas the brain burdens of IL-4Rα$^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$ mice were significantly lower as compared with wild-type mice. On infection, 68% of wild-type mice and 88% of IL-13-overexpressing IL-13$^{T/+}$ mice developed significant cerebral lesions. In contrast, only a few IL-4Rα$^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$ mice had small lesions in their brains. Furthermore, IL-13$^{T/+}$ mice harbored large pseudocystic lesions in the central nervous system parenchyma, bordered by voluminous foamy alternatively activated macrophages (aaMphs) that contained intracellular cryptococci, without significant microglial activation. In wild-type mice, aaMphs tightly bordered pseudocystic lesions as well, and these mice, in addition, showed microglial cell activation. Interestingly, in resistant IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-4Rα$^{-/-}$ mice, no aaMphs were discernible. Microglial cells of all mouse genotypes neither internalized cryptococci nor expressed markers of alternative activation, although they displayed similar IL-4Rα expression levels as macrophages. These data provide the first evidence of the development of aaMphs in a central nervous system infectious disease model, pointing to distinct roles of macrophages versus microglial cells in the central nervous system immune response against *C. neoformans*. *(Am J Pathol 2009, 174:486–496; DOI: 10.2353/ajpath.2009.080598)*

The opportunistic pathogenic yeast *Cryptococcus neoformans* causes life-threatening fungal infections of most internal organs including the central nervous system (CNS), primarily in patients affected by immunodeficiency syndromes such as AIDS.[1] The pathogenesis of cryptococcosis is not fully understood, however, especially in cases of different levels of immunocompetence. It is generally accepted that the fungus first invades the respiratory system, where it leads to relatively mild or asymptomatic bronchopneumonia in the immunocompetent.[2–5] Fungemia with generalization of the infection may result from reduced immunological control mechanisms.[6–9] Invasion of the CNS with subsequent development of meningoencephalitis is the major cause of death during cryptococcosis.[10,11]

The precise reaction pattern of recruited inflammatory cells, especially monocytes/macrophages, due to fungal invasion of the CNS parenchyma has been addressed

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mainly via analysis of helper T cell (Th)1 responses.[12] In this context, in addition to protective Th1-driven immune responses, the role of Th2 cytokines has gained interest recently.[13] The major Th2 cytokines interleukin (IL)-4 and IL-13 act via the IL-4Rα chain together with the γ_c chain or the IL-13Rα1/2 chains, and regulate macrophage functional status.[14] IL-4 has been shown to be detrimental in murine models of systemic and pulmonary cryptococcosis,[6,15–18] and we have recently illustrated the role of IL-13 in inducing the formation of alternatively activated macrophages (aaMphs) in murine pulmonary cryptococcosis.[19]

The activation phenotype of macrophages may critically influence the regulatory mechanisms by which inflammation and infection in the CNS are controlled. According to the current paradigm, classically activated macrophages are primed by interferon-γ and produce tumor necrosis factor, IL-1, oxygen and nitrogen radicals,[20] thereby producing proinflammatory cytokines that regulate the Th1 immune response. In contrast, aaMph[21] develop in response to Th2 cytokine stimulation such as IL-4 and IL-13 and are characterized by expression of genes associated with endocytosis and tissue repair such as arginase-1, mannose receptor (CD206), found-in-inflammatory-zone (FIZZ), and chitinase 3-like 3 (YM1) and largely fail to produce nitric oxide (NO) due to their induction of arginase.[22] As such, they are thought to be involved in tissue repair and remodeling,[22,23] in protection against diet-induced obesity,[24,25] and schistosomiasis,[26] but they may also elicit adverse tissue processes such as pulmonary or liver fibrosis.[27–31] In particular, their development renders the host vulnerable to infection with pathogens where macrophage activation and killing functions are required.[32]

In murine models of pulmonary *C. neoformans* infection, aaMph have been shown to be associated with uncontrolled lung infection.[18,19] The role of aaMph versus classically activated macrophage in the CNS due to pulmonary infection with the neurotropic pathogen *C. neoformans* has not been defined yet. In this study, we aimed to characterize the morphology and functional status of CNS macrophages in cerebral cryptococcosis following intranasal infection of susceptible wild-type and IL-13-transgenic BALB/c mice. Moreover, using mice unable to produce IL-4 or IL-13 or respond to both (IL-4R$\alpha^{-/-}$ mice), we show that abrogation of CNS aaMph development is associated with controlled infection.

Materials and Methods

Mice

Six to ten-week-old female wild-type, IL-4R$\alpha^{-/-}$,[33] IL-4$^{-/-}$,[34] IL-13$^{-/-}$,[35] as well as IL-13$^{T/+}$,[36] mice on BALB/c background were maintained in an IVC-Caging system under specific pathogen-free conditions and in accordance with the guidelines approved by the Animal Care and Usage Committee of the 'Regierungspräsidium Leipzig.' Sterile food and water were given *ad libitum*. The mice were tested periodically for pathogens in accordance with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations (FELASA) accreditation board. All mice were tested negative for pinworms and other endo- and ectoparasites.

Intranasal Infection of Mice with C. neoformans

Encapsulated *C. neoformans*, strain 1841, serotype D was kept as a frozen stock in skim milk and was grown in Sabouraud dextrose medium (2% glucose, 1% peptone, Sigma, Deisenhofen, Germany) overnight on a shaker at 30°C. Cells were washed twice in sterile PBS, resuspended in PBS, and counted in a hematocytometer. Inocula were diluted in PBS to a concentration of 2.5×10^4/ml for intranasal (i.n.) injections. Mice were infected by i.n. application of 10 μl volumes per nostril containing a total of 500 colony-forming units. For the intranasal infection, mice were anesthetized i.p. with a 1:1 mixture of 10% ketamine (100 mg/ml; Ceva Tiergesundheit, Düsseldorf, Germany) and 2% xylazine (20 mg/ml; Ceva Tiergesundheit).

Determination of Survival Rate and CNS Fungal Burden

Infected mice were monitored daily for survival and morbidity. Fungal burden was determined after sterile removal of the CNS from sacrificed mice and homogenization in 1 ml PBS with an Ultra-Turrax (T8; IKA-Werke, Staufen, Germany). Serial dilutions of the homogenates were plated on Sabouraud dextrose agar plates and colonies were counted after an incubation period of 72 hours at 30°C.

CNS Tissue Processing for Immunohistological Analysis and Electron Microscopy

On the indicated days post infection (p.i.), *C. neoformans*-infected wild-type, IL-4R$\alpha^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$, or uninfected mice, were perfused intra-cardially with 0.9% saline under CO_2 asphyxia. The brains of the animals were sterilely removed, mounted on thick filter paper with Tissue Tek optimal cutting temperature compound (Miles Scientific, Naperville, IL), snap-frozen in isopentane (Fluka, Neu-Ulm, Germay) pre-cooled on dry ice, and stored at −80°C.

For immunohistochemistry, 10-μm frozen sections were prepared in a serial fashion (30 transversal sections on six consecutive levels per CNS). The following rat anti-mouse monoclonal antibodies, obtained as hybridomas from the ATCC (Manassas, VA), were used for staining procedures: CD4 (clone G.K.1.5), CD8 (clone 2.43), CD45 (LCA, clone M1/9.3.4.HL.2), F4/80 (F4/80), MHC class II (I-A^{b,d,q} haplotypes, clone M5.114.15.2), and Ly6-G (GR1; clone RB6−8C5). Additionally, 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 for staining of alternatively activated macrophages.

Immunohistochemistry was performed by use of the Vectastain Elite ABC-Kit (Vector, Burlingame, CA) with appropriate biotinylated secondary antibodies. The peroxidase reaction product was visualized using 3,3′-diaminobenzidine (Sigma) as chromogene and H_2O_2 as cosubstrate. Negative controls, without application of the primary antibody, confirmed the specificity of the reactions. Glucuronoxylomannan (GXM) immunostaining was performed using mab 18B7 (kindly provided by Dr. Arturo Casadevall, Albert Einstein College of Medicine, New York, NY), and the Vector MOM Fluoresceine KIT (Vector). Histopathological alterations were microscopically evaluated on H&E-stained, and immunostained horizontal brain sections. The samples prepared for electron microscopy analysis were fixed in 2.5% cacodylate-buffered glutaraldehyde (pH 7.35). Following postfixation in phosphate-buffered 1.3% osmium tetroxide, the tissues were rinsed in buffer, dehydrated in graded alcohols and processed into polymerized blocks of Epon resin. Sections for initial light microscopy to determine tissue quality and architecture suitable for ultrastructure were cut at 0.5 μm and stained with toluidine blue. For electron microscopy, thin sections were cut with a diamond knife, mounted on copper grids, stained with alkaline lead citrate and 8% uranyl acetate, and examined using a Philips EM 208 electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV. Photography was performed with a Morada digital camera (Olympus SIS, Munster, Germany).

RNA Extraction

Total RNA was extracted from brain tissue samples of the respective time points using the trizol/chloroform method according to the manufacturer's instruction (Invitrogen Carlsbad, CA), and resuspended in 60 μl DEPC-treated water. The concentration of total RNA was determined using the Qubit fluorometer according to the manufacturer's protocol (Invitrogen, Eugene, OR).

Quantitative Real-Time PCR

The RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol using 1 ng total RNA per sample as described.[37] Briefly, the expression level of arginase-1, YM1, and the endogenous control gene, hypoxanthin-guanine-phosphoribosyl-transferase (*hprt*), in the specimens was analyzed by real-time quantitative reverse transcriptase (RT)-PCR using the 5′-nuclease technology on an ABI PRISM 7300HT Sequence Detection System and the Mouse TaqMan pre-developed assay reagents (both: Applied Biosystems). The assay identification numbers are as follows: IL-4, Mm00445259_m1; IL-13, Mm00434204_m1; Arginase-1, Mm01190441_g1; YM1, Mm00657889_mH, and hypoxanthine-guanine-phosphoribosyl-transferase (HPRT), Mm00446968_m1. PCR reactions were prepared in a final volume of 20 μl, with final concentrations of 1x TaqMan Universal PCR Master Mix (Applied Biosystems), and a cDNA equivalent of 5 ng RNA. All analyses were performed in triplicate, and the threshold cycle (C_t) was determined. Gene expression was concomitantly measured in naive (non infected) murine brain as calibrator, to allow comparison between five different samples of wild-type, IL-4R$\alpha^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice, using the $\Delta\Delta C_t$-method.[38]

Cytokine and Antibody Analysis

Cytokine concentrations were determined by sandwich enzyme-linked immunosorbent assay systems with unlabeled capture antibodies (Abs), and labeled detection Abs. To determine the concentration of IL-4, mAb 11B11 was used as the capture Ab and biotin-labeled BVD6-24G2 (BD Pharmingen) was used as the detection Ab followed by incubation with peroxidase-labeled streptavidin. The concentration of IL-13 was detected with the R&D Systems Duoset kit.

Flow Cytometry

Cerebral leukocytes were isolated from brains after perfusion with 0.9% NaCl. Brain tissue was minced through a 100 μm-mesh sieve Becton-Dickcinson (BD), leukocytes were separated by Percoll gradient centrifugation (Amersham-Pharmacia, Freiburg, Germany),[39] and brain-derived leukocytes were subjected to double or triple immunofluorescence staining followed by flow cytometry as described.[37,40] Macrophages were distinguished from microglial cells according to their higher levels of CD45.[41] Neutrophils, which were LCA^{high}Gr-1^{high}, could be distinguished from macrophages (LCA^{high}Gr-1^{-} and LCA^{high}Gr-1^{dim}). The expression level of IL-4Rα on cells was analyzed by using a biotinylated antibody against murine CD124 (clone mIL4R-M1; BD), combined with streptavidin-allophycocyanine. Brains of 5 mice were cut into halves, while 1 ml of each 5 halves were pooled and subjected to flow cytometry, the other 5 halves were cryopreserved individually. Before pooling these halves, 1 ml of the homogenized brain tissue (each half was passed through a 100 μm sieve [BD] and suspended in 2 ml of PBS) was subjected to colony forming units analysis.

Statistical Evaluation

The one-tailed Mann-Whitney test was performed to determine the significance of differences in the intracerebral fungal load, and the two-tailed Mann-Whitney test for the number of intracerebral leukocytes between wild-type, IL-4R$\alpha^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice, and quantitative differences in mRNA transcripts. Data are presented as means ± SD. The level of confidence for significance was $P < 0.05$. All experiments were performed in triplicate. A representative experiment is shown in each figure.

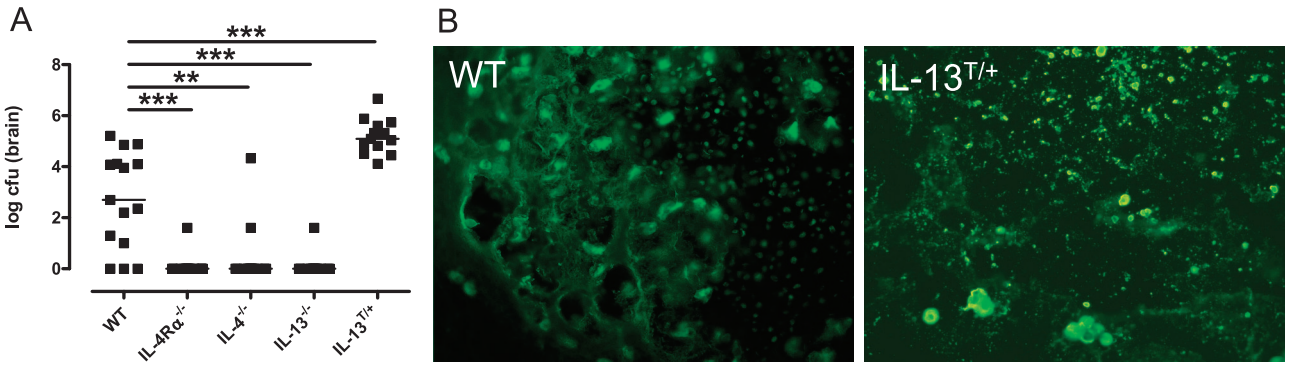

Figure 1. IL-4/IL-13 expression leads to elevated intracerebral fungal load in mice infected i.n. with *C. neoformans* for 60 days. **A:** The intracerebral fungal load of wild-type (WT), IL-4Rα$^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice was counted after sterile removal of the brain (n = 5 animals/genotype) and data from four experiments were pooled. Data represent the median of 15 animals per group. Statistical analysis between wild-type, IL-4Rα$^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice was performed by use of the Mann-Whitney-U Test. $^{**}P < 0.01$; $^{***}P < 0.001$. **B:** In wild-type mice, huge amounts of anti-GXM positive cryptococci and yeast fragments can be found within a pseudocystic lesion. In IL-13$^{T/+}$ mice, a large pseudocystic lesion, harboring densely packed masses of small yeast fragments, and large grouped intact yeasts are shown. Day 60 p.i.; Anti-GXM immunostaining magnification = original ×400.

Results

Elevated Cerebral Fungal Load in the Presence of IL-4 and IL-13 in Mice Infected Intranasally with C. neoformans *for 60 Days*

Following pulmonary infection of wild-type and the type 2 cytokine mutant mice, IL-4Rα$^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice, with the highly virulent strain *C. neoformans* 1841, dissemination to the CNS becomes detectable after about 40 to 50 days of infection. At 60 dpi the expression of IL-4 or IL-13 in wild-type mice is associated with high organ burdens of *C. neoformans* within the CNS, and over-expression of IL-13 in IL-13$^{T/+}$ mice leads to even higher levels of cryptococci (Figure 1A). In contrast, brain infection was undetectable in almost all of the intranasally infected IL-4Rα$^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$ mice, indicating protection from cerebral cryptococcosis in the absence of IL-4/IL-13 signaling (Figure 1A).

As shown in Figure 1B, deposition of the polysaccharide GXM associated with the capsule of *C. neoformans* was found abundantly in wild-type and IL-13$^{T/+}$ mice. In wild-type mice (Figure 1B), GXM-positive cryptococci were mostly found intact at the border of the lesion and fragmented within the center of the lesion. In IL-13$^{T/+}$ mice (Figure 1B), intense GXM immunostaining revealed a fragmented pattern with only a few intact yeasts. However, GXM immunoreactivity was absent in resistant IL-4Rα$^{-/-}$ mice, highly restricted to local lesions in (2 out of 25) IL-4$^{-/-}$ mice, and minimal in one (out of 25) IL-13$^{-/-}$ mouse (data not shown).

IL-4 and IL-13 Profoundly Impact Lesion Development within the CNS Induced by C. neoformans

Eighty-eight percent (ie, 22 out of 25) of IL-13$^{T/+}$ mice and 68% (ie, 17 out of 25) of wild-type mice showed intracerebral lesions, while only in 4% (ie, 1 out of 25) of IL-4Rα$^{-/-}$ mice, 8% (ie, 2 out of 25) of IL-4$^{-/-}$ mice, and 4% (ie, 1 out of 25) of IL-13$^{-/-}$ mice focal CNS lesions were detectable. The morphological reaction pattern of intracerebral inflammation by *C. neoformans* differed fundamentally in the different mouse strains. IL-13$^{T/+}$ mice harbored larger lesions, which were less well demarcated and contained more fungi or fungal fragments in their brains as compared with wild-type mice (Figure 2, E versus A). In comparing the susceptible genotypes with the resistant genotypes, the lesions had a pseudocystic morphology (Figure 2, A and E). These pseudocystic lesions, harboring fungal accumulations, were diffusely disseminated in all regions of the CNS, including the

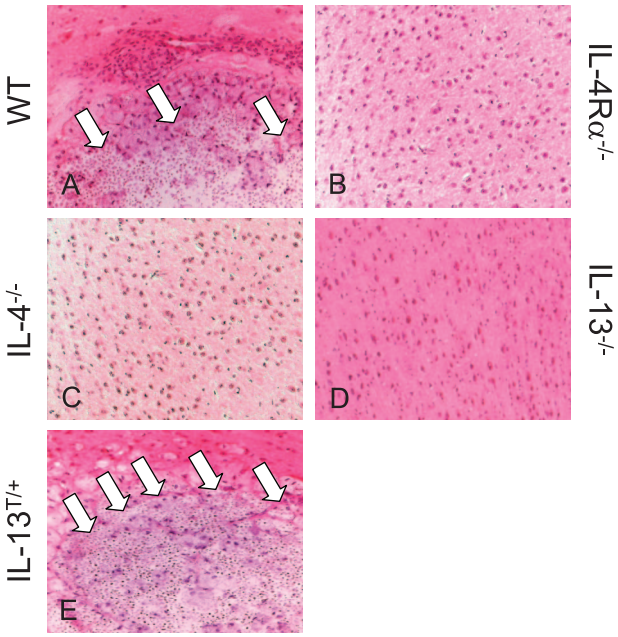

Figure 2. Development of pseudocystic lesion in large scale in the brains of susceptible *C. neoformans*-infected wild-type and IL-13$^{T/+}$ mice, but the absence of IL-4/−13 is associated with occasional formation of microglial nodules, granulomas, or only small pseudocystic lesions. Shown are representative micrographs (see supplemental figures). **A–E:** Day 60 pi; H&E staining of the brain of wild-type (WT), IL-4Rα$^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice; magnification = original ×400. In wild-type and IL-13$^{T/+}$ mice large foamy macrophages bordering pseudocystic lesions filled with cryptococci (**white arrows**) could be found, whereas the brain parenchyma of IL-4Rα$^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$ mice is unremarkable.

supratentorial lobes, the basal ganglia and the cerebellum in IL-13$^{T/+}$ mice. In contrast to IL-13$^{T/+}$ mice, in wild-type mice the semioval center white matter was exclusively affected. The CNS parenchyma of IL-4R$\alpha^{-/-}$ mice, IL-4$^{-/-}$ mice, and IL-13$^{-/-}$ mice was largely unremarkable (Figure 2, B–D). Two small microglial nodules were detected in the left frontal white matter in a single IL-4R$\alpha^{-/-}$ mouse, but fungal masses or fragments were consistently absent (supplemental Figure S1A, see *http://ajp.amjpathol.org*). In contrast, in two IL-4$^{-/-}$ mice (supplemental Figures S1B and S1C, see *http://ajp.amjpathol.org*), the lesion morphology was characterized by typical granuloma formation with significant accumulations of compact macrophages, with partial epitheloid appearance, and lymphocytes (supplemental Figure S1B, see *http://ajp.amjpathol.org*). Yeast accumulated focally in the center of the lesion but not in the cytoplasm of macrophages (supplemental Figure S1C, see *http://ajp.amjpathol.org*). In a single IL-13$^{-/-}$ mouse, a minor accumulation of *C. neoformans* within a small pseudocystic lesion was detected (supplemental Figure S1D, see *http://ajp.amjpathol.org*), while all of the other IL-13$^{-/-}$ mice did not show any inflammatory foci. *C. neoformans* mainly localized to the cytoplasm of macrophages situated at the border between CNS parenchyma and centrally pseudocystic lesion of wild-type and IL-13$^{T/+}$ mice (Figure 2, A and E). In comparison with the lung, where large encapsulated cryptococcal yeasts are detectable mainly extracellularly (Stenzel and Köhler, unpublished observation), huge masses of fungi and fungal fragments with variable size were apparently localized within the macrophages of the CNS in wild-type and IL-13$^{T/+}$ mice. Thus, expression of IL-4 and IL-13 correlates with loss of fungal control, and a specific reaction pattern of the CNS parenchyma leading to large pseudocystic lesions bordered by voluminous foamy macrophages filled with yeast. While the absence of IL-4 may rarely enable formation of granulomas, loss of IL-13 is associated with appearance of small pseudocystic lesions in rare cases, and concomitant loss of IL-4 and IL-13 signaling (ie, IL-4Rα deficiency) apparently suppresses cryptococcal invasion very effectively, with only scarce formation of microglial nodules.

In the Presence of IL-4 and IL-13, Elevated Numbers of Macrophages Rather than Microglial Cells in the Brain following Intranasal Infection with C. neoformans

Macrophages are central effector cells in cryptococcosis.[42] Quantitative analysis of brains by flow cytometry at day 60 p.i., revealed that CD11b^{+}CD45^{high} macrophages, and also Gr-1^{+}CD11b^{+}CD45^{high} neutrophils (data not shown), were found at the highest numbers in susceptible genotypes (ie, IL-13$^{T/+}$, and wild-type mice), whereas only low numbers of these cell types were found in IL-4R$\alpha^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$ mice (Figure 3A). Following infection of mice with *C. neoformans,* it was specifically the number of macrophages that increased in

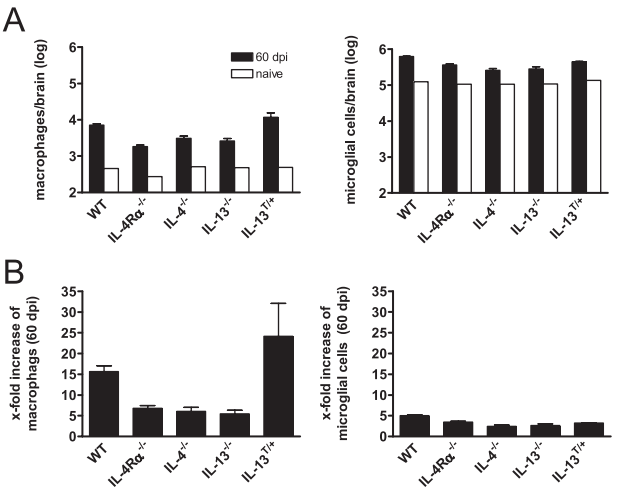

Figure 3. High numbers of macrophages infiltrate the CNS in the presence of IL-4/IL-13 following i.n. infection with *C. neoformans.* For fluorescence-activated cell sorting analysis of intracerebral leukocytes, brains of five mice per genotype were pooled following i.n. infection with *C. neoformans* in BALB/c wild-type (WT), IL-4R$\alpha^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice (**A**). Three similar experiments from infected mice and one experiment from naïve mice are shown. Cells were isolated, stained for Gr-1, CD11b, CD45, and analyzed by flow cytometry. Data are expressed as numbers of cells of the respective cell type per brain. Numbers of the respective cell types of naïve mice are shown in open bars. In (**B**) the x-fold increase of macrophages and microglial cells during cryptococcosis (60 dpi) in comparison with naïve mice is shown.

brains of wild-type and IL-13$^{T/+}$ mice 15- and 24-fold, respectively. In contrast, there was only a relatively small increase in microglial cells (Figure 3B). Together, in susceptible IL-4 and IL-13 expressing wild-type and IL-13$^{T/+}$ mice, development of high cryptococcal burdens in the brain is associated with greatly enhanced numbers of macrophages but to a lesser degree with microglial cells.

In the Presence of IL-4 and IL-13, Large Foamy Macrophages Appear in the CNS after Cryptococcal Infection and Are, in Contrast to Microglial Cells, aaMphs

The CNS immune response to *C. neoformans* is primarily monocytic.[10,43] Moreover, in recent years classical versus alternative activation of macrophages have been shown to crucially affect the type of ensuing immune responses after infection.[22,24] In *C. neoformans* infection of the lung, development of aaMphs leads to increased mortality due to excessive immunopathology.[19,44,45] In light of elevated numbers of brain macrophages observed (Figure 3, A and B), we were interested in characterizing their activation status. Strikingly, in susceptible wild-type and IL-13$^{T/+}$ mice, large foamy macrophages strongly expressing CD206 and YM1 appeared (Figures 4 and 5). In parallel, expression of YM1, and less importantly arginase-1, mRNA was significantly elevated in the brains of infected wild-type and IL-13$^{T/+}$ mice (Figure 6). Numerous activated (ie, enlarged and ramified, see white arrow heads in Figure 4A) MHC class II microglial cells were detected in the vicinity of large pseudocystic lesions of wild-type mice. However, microglial cells were

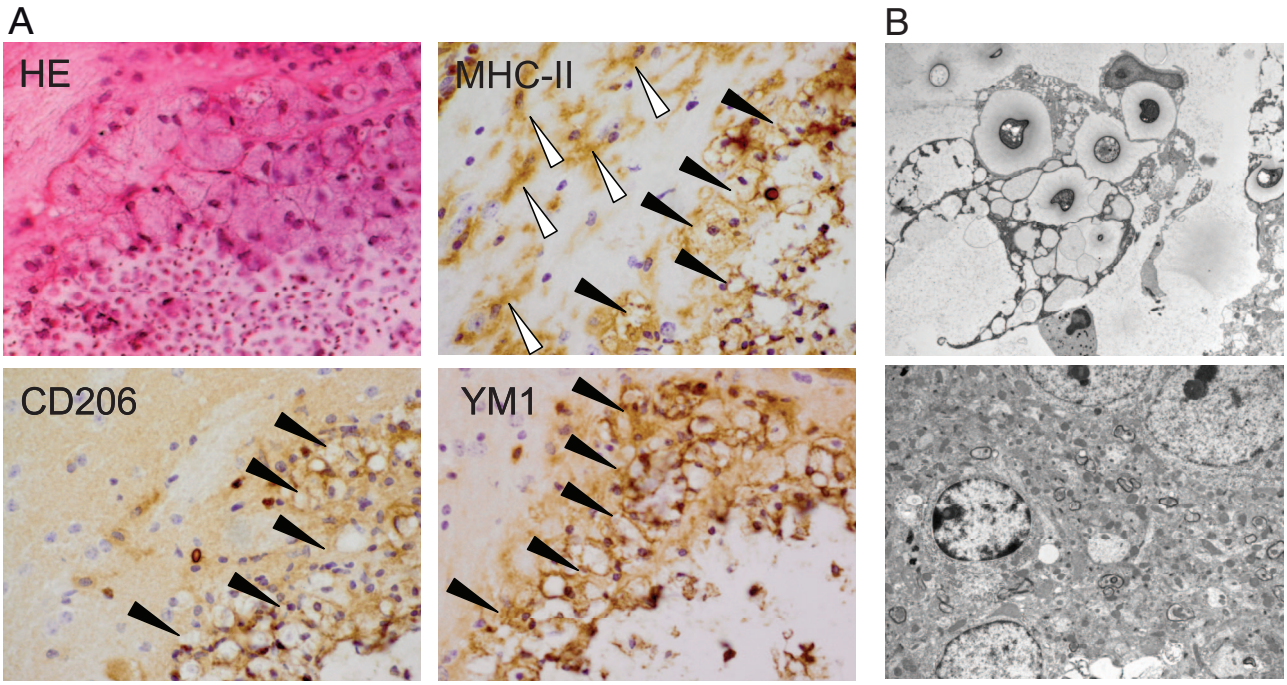

Figure 4. In the presence of IL-4 and IL-13, macrophages but not microglial cells expressing markers of alternative activation are detected in the brains of susceptible BALB/c wild-type mice at 60 d.p.i. **A:** Wild-type (WT) mice show that large pseudocystic lesion, harboring many small yeasts and yeast fragments, is bordered by inflammatory leukocytes, consisting mainly of large rounded foamy macrophages (**black arrowheads**). H&E staining, magnification = original ×600. The large foamy rounded cells are identified as MHC class II$^+$ macrophages tightly bordering the lesion. Activated MHC class II$^+$ ramified microglial cells (**white arrowheads**) are demonstrated in the outer vicinity of the lesion. Anti-MHC class II immunostaining, magnification = original ×600. Large round cells strongly express the CD206 antibody, whereas the antibody against the mannose receptor did not detect ramified microglial cells. Anti-CD206 immunostaining; magnification = original ×600. Many round cells, but not microglial cells, bordering the lesion strongly express the YM1 antibody. Anti-YM1 immunostaining; magnification = original ×600. **B: Upper panel:** Ultrastructural analysis of the identified type of macrophages reveals that they harbor yeasts and yeast fragments within their cytoplasm, and that they also contain numerous small vacuoles corresponding to their 'foamy' appearance, which are phagolysosomes. **Lower panel:** The ultrastructural analysis of microglial cells identifies a cell with dense-staining nucleus and marginated chromatin free of cryptococci or vacuolated lysosomes. Electron microscopy; magnification = original ×3000.

not found to express markers of alternative activation in this specific localization, indicating that the development of alternatively activated cells is restricted to macrophages but not microglial cells. The particular morphology of the large foamy macrophages was further characterized by electron microscopy of typical lesions found in wild-type mice (Figure 4B, upper panel). The electron micrograph demonstrates a giant foamy macrophage with intracellular yeasts also containing intracellular vacuoles. In contrast, microglial cells of the same animal are not rounded and do not contain yeast, yeast fragments, intracellular vacuoles, or phagolysosomes (Figure 4B, lower panel). Since microglial cells did not express markers of alternative activation, we wished to determine whether they express IL-4Rα, which is essential for alternative activation of macrophages (Figure 5).[22] To approach this question, CD11b$^+$CD45^{high} macrophages and CD11b$^+$CD45^{dim} microglial cells of wild-type animals were stained for the presence of the IL-4Rα chain on their surface. Wild-type macrophages and microglial cells exhibited a median fluorescence intensity of 65.1, and 35.3, respectively. However, in IL-4R$\alpha^{-/-}$ macrophages and microglial cells used as negative controls, background median fluorescence intensity levels of 14.6 and 4.4, respectively, were found (data not shown). These results indicate that both cell types found in brains of infected susceptible wild-type mice express the IL-4Rα and, thus both cell types could be responsive to IL-4/IL-13. However, we did not detect any IL-4 and IL-13 protein in the brains of the mice of different genotypes examined by enzyme-linked immunosorbent assay (data not shown). Brain IL-4 production was also not detected by real-time PCR (Figure 6), while IL-13 was only detected at significant levels in the CNS of IL-13$^{T/+}$ mice. Since these cytokines were clearly expressed in the lungs of *Cryptococcus*-infected mice as shown previously,[19] we therefore conclude that macrophages are alternatively activated in the periphery (ie, lung), and enter the CNS subsequently. This cascade would explain why only macrophages, and not microglial cells, show an alternative activation status following i.n. infection of mice with *C. neoformans*. These data provide evidence in brains of mice for IL-4/IL-13-dependent alternatively activated macrophages, which are associated with uncontrolled infection.

Loss of Fungal Control in Mice Expressing IL-4 and IL-13 Is Associated with Development of aaMph

The development of aaMph has been shown to depend on IL-4 or IL-13.[22] To analyze whether aaMph found in susceptible wild-type mice (Figure 4) correlated with the presence of IL-4/IL-13, wild-type and Th2 mutant mice

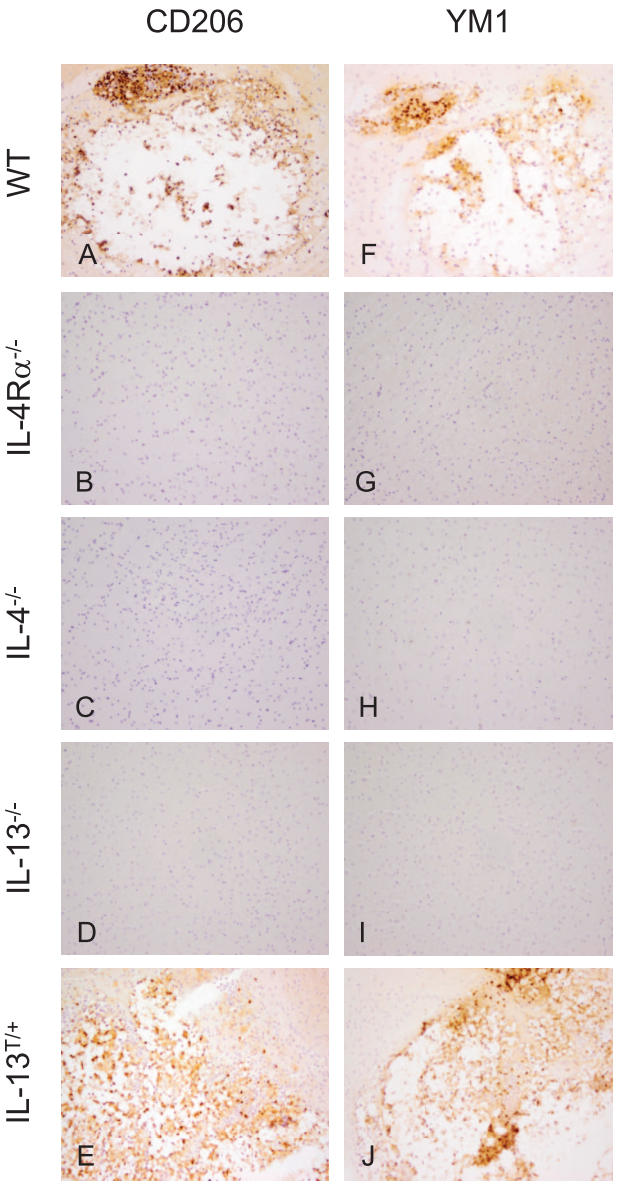

Figure 5. Alternatively activated macrophages develop in the CNS of susceptible wild-type, and IL-13$^{T/+}$ mice after i.n. infection with *C. neoformans*. **A–E:** Mannose receptor (CD206) expression as a marker for alternative activation of macrophages in the brain (60 dpi). In wild-type (WT) and IL-13$^{T/+}$ mice, macrophages show a strong expression of CD206, whereas the receptor is absent on cells of IL-4Rα$^{-/-}$ (**B**), IL-4$^{-/-}$ (**C**), and IL-13$^{-/-}$ (**D**) mice. In no case were microglial cells CD206 positive. Anti-CD206 immunostaining, magnification = original ×400. **F–J:** Chitinase YM1 expression as a marker for alternative activation of myeloid leukocytes in the brain (60 dpi). In wild-type and IL-13$^{T/+}$ mice, macrophages show a strong expression of YM1, whereas the molecule is absent in cells of IL-4Rα$^{-/-}$ (**G**), IL-4$^{-/-}$ (**H**), and IL-13$^{-/-}$ (**I**) mice. In no case microglial cells were YM1 positive. Anti-YM1 immunostaining; magnification = original ×400.

were compared for expression of CD206 and YM1. In wild-type mice large focal cystic lesions were tightly bordered by vacuolated voluminous macrophages, while in IL-13$^{T/+}$ mice these voluminous macrophages spread more diffusely into the adjacent CNS parenchyma (Figures 2A and 7A versus Figures 2E and 7E). In wild-type and IL-13$^{T/+}$ mice, these macrophages (Figure 7, A and E) express markers for aaMph, CD206, and YM1 (Figure 5, A, E, F, J). In contrast, in IL-4Rα$^{-/-}$, IL-4$^{-/-}$, and

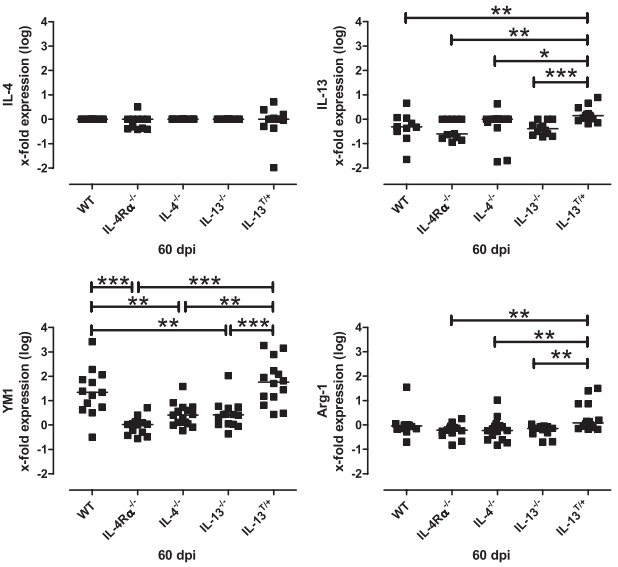

Figure 6. Differential transcription of IL-4, IL-13, YM1, and arginase-1 in wild-type, IL-4Rα$^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice after i.n. infection with *C. neoformans*. YM1 mRNA expression in the brains of wild-type (WT) and IL-13$^{T/+}$ mice (60 dpi) was significantly elevated as compared with non-susceptible Th2 mutant mice. Arginase-1 mRNA expression in the brains of IL-13$^{T/+}$ mice (60 dpi) was significantly elevated as compared with non-susceptible Th2 mutant mice. No IL-4 expression was detected in the CNS of all genotypes. IL-13 expression was not detected in all genotypes except for IL-13$^{T/+}$ mice. Surprisingly, we detected some mRNA in IL-13 mutant mice using the primers described above, which results from the use of the IL-13 KO targeting vector.[35] Data from 8 to 12 representative mice per genotype are shown in the graph. Statistical analysis between wild-type, IL-4Rα$^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice was performed by use of the Mann-Whitney-U Test, with: $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

IL-13$^{-/-}$ mice (Figure 7, B–D), cells expressing YM1 or CD206, markers indicating IL-4/IL-13-dependent development of aaMph (Figure 5, B–D and G–I) were lacking. Further, microglial cells did not show markers of alternative activation in any of these genotypes. In the single mice that showed inflammatory foci among the resistant IL-4Rα$^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$ mice, no markers of alternative activation were detected either (supplemental Figure S2, A–F, see *http://ajp.amjpathol.org*).

Additional analysis of the alternative versus classical macrophage activation involved the detection of YM1 and arginase-1 mRNA by *real-time* PCR. In accordance with the immunohistochemical data showing cell-specific expression of high levels of YM1 at the protein level, significantly higher levels of YM1 mRNA were expressed in wild-type and IL-13$^{T/+}$ mice as compared with the non-susceptible genotypes. Arginase-1 mRNA was expressed at significantly higher levels in brains of infected IL-13$^{T/+}$ mice than in resistant mice. Low or no levels of YM1 and arginase-1 mRNA were expressed in IL-4Rα$^{-/-}$, IL-4$^{-/-}$, or IL-13$^{-/-}$ mice (Figure 6).

Microglial Cells Become Activated on Infection with C. neoformans *but, in Contrast to Macrophages, Do Not Contain Intracellular* C. neoformans

In the CNS, macrophages recruited from the periphery are important effector cells in cerebral *C. neoformans*

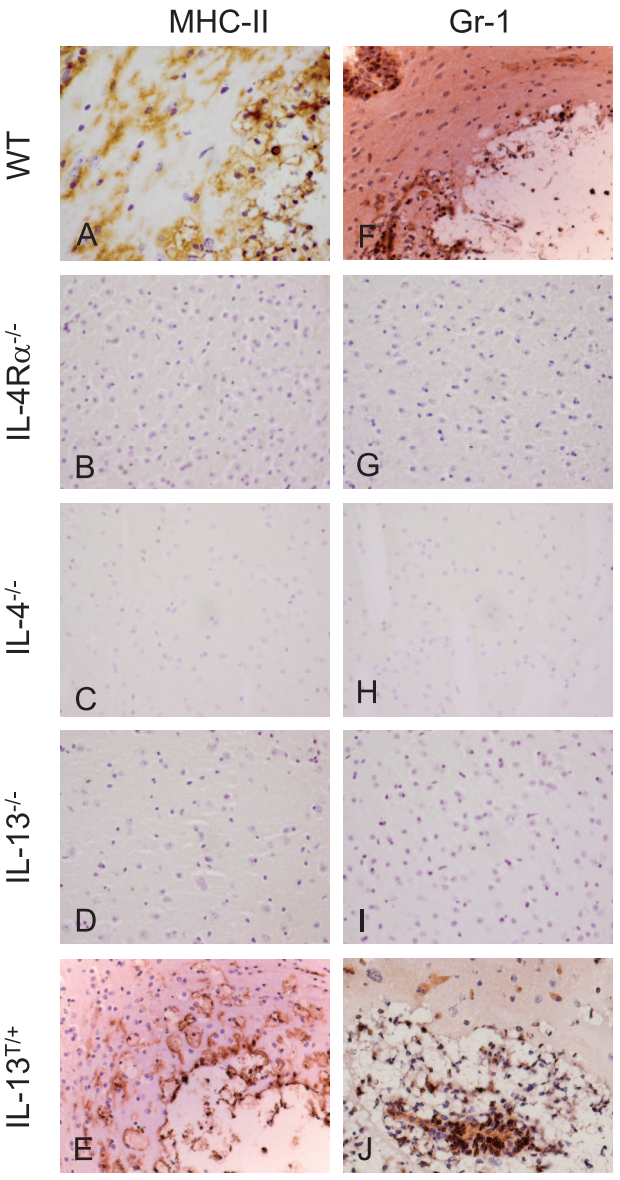

Figure 7. Innate cellular immune reaction pattern in wild-type, IL-4R$\alpha^{-/-}$, IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-13$^{T/+}$ mice after i.n. infection with *C. neoformans*. **A–E:** In a BALB/c wild-type (WT) mouse (**A**), foamy rounded MHC class II$^+$ macrophages harboring yeast fragments are tightly bordering the lesion. In an IL-13$^{T/+}$ mice (**E**), voluminous macrophages harboring yeast fragments within their cytoplasm are demonstrated at the border of a pseudocystic lesion. In comparison with the wild-type (**A**), the lesion is less well demarcated by macrophages, which rather diffusely infiltrate the parenchyma. Microglial activation is a less prominent feature in an IL-13$^{T/+}$ mice, as compared with the wild-type (**A**). MHC class II$^+$ cells are generally absent in the CNS of IL-4R$^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$ mice (**B–D**). Day 60 p.i.; Anti-MHC class II immunostaining; magnification = original ×400. **F–J:** In a BALB/c wild-type mouse (**F**), Gr-1$^+$ granulocytes are detected at the border and in the center of the lesion. Granulocytes also dissociate the vessel wall, which is localized at the **upper left**. In an Il-13$^{T/+}$ mice (**J**), many Gr-1$^+$ granulocytes are stained in the center and at the border of the lesion. Gr-1$^+$ cells are generally absent in the CNS of IL-4R$^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$ mice (**G–I**). **F–J:** Day 60 p.i.; Anti-Gr-1 immunostaining; magnification = original ×400.

infection.[12,46–48] Moreover, expansion and proliferation of microglial cells is a crucial feature of a number of neuroinflammatory, traumatic, and neurodegenerative diseases.[49–52] In addition to F4/80$^+$ and MHC-II$^+$ macrophages, microglial cells were prominently activated in the vicinity of the lesions in *Cryptococcus*-infected wild-type mice (Figures 4A, 7A), whereas microglial activation was not as prominent in IL-13$^{T/+}$ mice (Figure 7E). By contrast, inflammatory leukocytes as stained by MHC class II or Gr-1 were generally absent in IL-4R$\alpha^{-/-}$, IL-4$^{-/-}$, or IL-13$^{-/-}$ mice (Figure 7, B–D, and G–I). In a single IL-4R$\alpha^{-/-}$ mouse, small microglial nodules and scarce Gr-1 positive cells were detected, but no round large or foamy macrophages were observed (supplemental Figure S3, A and D, see *http://ajp.amjpathol.org*). In two IL-4$^{-/-}$ mice, the granuloma consisted of compact F4/80$^+$ and MHC class II$^+$ macrophages and microglial cells (supplemental Figure S3B see *http://ajp.amjpathol.org*) as well as Gr-1$^+$ cells (supplemental Figure S3E see *http://ajp.amjpathol.org*), while multinucleated giant cells were absent and fibrous tissue as stained by Elastica van Gieson was not detected either (data not shown). A small fungal accumulation in a single IL-13$^{-/-}$ mouse was bordered by single F4/80$^+$ and MHC class II$^+$ macrophages, and discrete microglial cell activation was detected additionally (supplemental Figure S3C see *http://ajp.amjpathol.org*). Thus, microglial activation and some expansion occur in all of the infected brains following *C. neoformans* infection. However, microglial cells do not appear to contact and phagocytose cryptococci as macrophages do.

In agreement with the quantification presented in Figure 3, the highest numbers of granulocytes were detected in IL13$^{T/+}$ mice (Figure 7J). In wild-type mice some Gr-1$^+$ cells bordered the lesion and even infiltrated the CNS parenchyma (Figure 7F), while in IL-4R$\alpha^{-/-}$ and IL-4$^{-/-}$ mice that showed cryptococci in the brain, granulocytes were confined to the inflammatory focus and appeared at low numbers (supplemental Figure S3, D and E, see *http://ajp.amjpathol.org*). In one IL-13$^{-/-}$ mouse that showed cryptococcal infection of the brain, granulocytes were barely detectable by immunohistochemistry (supplemental Figure S3F, see *http://ajp.amjpathol.org*), consistent with the data generated by flow cytometry analysis (data not shown). Thus, in addition to aaMph, granulocytes are also detectable in the brains of susceptible genotypes during cryptococcal infection.

Discussion

In the present study, we analyzed the roles of the Th2 cytokines IL-4 and IL-13 in cerebral cryptococcosis following intranasal infection, and found that mice defective in one or both cytokines were largely resistant to the infection. These mice harbored low or no fungi within their brains, and recruited low numbers of leukocytes. In rare cases of infection of their brains, they developed lesions characterized by a classical cellular immune response such as microglial nodules or granuloma formation. In contrast, susceptible wild-type and IL-13 transgenic BALB/c mice harbored high fungal burdens leading to lesion morphology, characterized by a pseudocystic appearance disseminated throughout the brain parenchyma. Most importantly, these inflammatory foci are characterized by large aaMph expressing CD206, YM1,

and arginase-1 and containing fungi. These cells appear in abundance and border the lesions. Activated microglial cells were also found at high numbers, but these cells did not express markers of alternative activation nor did they contain fungi. Collectively this shows that development of aaMph in the CNS after pulmonary *C. neoformans* infection and dissemination to the CNS is crucially dependent on IL-4 and IL-13, and these macrophages are unable to control cerebral fungal growth. Conversely, no aaMph developed in the absence of IL-4 and/or IL-13. Also, neither morphologically resting nor activated microglia did show any expression of markers typical of alternative activation (neither CD206 nor YM1).

Cerebral cryptococcosis in *C. neoformans*-infected BALB/c mice is characterized by both the production of Th1 and Th2 cytokines. Th1 cytokines such as interferon-γ, IL-12 and tumor necrosis factor have been demonstrated to be crucially involved in protection from lethal cryptococcosis.[17–19,53–55] However, in a model of cerebral cryptococcosis Maffei et al[43] did not describe a polarization of Th1 or Th2 responses by detecting transcripts of tumor necrosis factor and inducible nitric oxide synthase in addition to IL-4, and IL-10. Others propose that Th1 cytokines are of prime importance since expression of these factors correlates with protection against *C. neoformans* in the CNS, whereas Th2 cytokines were not elevated in a murine model of *C. neoformans* infection of the CNS with a prior peripheral immunization.[55,56] Huffnagle et al stated that the host defense mechanisms that clear *C. neoformans* from the CNS appear to be similar to those in the lungs[17] ie, via a Th1 cell-mediated inflammatory response, and that Th2 type immunity is ineffective at eliminating the infection in the brain and results in decreased survival. Extending these results, we show that Th2 cytokines highly impact the inflammatory response that develops within the CNS by altering macrophage activation. Overexpression of IL-13 in IL-13$^{T/+}$ mice leads to the most severe inflammatory phenotype with the highest fungal burdens, highest frequency of CNS involvement following pulmonary infection, largest inflammatory foci, poor demarcation by macrophages at the lesion border, and highest numbers of leukocytes infiltrating the parenchyma. These findings were in contrast to Olszewski et al who used the *C. neoformans* strain (serotype A) H99 in a model of intratracheal infection in C57BL/6 × 129 F2 mice.[12] Olszewski et al showed that the intracranial inflammatory response after transcapillary invasion of the fungi was minimal without local leukocyte accumulation, and only scarce neutrophils were detected at 4–6 weeks post infection. Interestingly these authors did not mention any macrophages in their model of cerebral cryptococcosis. These differences in the immune response of wild-type animals in comparison with the results presented here may be due to differences in the cryptococcal strain and also the host genetic background.

Microglial cell activation and expansion was observed not only in susceptible genotypes, but also in resistant IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice, where, although in rare cases, these hosts showed microglial cell activation and expansion with a highly specific morphological pattern. Studying the role of microglial cells in cerebral cryptococcosis *in vitro*, using human fetal microglial cultures, Lee et al[57] showed that microglial cells initially internalized, and contained yeasts within phagolysosomes. Yeast were observed growing extracellularly 16 to 24 hours after being phagocytosed.[57] Based on morphological analysis, the authors differentiated between two types of phagosomes in microglia: spacious phagosomes and close-fitting phagosomes, and suggest that in human microglia, *C. neoformans* survive and replicate within spacious phagosomes. This study was undertaken *in vitro* and with human fetal microglia cells that on stimulation with *C. neoformans*, may have differentiated into phagocytes. We believe that this may be the first thorough description of aaMph, and not genuine microglial cells, in cerebral cryptococcosis, although the concept of different macrophage activation status[58,59] had not been put forward yet. *In vitro* and *in vivo* observations on *C. neoformans* interaction with alveolar macrophages were demonstrated to be critical for containing the infection via a unique intracellular pathogenic strategy involving cytoplasmic accumulation of polysaccharide-containing vesicles and intracellular replication leading to the formation of spacious phagosomes in which multiple cryptococcal cells are present.[60–62] Reports on the alternative activation status of these alveolar macrophages in the context of Th2-mediated immune responses in bronchopulmonary cryptococcosis have pointed out their unique roles in disease.[19,44]

Markers of alternative activation have been studied in CNS diseases only recently. For example, in a mouse model of Alzheimer disease, YM1 mRNA was reported to increase, and in the brains of Alzheimer patients chitinase 3-like-1 and -2 mRNA levels were significantly elevated, although the exact cellular source of the increased transcription of these markers was not determined.[63] In an experimental autoimmune encephalomyelitis model, microglial cells in the resting and the activated state were shown to produce YM1 and lacked the production of NO in an IL-4-dependent manner. Using bone marrow chimeras, the authors concluded that macrophages entering the CNS from the periphery exhibit a dual phenotype (classical Mph as well as aaMph) since these cells, in contrast to microglial cells, also produced inducible nitric oxide synthase.[64] Interestingly these authors found YM1 mRNA in resting microglia, and YM1 expression as measured by fluorescence-activated cell sorting analysis in CD45^{low} and CD45^{high} expressing 'microglia' and in peripheral macrophages. In our study CD11b^{+}CD45^{high} expressing cells were interpreted as macrophages according to the original description of this technique,[41,65] and we did not detect YM1 expression by immunohistochemistry on resting or activated microglial cells in susceptible mice. Because of the obstacle of differentiating *in situ* between blood-derived macrophages entering the inflamed CNS and microglial cells solely by immunohistochemical techniques, we have added fluorescence-activated cell sorting analysis and ultrastructural analysis to strengthen our findings. In agreement with our results, Ponomarev et al were unable to detect YM1 expression in IL-4- or IL-4Rα- deficient mice.[64] Since we did not detect

any IL-4 and IL-13 protein or mRNA in the CNS of wild-type mice infected with *C. neoformans*, we believe that macrophages are alternatively activated in the periphery (ie, the lung), and this may be the reason why only macrophages, and not microglial cells show an alternative activation status.

We believe that it is of utmost importance to dissect the function and morphology of macrophages and microglial cells in cerebral cryptococcosis in particular in view of possible differences in immune reactions from individuals with differing susceptibility. Shibuya et al have examined the morphological characteristics of macrophages in bronchopulmonary cryptococcosis in immunocompetent individuals,[66] in patients with AIDS, and in patients undergoing highly active antiretroviral therapy (HAART). They found that immunodeficiency was associated with histiocytic, minor lymphocytic and granulocytic response, while HAART induces a massive histiocytic and lymphocytic involvement. In a murine model of cerebral cryptococcosis using i.v. infection with a human isolate Chretien et al localized the fungi to mononuclear cells in cerebrovascular capillaries, in endothelial cells and in vacuolated macrophages within the CNS parenchyma in cystic lesions.[10] Furthermore, a reaction pattern in HIV-negative patients similar to mice recovering from cryptococcosis with a granulomatous pattern was also described.[10] Our results show a strict IL-4- and IL-13-dependency of macrophage frequency, morphology, and functional status leading to distinct lesion morphology. Our data in non-susceptible IL-4- and IL-4Rα-deficient mice also point to a protective granulomatous reaction pattern, which may be an interesting model to further study subtle protective genetically determined effects in cerebral cryptococcosis.

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3.5 Einleitung zum Manuskript „Lack of IL-4 receptor expression on T helper cells reduces T helper 2 cell polyfunctionality and confers resistance in allergic bronchopulmonary mycosis"

In den vorangegangenen Arbeiten fiel immer wieder die Bedeutung der alternativen Aktivierung von Makrophagen auf. Diese ist abhängig von IL-4 und IL-13 (150). Da gezeigt werden konnte, dass Th-Zellen wichtige IL-4-Produzenten sind und der IL-4R wichtig für die Induktion der Immunpathologie in der pulmonalen Kryptokokkose ist, wurden Th-zellspezifische IL-4R-defiziente Mäuse generiert (161), um die Wirkung des IL-4R auf die Th-Zellen zu analysieren und die Bedeutung der Th-Zellen in der Immunpathologie der pulmonalen Kryptokokkose abschließend zu untersuchen.

Hauptergebnisse

- **Th-zellspezifische IL-4R-defiziente Mäuse sind in der pulmonalen Kryptokokkose resistenter als nicht transgene Wurfgeschwister. Die Überlebensrate ist signifikant höher, einhergehend mit einer geringeren Organlast in der Lunge.**
- **Die Eosinophilie und die Zahl an schleimbildenden Becherzellen in der Lunge ist in den Th-zellspezifischen IL-4R-defizienten Mäusen im Vergleich zu Kontrolltieren signifikant verringert.**
- **Die Th2-Antwort ist in Th-zellspezifischen IL-4R-defizienten Mäusen verringert, aber vorhanden und somit teilweise IL-4R-unabhängig.**
- **Th-zellspezifische IL-4R-defiziente Mäuse zeigen eine geringere Produktion von Th2-Zytokinen und damit einhergehend eine Verringerung der Anzahl an Th2-Zellen**
- **Die Zahl an antigenspezifischen Th-Zellen, welche die Zytokine IL-4, IL-5 oder IL-13 produzieren ist in Th-zellspezifischen IL-4R-defizienten Mäusen gegenüber Kontrollen verringert.**
- **Empfängliche Kontrolltiere zeigen verstärkt antigenspezifische Th2-Zytokin-Mehrfachproduzenten (also polyfunktionale Th2-Zellen, die gleichzeitig IL-4, IL-5, IL-13 bilden). Diese Mehrfachproduzenten produzieren je Zelle größere Mengen der einzelnen Zytokine als Einfachproduzenten.**
- **In Th-zellspezifischen IL-4R-defizienten Mäusen ist einhergehend mit der geringeren Zahl an polyfunktionalen Th2-Zellen die Zahl an alternativ aktivierten Makrophagen signifikant reduziert.**
- **Mit dieser Studie sind erstmals polyfunktionale Th2-Zellen und deren Bedeutung für Immunpathologien nachgewiesen worden.**

Schlussfolgerungen

Bei der Ausprägung der Immunpathologie in der pulmonalen Kryptokokkose spielt nicht nur die Zahl an Th2-Zellen eine Rolle, sondern auch der Phänotyp jeder einzelnen Zelle. Wie hier gezeigt werden

konnte, korreliert die Zahl an polyfunktionalen Th2-Zellen mit der Ausprägung der Immunpathologie. Diese polyfunktionalen Th2-Zellen eignen sich zukünftig somit als diagnostischer Marker, um die Krankheitsentwicklung in der Kryptokokkose (und evtl. darüber hinaus, evtl. auch im Bereich von asthmatischen Fragestellungen) frühzeitig bestimmen zu können (243).

Tierexperimente

Die Mäuse in der vorliegenden Arbeit wurden im Rahmen der Tierversuchsvorhaben Az. 24-9168.-36/04 und 24-9168.11/14/1 (Landesdirektion Sachsen – Dienststelle Leipzig) verwendet.

3.5.1 Publikation 5: Polyfunktionale Th2-Zellen sind wichtige Effektorzellen in der Immunpathologie der pulmonalen Kryptokokkose

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

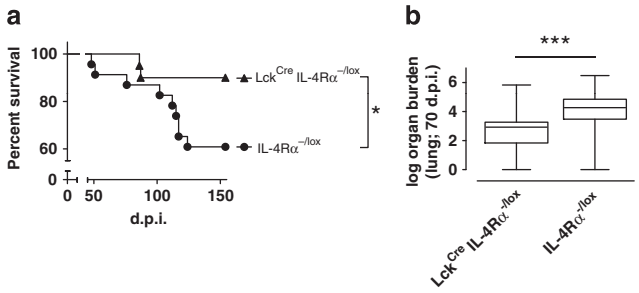


Figure 1 Enhanced survival rate and lower lung burden of mice with T helper (Th)-specific interleukin (IL)-4R deficiency in contrast to littermate controls. Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice and their littermate controls (IL-4R$\alpha^{-/lox}$) were compared for survival rate (**a**) and lung burden (**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

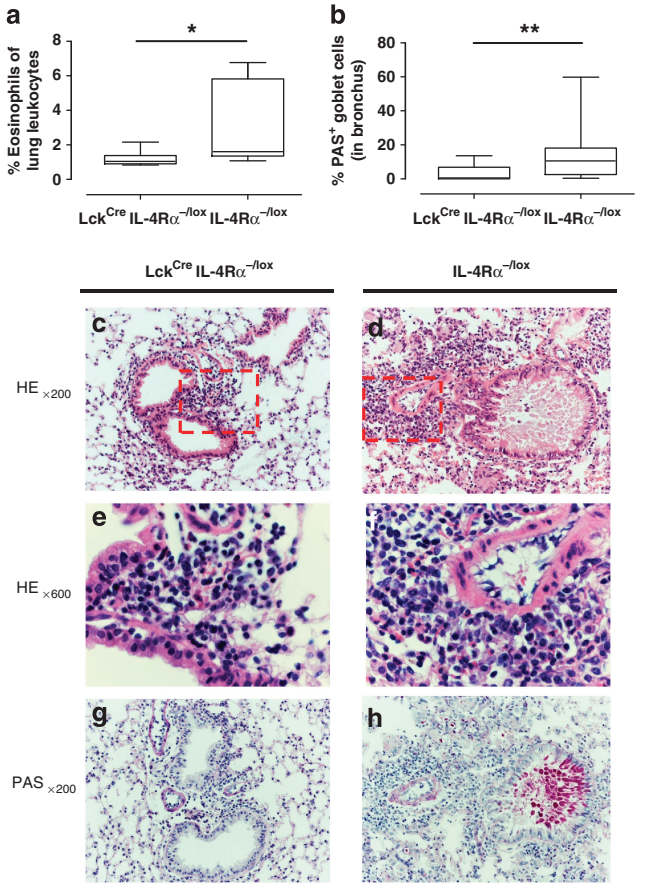


Figure 2 Diminished eosinophil recruitment and goblet cell mucus production in mice with T-helper (Th)-specific IL-4R deficiency in contrast to littermate controls. Comparison of eosinophil proportions and mucus production in the lung of infected Lck^{Cre}IL-4Rα$^{-/lox}$ mice and littermate controls (IL-4Rα$^{-/lox}$) 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-4Rα$^{-/lox}$ mice are depicted, whereas a cluster of eosinophils is shown in littermate controls (IL-4Rα$^{-/lox}$). 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-4Rα$^{-/lox}$, lacking IL-4Rα on CD4$^+$ T cells; IL-4Rα$^{-/lox}$, 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ mice vs. 0.187% in IL-4Rα$^{-/lox}$ 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.

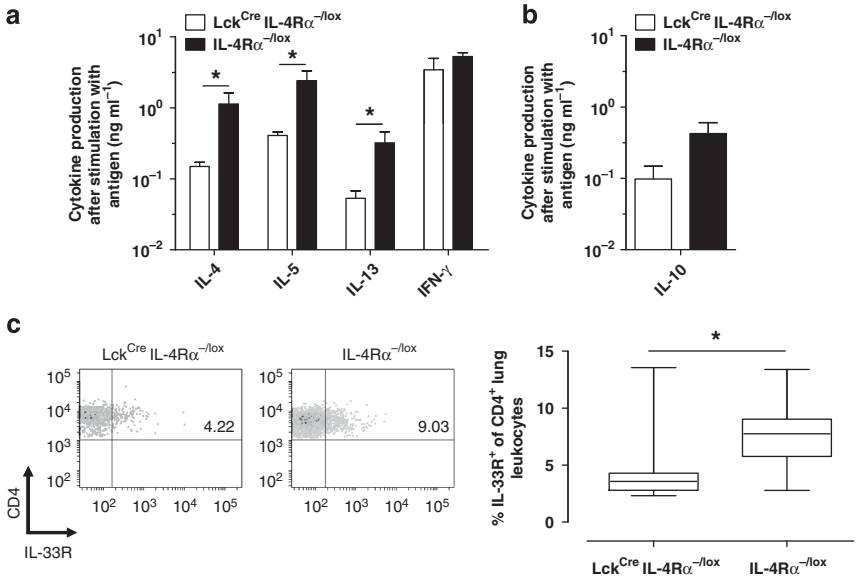


Figure 3 Reduced T helper (Th)2 cytokine production and reduced Th2 cell proportion in mice with Th-specific IL-4R deficiency in contrast to littermate controls. Comparison of the cytokine profile after re-stimulation of purified lung leukocytes with cryptococcal antigen. Cytokine production and Th2 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

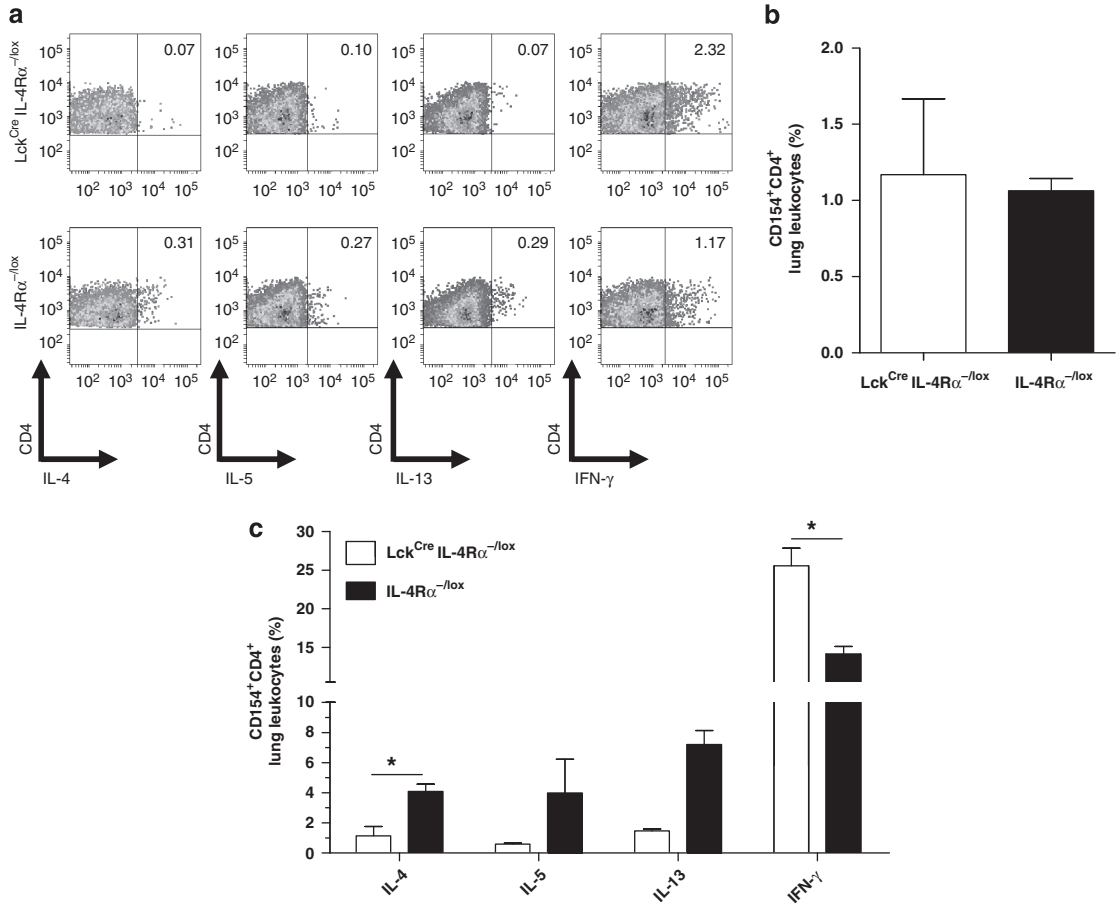


Figure 4 Diminished proportions of T helper (Th)2 cells in mice with Th-specific IL-4R deficiency in contrast to littermate controls. Comparison of total ($CD4^+$) and antigen-specific Th cell proportions ($CD4^+CD154^+$) and their cytokine profile in $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice vs. littermate 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

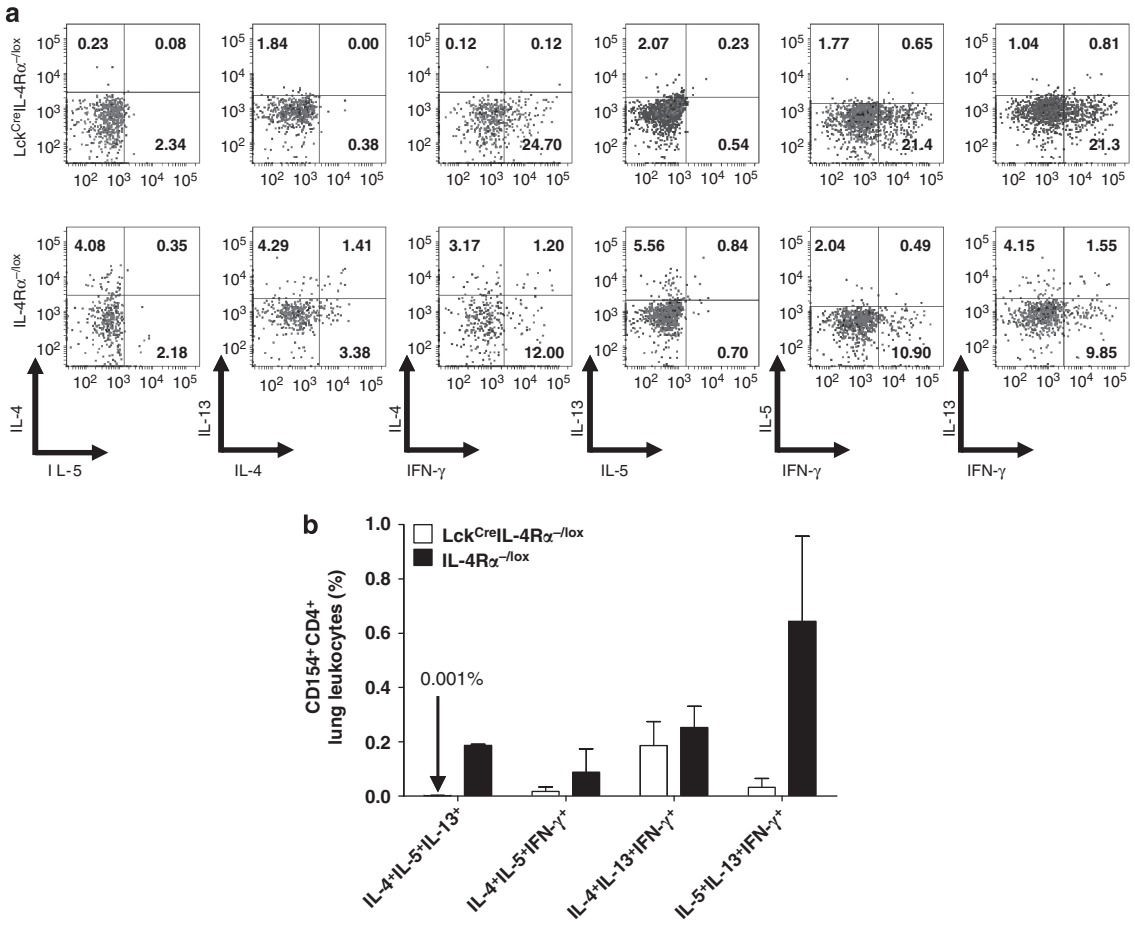


Figure 5 Reduced polyfunctional antigen-specific Th2 cell response in mice with T helper (Th)-specific interleukin (IL)-4R deficiency in contrast to littermate controls. Comparison of antigen-specific Th cell proportions ($CD4^+CD154^+$) and their polyfunctional cytokine profile ($Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ 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.

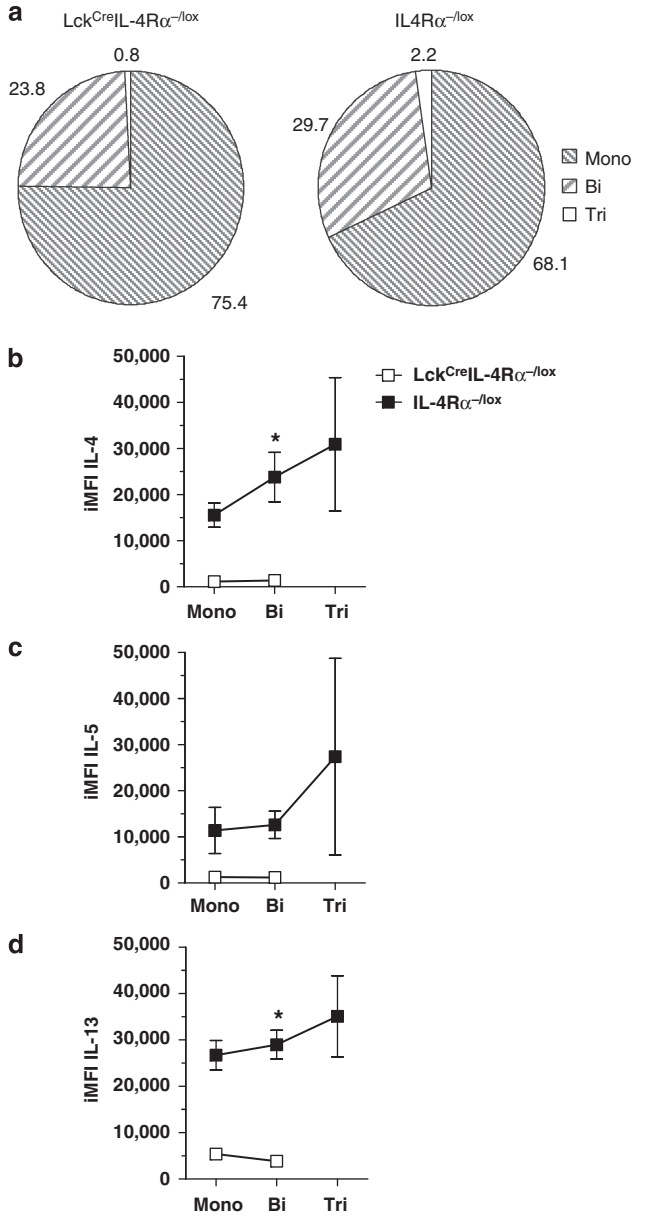
effect for expression of IL-4Rα.[25,46] Interestingly, gradual expression of IL-4Rα resulted in gradual susceptibility to infection with *C. neoformans*.[25] This argues for a sensitive low-level interaction of IL-4/IL-13 with IL-4R in our model of pulmonary cryptococcosis.[25] To deepen the functional 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.

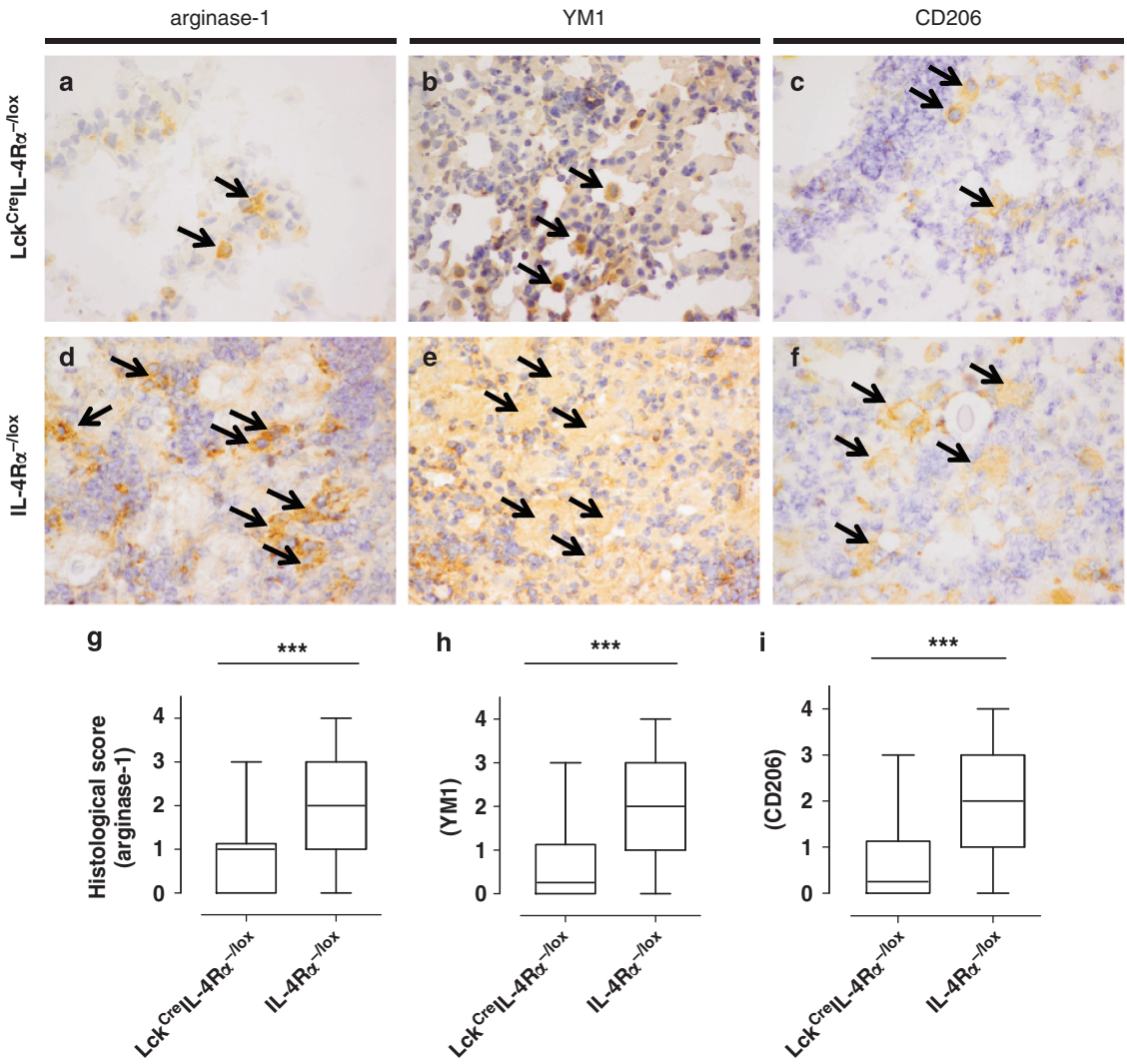


Figure 7 Diminished alternative activation of pulmonary macrophages in mice with T-helper (Th)-specific interleukin (IL)-4R deficiency in contrast to littermate controls. Analysis of the alternative activation status of pulmonary macrophages in lungs of *C. neoformans*-infected Lck^{Cre}IL-4Rα$^{-/lox}$ 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

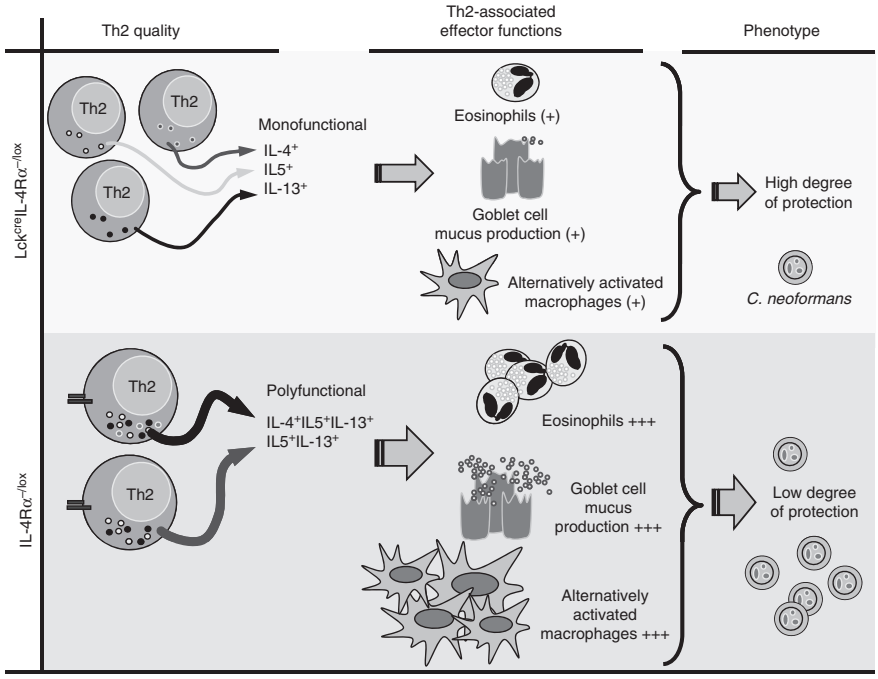


Figure 8 Polyfunctional T-helper (Th)2 cells in pathology of pulmonary cryptococcosis — a simplified scheme. Interleukin (IL)-4R expression on Th cells determines the quality (mono-functional vs. polyfunctional) and the magnitude of the Th2 response. IL-4R-dependent polyfunctional Th2 cells lead to a 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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4 Diskussion

Da die im Kapitel 3 aufgeführten Publikationen bereits Diskussionsteile enthalten, soll an dieser Stelle eine übergreifende Diskussion wesentlicher Studienergebnisse erfolgen, die vor allem Therapieausblicke bietet.

Insbesondere konnten durch die vorliegende Arbeit immunpathologische Mechanismen der pulmonalen Kryptokokkose aufgeklärt werden. Diese Befunde liefern Grundlagen zur Entwicklung von Therapien, die nicht auf der direkten Bekämpfung des Erregers beruhen, sondern die Immunantwort modulieren könnten. Somit könnte über die Kryptokokkose hinaus bei ähnlich verlaufenden Immunpathologien, wie wie sie in Teilaspekten z.B. bei Asthma bronchiale vorliegt, eine solche Therapie greifen.

4.1 Kryptokokkose des Menschen – Aktuelle epidemiologische und therapeutische Situation

Der opportunistische Erreger *Cryptococcus neoformans* führt insbesondere bei immungeschwächten Personen, wie z.B. bei HIV-Patienten zu gravierenden Komplikationen, die unbehandelt durch auftretende Meningitiden letal verlaufen können (Mortalität 6-25%) (82). Seit der Einführung der sogenannten hochaktiven antiretroviralen Therapie (HAART) hat sich die Lage in Industrieländern allerdings verbessert: Die Zahl der Rezidive ist gesunken, wohingegen die Frühmortalität unverändert blieb (82).

In Entwicklungsländern, in denen eine HAART nicht in ausreichendem Maße zur Verfügung steht, sieht die Situation allerdings dramatischer aus (4). Alleine südlich der Sahara sterben jährlich mehr als eine halbe Millionen HIV-Infizierte an einer zerebralen Kryptokokkose. Zudem ist die Kryptokokkose die dritthäufigste Pilzerkrankung bei Transplantatempfängern. Rund 60% aller Kryptokokkosen von nicht-HIV-assoziierten Infektionen fallen in diesen Bereich (244,245).

Die Kryptokokkose manifestiert sich in immunsupprimierten Patienten hauptsächlich in zwei Krankheitsmerkmalen. Nach Inhalation von eingetrockneten Pilzen oder Sporen aus kontaminiertem Vogelkot kann es zu einer pulmonalen Form kommen, die sich in einer atypischen Pneumonie äußert (82). Nach Vermehrung in der Lunge kommt es häufig durch hämatogene Streuung zu einem Befall des zentralen Nervensystems mit einem enzephalitischen Bild (Hirndruck ist erhöht) und einer Ausbildung von lebensbedrohlichen Meningitiden (4). In immunkompetenten Individuen hingegen kommt es zu einer persistierenden latenten Infektion. So konnte z.B. nachgewiesen werden, dass AIDS-Patienten an einer Kryptokokkose erkrankt waren, diese nicht durch Neuinfektion erworben hatten, sondern durch den Ausbruch von latent persistierenden Erregern (5).

4.2 Pathogenese der pulmonalen Kryptokokkose

Für unsere Untersuchungen zur Klärung der Pathogenese der pulmonalen sowie der zerebralen Kryptokokkose haben wir ein Tiermodell etabliert, welches dem Bild im menschlichen

immunsupprimierten Patienten ähnelt. Hierzu haben wir ein Nagermodell mit Balb/c Mäusen aufgebaut, die intranasal mit einem kleinen Inokulum (500 KbE) eines hochvirulenten *C. neoformans*-Stammes infiziert werden. Die Art der Infektion kommt dabei vermutlich der natürlichen Situation sehr nahe (238). Ähnliche Modelle wurden von diversen Arbeitsgruppen, die die pulmonale Kryptokokkose untersuchen, etabliert. Diese verwenden allerdings häufig ein intratracheales Modell, wobei die Pilze direkt in die Trachea appliziert werden (220,246). Die Infektion der oberen Atemwege und der Lunge stellt ein gutes Modell für die natürliche Infektion dar. Der Pilz etabliert sich im murinen Infektionsmodell in der Lunge, dem primär infizierten Organ in vielen Spezies (20,27,247) und vermehrt sich dort. In dem etablierten murinen pulmonalen Infektionsmodell kommt es innerhalb der ersten 42 Tage nur zu einer geringen Streuung, die dann nach 6 Wochen deutlich zunimmt. Die Infektion wird mit einem klinischen Isolat, das aus einem AIDS-Patienten aus der Schweiz stammt, durchgeführt. Der Zeitpunkt der Dissemination hängt dabei vom Kryptokokkenstamm ab.

Es gibt Stämme, die im Mausmodell innerhalb von wenigen Tagen in die Peripherie disseminieren (86,137). Diese schnelle Dissemination entspricht nach dem Stand der Forschung aber nicht den Gegebenheiten im Menschen (5). Einhergehend mit der Streuung lassen sich im murinen Mausmodell auch deutliche immunologische/pathologische Veränderungen feststellen. Insbesondere sind nach der Streuung Kryptokokkome im Gehirn der Mäuse feststellbar (242). Der Pilz wird hämatogen gestreut. Wie nachgewiesen werden konnte, spielen Makrophagen bei der Dissemination eine Rolle (242,248). Zu diesem Zeitpunkt lässt sich Kapselmaterial (GXM) per ELISA im Serum nachweisen. Wie die Ergebnisse nahelegen existiert ein *Shuttle*-Mechanismus in periphere Organe, vor allem in das Gehirn, an dem Makrophagen beteiligt sind. Diese Makrophagen haben einen alternativ aktivierten Phänotyp. Dieser *Shuttle*-Mechanismus wird ebenfalls von anderen Gruppen postuliert (84,85). Der Weg der ZNS-Infektion hängt aber wahrscheinlich vom Kryptokokkenstamm ab, da andere Arbeitsgruppen eine direkte Infektion ohne Makrophagenbeteiligung nachweisen konnten (52,80,83,222). In dem in der vorliegenden Arbeit beschriebenen Modell erliegen die Tiere zwischen der 10. und 12. Woche der Infektion, wobei ein starker Befall der Lunge (Ausbildung von massiven Läsionen, sogenannten Kryptokokkomen) sichtbar wird und Entzündungen der Hirnhäute festzustellen sind (238,242). Diese Symptome werden auch von anderen Autoren in pulmonalen Infektionsmodellen beschrieben (85,184,246). Ähnliche Symptome sind auch bei Patienten mit AIDS dokumentiert, die an einer Kryptokokkose erkrankt sind (82).

4.3 Die T-Zell-Dichotomie in der pulmonalen Kryptokokkose

Im Mausmodell der pulmonalen Kryptokokkose existieren zwei widerstreitende Immunreaktionen. Auf der einen Seite existiert eine schützende Immunantwort, die mit der Bildung von IFN-γ durch Th1-Zellen einhergeht. IFN-γ ist ein Botenstoff, der die Aktivierung $CD8^+$ T-Zellen fördert. Die entstehenden

Effektor-T-Zellen sind zytotoxisch wirksam (117,236). Neben der Aktivierung von zytotoxischen Zellen (115,249) werden zudem Makrophagen zur Bildung von Effektorsubstanzen, wie z.B. Sauerstoffradikalen und Stickstoffintermediaten, angeregt (184). Da aufgenommene Kryptokokken vor allem intrazellulär vorkommen, ist es für zytotoxische T-Zellen möglich, die Wirtszellen und damit einhergehend auch den Keim abzutöten (70,207). Makrophagen hingegen können aufgenommene Erreger nach Aktivierung durch IFN-γ (caMϕ, Abb. 4.1) direkt in den Phagolysosomen abtöten (184). Auf diese Weise können somit IFN-γ-produzierende Th1-Zellen das Wachstum des Pilzes reduzieren und die Ausbreitung verhindern. Auf der anderen Seite existiert eine pathologische Immunantwort. Kryptokokken induzieren, auf bis heute noch nicht vollständig verstandene Weise (124), eine schwache Th2-Antwort. In diesem Fall werden naive Th-Zellen durch den Botenstoff IL-4 so beeinflusst, dass sie zu Th2-Zellen ausdifferenzieren. Th2-Zellen sezernieren u.a. die Zytokine IL-4, IL-5 und IL-13, die in der Lunge dazu führen, dass Makrophagen alternativ aktiviert (aaMϕ, Abb. 4.1) werden (IL-4). Wobei diese Zellen keine antimikrobiellen Aufgaben wahrnehmen, sondern der Geweberegeneration dienen (150), Becherzellen zur Schleimbildung anregen (IL-13) (238) und die Einwanderung von Eosinophilen in das Gewebe fördern (IL-5) (239,243). Durch die fehlende Kontrolle des Erregerwachstums kommt es in der Regel zu einer hämatogenen Ausbreitung des Erregers in periphere Organe. Durch den ausgeprägten Neurotropismus der Kryptokokken wird insbesondere das Gehirn befallen.

4.4 Die zerebrale Kryptokokkose

Wie die vorliegende Arbeit gezeigt hat, wird die Infektion des Gehirns durch alternativ aktivierte Makrophagen gefördert, die den Erreger aufnehmen und in das Gehirn transportieren (*Shuttle*) (242). Dieser Mechanismus wird auch durch Studien anderer Arbeitsgruppen gestützt (80,84). Die Infektion des Gehirns führt allerdings nicht zu einer alternativen Aktivierung der sessilen Mikrogliazellen, in denen auch keine Kryptokokken nachgewiesen werden können. Der Befall des Gehirns verursacht letztendlich, wie bereits beschrieben, eine Entzündung der Meningen. Die im Infektionsmodell beobachteten Symptome der Entzündungsreaktionen sind auch in menschlichen Patienten zu finden. Entzündungsreaktionen der Meningen resultieren in häufig letal verlaufenden Krankheitsbildern (4,82,119). Da in AIDS-Patienten auch eine Verschiebung der T-Helferzellantwort hin zu einer Th2-Reaktion beschrieben ist (125), und Vergleichbares in dem hier vorgestellten Infektionsmodell in empfänglichen Mäusen abläuft, ist ein ähnliches Infektionsgeschehen im Menschen durch alternativ aktivierte Makrophagen vorstellbar. Durch die Verschiebung der Th-Antwort in Richtung Th2 (125,126) ist eine Kontrolle des Erregers nicht möglich und eine Therapie notwendig, um die Infektion zu kontrollieren.

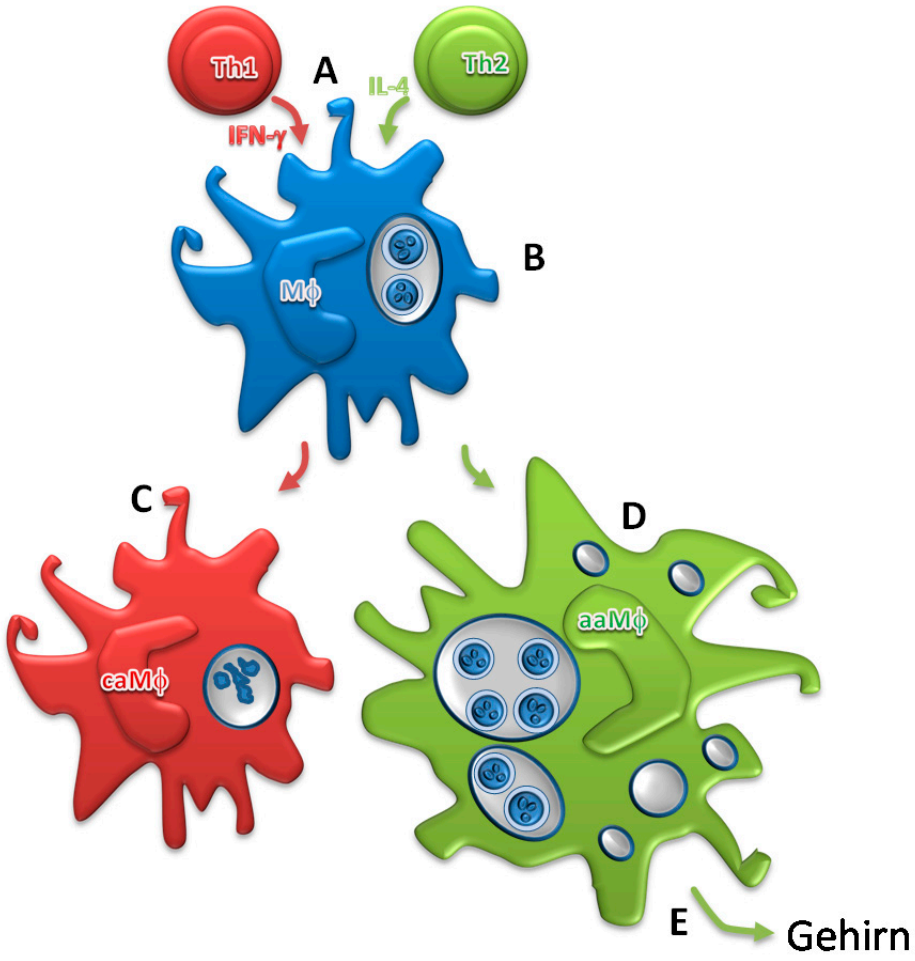

Abb. 4.1: Modell der Makrophagenaktivierung und die daraus resultierende Kontrolle der Kryptokokken. Th-Zellen sezernieren Zytokine (A) und resultieren in einer Aktivierung von Makrophagen (Mϕ) (B). Th1-Zellen produzieren IFN-γ (A) und führen zu einer klassischen Aktivierung von Makrophagen (caMϕ) (C). Aufgenommene Kryptokokken werden im Phagolysosom abgebaut. Im Gegensatz dazu sezernieren Th2-Zellen IL-4 (A) und aktivieren die Makrophagen alternativ (aaMϕ) (D). Die so aktivierten Makrophagen werden zu schaumzelligen Makrophagen, in denen sich Kryptokokken vermehren können. Die Kryptokokken werden per Makrophagen-*Shuttle* ins Gehirn transportiert (E).

4.5 Therapieansätze gegen die Kryptokokkose

Die häufig tödlich verlaufende Meningitis muss mit fungiziden und fungistatischen Mitteln therapiert werden, die allerdings eine Vielzahl von Nebenwirkungen aufweisen (82). Studien zu Immuntherapien – z.B. durch Gabe von monoklonalen Antikörpern gegen Kapselmaterial des Pilzes – sind zum jetzigen Zeitpunkt nur im Tiermodell getestet worden (142,143). Für die Etablierung der Immuntherapien/Vakzinierungen werden gegenwärtig einige Probleme gesehen: so ist beispielsweise nicht bekannt, ob eine antikörpervermittelte oder eine zellvermittelte Immunantwort den besten Schutz gegen eine Kryptokokkose im Menschen bietet (250). Aber aus den Vakzinierungstudien in der Maus ist

abzulesen, dass eine Immunisierung einige Vorteile brächte. Allerdings ist der Schutz, vermittelt durch Antikörper und Immunzellen, noch nicht hinreichend verstanden (250). Bekannt ist, dass einige Konjugatimpfstoffe keine schützenden Antikörperantworten induzieren und einige Impfungen sogar zu einer Verschlechterung des Zustandes durch pathologisch wirksame zelluläre Immuneffekte führen. Die hier vorliegende Arbeit soll in diesem Zusammenhang helfen, die pathologischen Faktoren der Immunantwort gegen eine Kryptokokkose zu identifizieren und Antworten auf die Frage geben, welche Zellen und Botenstoffe für die Immunpathologie von besonderer Bedeutung sind. Wie gezeigt werden konnte, ist eine Th1-Antwort und die damit einhergehende zelluläre Immunreaktion essentiell für den Schutz vor den pathologischen Auswirkungen einer pulmonalen und zerebralen Kryptokokkose. Durch die Blockade von Th2-Zytokinen bzw. ihrer Rezeptoren ist eine Induktion der schützenden Th1-Antwort möglich.

Als Therapie wird eine Kombination aus drei Antimykotika (Deutschland), bestehend aus Amphotericin B, Flucytosin und Fluconazol, bzw. eine Kombination aus zwei Antimykotika (USA), Amphotericin B und Fluconazol, verwendet (82). Vor allem Amphotericin B und Flucytosin weisen eine Reihe von starken Nebenwirkungen auf (u.a.Nephrotoxizität, Leberschädigungen, Anämien und myokardiale Toxizität (82,251)). Aufgrund der starken Nebenwirkungen und der intravenösen Darreichungsform wird eine stationäre Therapie empfohlen. Neben diesen Problematiken mit therapeutischen Ansätzen kommt noch hinzu, dass es, wie erwähnt, bis zum heutigen Tage keine wirkungsvolle Impfung gegen *C. neoformans* gibt und somit prophylaktisch im Falle einer HIV-Infektion nur auf eine Behandlung mit Fluconazol zurückgegriffen werden kann, wobei allerdings auch Resistenzen gegenüber diesen Mitteln auftreten (252). Die Problematik mit der antifungalen Therapie und einer wirkungsvollen Prophylaxe, auf der einen und Resistenzbildung auf der anderen Seite, macht es notwendig nach Alternativen zu suchen.

4.6 Immuntherapeutische Zielmoleküle

Ein alternativer Therapieansatz wäre eine Immuntherapie. Diese Therapieform hätte ggf. mehr Erfolg als ein direktes Vorgehen gegen die Kryptokokken. Die in der Habilitationsschrift gezeigten Daten legen dar, dass IL-4 und IL-13 entscheidend an der Pathologie der pulmonalen Kryptokokkose beteiligt sind. Die Signaltransduktion erfolgt mittels der Typ 1- oder Typ 2-IL-4-Rezeptoren (s. Abb. 2.3). Die IL-4Rα-Untereinheit ist von besonderer Bedeutung, da in der vorliegenden Arbeit gezeigt werden konnte, dass normalerweise empfängliche Mäuse, die zu 100% einer pulmonalen Kryptokokkose erliegen, durch Defizienz der IL-4Rα-Untereinheit resistent werden. Diese Tiere zeigen im Gegensatz zu den empfänglichen Mäusen eine gute Kontrolle der Erreger mit nur geringer Dissemination in die peripheren Organe. Damit einhergehend findet sich eine ausgeprägte Th1-Antwort, die u.a. durch das Auftreten

klassisch aktivierter Makrophagen gekennzeichnet ist. Im Unterschied zu den empfänglichen Mäusen weisen IL-4Rα-defiziente Mäuse auch keine Hyperreagibilität der Atemwege auf (241).

Die Bedeutung des IL-4R für -Resistenz oder Suszeptibilität gegenüber intrazellulären Erregern hängt von dem Erreger selbst ab. Während z.B. $CD8^+$ Gedächtniszellen in nicht lymphoiden Organen ohne den IL-4R keine stabile Population bilden können und somit die schützende Immunantwort gegen Leberstadien des Parasiten *Plasmodium yoelii* gestört ist (253), sind IL-4R-defiziente Mäuse gegenüber *Plasmodium berghi* durch eine frühe NK-Zellantwort und einer erhöhten iNOS-Aktivität besser geschützt als die Wildtyp-Kontrolltiere (160). Der Parasit *Toxoplasma gondii* induziert zur Etablierung in Phagozyten nach Infektion von Makrophagen die Phosphorylierung und Kerntranslokation von STAT6 und löst die Expression IL-4-abhängiger Genprodukte aus, ohne externe Stimulation durch IL-4. Die STAT6-Aktivierung führt u.a. zu einer vermehrten Expression des IL-4R und zur Sezernierung von Th2-Chemokinen, wie z.B. den Liganden für CCR3 und CCR4, durch die Th2-Zellen angelockt werden. Der Parasit schafft sich durch diesen Evasionsmechanismus ein Th2-Milieu, in dem er überleben und die für ihn schädliche Wirkung von IFN-γ reduzieren kann (254). IL-4 ist über den IL-4R in der Toxoplasmose auch an der Kontrolle des antiparasitisch wirksamen Enzyms Indoleamin 2,3-dioxygenase (IDO) beteiligt, welches Tryptophan aus infizierten Zellen entfernen kann und somit das Wachstum des Parasiten inhibiert. IDO wird durch IFN-γ-Stimulation in Zellen heraufreguliert und fördert somit die Parasitenkontrolle. Diesem Effekt wird durch IL-4 und IL-13 entgegengewirkt. Beide Zytokine begünstigen somit das Parasitenwachstum (255).

In der Pilzinfektion mit *Candida albicans* spielen IL-4 und der IL-4R ebenfalls eine wichtige Rolle. So konnte gezeigt werden, dass humane mononukleäre Phagozyten eine geringere Aufnahme von Blastokonidien unter IL-4-Gabe in Anwesenheit von humanem Serum zeigen. In Abwesenheit ist die Aufnahme von Konidien über Mannoserezeptoren hingegen erhöht, die antifungale Antwort ist aber in beiden Fällen gestört. IL-4 scheint somit an einer Verschlimmerung des Krankheitsbildes beteiligt zu sein (256).

In einem weiteren Parasitenmodell, der murinen Leishmaniose, konnte gezeigt werden, dass die Wirkung von IL-4 und IL-13 zwiespältig sein kann. In diesem Modell führt die Infektion mit dem Erreger *Leishmania major* in Balb/c-Mäusen zu einer nicht kontrollierten Infektion durch Induktion einer Th2-Antwort, während C57BL/6-Mäuse eine schützende Th1-Antwort ausbilden und resistent sind. IL-4-defiziente Mäuse auf dem empfänglichen Balb/c-Hintergrund können den Erreger nicht vollständig eliminieren, aber nach Injektion in die Hinterpfote den injizierten Erreger an seiner Ausbreitung hemmen. Bei IL-4R-defizienten Mäusen hingegen kommt es spät zu einer Dissemination des Erregers und zu ulzerativen Veränderung der Pfote. Diese Tiere können die Infektion somit im späteren Verlauf der Infektion nicht mehr kontrollieren. Diese Ergebnisse deuten auf einen schützenden Effekt durch IL-13 hin (257). Zudem ist in den IL-4R-defizienten Mäusen in der Leishmaniose eine deutliche Th2-

Polarisation der Th-Zellen zu finden, d.h. es kommt zu einer IL-4R-unabhängigen Th2-Entwicklung während der Infektion (155).

Der Befund des in dieser Arbeit zugrunde liegenden Infektionsmodells zeigt hingegen eindeutig, dass der IL-4R und die Zytokine IL-4 und IL-13 in der pulmonalen Kryptokokkose starke immunpathologische Effekte haben und somit als Ziel einer möglichen Immuntherapie in Frage kommen.

Durch weitere Analysen konnte darüber hinaus in dem Modell der pulmonalen Kryptokokkose das Vorliegen eines Gendosis-Effektes der IL-4Rα-Untereinheit nachgewiesen werden (241). Diese Tatsache war bis dato nicht bekannt und wurde zeitgleich von einer anderen Arbeitsgruppe bestätigt (258). Tiere, die heterozygot für die IL-4Rα-Untereinheit sind, welche ubiquitär auf allen Immunzellen und Epithelien zu finden ist, zeigen nur ungefähr 50% des IL-4Rα auf der Oberfläche der Zellen. Dieser Effekt ist in Modellen mit einer stärkeren IL-4-Antwort nicht erkennbar. So unterscheiden sich die Phänotypen der Wildtyp- und heterozygoten Tieren in Wurminfektionen (z.B. Infektionen mit Trematoden (*Schistosoma mansoni*) (159) oder Nematoden (*Nippostrongulus brasiliensis*) (159)) nicht voneinander. Dieser Zusammenhang zwischen der Stärke der IL-4-Antwort und der Expression des IL-4R konnte *in vitro* nachgebildet werden (unpublizierte Daten aus dem Institut für Immunologie und (240)). Während in Wurminfektionen hohe IL-4-Spiegel zu finden sind, die man auch im Serum nachweisen kann, ist der IL-4-Spiegel während der pulmonalne Kryptokokkose, vergleichbar mit dem Geschehen bei Asthma, nur sehr gering. Diese geringen IL-4-Spiegel in der pulmonalen Kryptokokkose beeinflussen die Expression des IL-4Rα auf der Oberfläche der Zellen nicht, im Gegensatz zu hohen Spiegeln, z.B. bei Wurminfektionen, die zu einer erhöhten Expression des IL-4Rα führen (240). Die verstärkte Expression verwischt die Unterschiede und in diesen Modellen verhalten sich die beiden Gruppen – Wildtyp- und IL-4R-heterozygote Mäuse – gleich (159).

Aufgrund des beschriebenen Gendosis-Effekts der IL-4Rα-Kette in der pulmonalen Kryptokokkose bietet sich dieses Protein als therapeutisches Zielmolekül an.

4.7 Therapieansätze gegen Th2-Faktoren des Immunsystems

Die vorliegende Arbeit hat Grundlagen für das Verständnis der immunpathologischen Mechanismen in der pulmonalen Kryptokokkose geschaffen. Darauf aufbauend sind Zielmoleküle charakterisiert worden, die, wie im vorherigen Absatz erläutert, immuntherapeutisch moduliert werden könnten. Die Therapie sollte unter optimalen Umständen lokal in der Lunge den für die Pathologie wichtigen IL-4/IL-13-IL-4Rα-Stat6-Signaltransduktionsweg ausschalten (169). Aus den Daten der vorliegenden Arbeit wird ersichtlich, dass bereits eine teilweise Reduktion der Expression des IL-4Rα, wie in IL-4R-heterozygoten Mäusen vorzufinden, deutliche protektive Effekte in der pulmonalen Kryptokokkose hat. Aus diesem Grunde ist eine lokale Blockade des IL-4R als Therapie durchaus in Betracht zu ziehen. Eine derartige Immuntherapie besteht für die pulmonale Kryptokokkose bisher nicht. Eine Therapie zur

Modulation/Inhibition des IL-4/IL-13-IL-4Rα-Stat6-Signaltransduktionsweges sollte im Frühstadium der Infektion auf die lokale Behandlung der Lunge beschränkt bleiben, um ungewollte Nebeneffekte (z.B. eine aus der Therapie resultierende verminderte Wurmabwehr des Darms) zu vermeiden. Das Asthma-Modell ist der pulmonalen Kryptokokkose in der Maus in Bezug auf die Hyperreagibilität, die Becherzellhyperplasie und Eosinophilenrekrutierung klinisch ähnlich (259). Aus diesem Grund wird im Anschluss aus Arbeiten zur Untersuchung von Asthma zitiert (Abb. 4.2). Eine Ähnlichkeit der Modelle legt nahe, dass Befunde, die in der Kryptokokkose erhoben wurden, auch auf das Immungeschehen in der Lunge während eines Asthma bronchiale angewendet werden könnten. Das Labormodell der pulmonalen Kryptokokkose könnte hierdurch eine Bedeutung erlangen, welche weit über die Untersuchung des Infektionsgeschehens gegen einen Pilz hinausgeht.

Die erste Möglichkeit zur Blockierung des IL-4R besteht in einer Behandlung mit blockierenden Antikörpern, welche die Aktivierung des Rezeptors verhindern. Der Antikörper würde sterisch die Bindung des Liganden (IL-4 oder IL-13) hemmen. Eine solche Therapie wurde schon in diversen Erkrankungen, z.B. Asthma getestet, bisher leider mit geringem Erfolg (241,260). In einer Studie mit einem monoklonalen anti-IL-4Rα-Antikörper zeigte dieser in hoher Konzentration nur in einer Subpopulation von Patienten Wirkung. So wiesen diese Patienten eine geringere Anzahl an asthmatischen Anfällen auf (261). Neben der Antikörpertherapie wurden auch lösliche IL-4R (sIL-4R Typ I und Typ II) verwendet, um die Zytokine IL-4 und IL-13 zu neutralisieren (262,263). Aber auch diese Therapie konnte bisher nicht überzeugen. In klinischen Studien zeigten die löslichen Rezeptoren keine positive Wirkung (264).

Eine weitere Strategie besteht in der direkten Blockierung des IL-4R durch einen modifizierten Liganden. Das Mutein Pitrakinra, bei dem es sich um ein modifiziertes IL-4-Molekül handelt, bindet an die IL-4Rα-Kette und verhindert dadurch die IL-4- und IL-13-vermittelte Signalweiterleitung. In Studien am Menschen zeigte sich, dass die behandelten Patienten im Gegensatz zur Placebo-Gruppe deutlich weniger Asthmaanfälle hatten (265). Die Wirkung des Muteins beruht darauf, dass es an die IL-4Rα-Kette bindet und hierdurch die Rezeptorkettenbindung an die γc-Kette bzw. die IL-13Rα1-Kette verhindert. Neben Asthma ist auch eine Behandlung gegen Ekzeme im Gespräch. Hierbei sollen als Zielzellen Th2-Zellen, B-Zellen und dendritische Zellen durch das Mutein moduliert werden (266).

Als weitere mögliche Therapieform gilt die RNA-Interferenz, bei der aus mRNA und einer komplementären RNA doppelsträngige RNA erzeugt wird, die im Anschluss durch interne Nukleasen abgebaut und somit inaktiviert wird. Hierbei gibt es verschiedene Vorgehensweisen. Es kann zum Einen direkt zum Genprodukt komplementäre RNA generiert werden, die über ein Transfersystem in die Zellen eingebracht wird (z.B. über Lipofektion). Diese komplementäre RNA kann dann an mRNA binden und somit RNasen aktivieren bzw. das Ablesen der Information sterisch hemmen (267). Des Weiteren werden sogenannte Ribozyme eingesetzt, RNA-Strukturen mit enzymatischen Eigenschaften. Hierbei

bindet das Ribozym komplementär an die Ziel-mRNA und schneidet diese (268). Bei einem weiteren gebräuchlichen Verfahren, wird zielsequenzspezifische dsRNA (doppelsträngige RNA) oder shRNA (*small hairpin* RNA) verwendet. Beide Varianten besitzen doppelsträngige Abschnitte, die durch ein Enzym names *Dicer* in kurze doppelsträngige RNA-Fragmente geschnitten und als siRNA (*small interfering RNA*) in ein Reparatursystem namens RISC-Komplex (*RNA-induced silencing complex*) eingebaut werden. Dieser Komplex ist daraufhin in der Lage, die Ziel-RNA zu erkennen und zu spalten, wodurch sie inaktiviert wird (267). Ein Nachteil ist für erste Studien im Tierexperiment die kurze Dauer der therapeutischen Wirkung von siRNAs, die sich aber im Menschen als nützlich erweist, da länger währende Unterdrückungen des IL-4R ausgeschlossen werden können. Für erste Untersuchungen im Tiermodell würde hingegen eher mit einem stabileren DNA-Vektor transfiziert werden, welcher für small hairpin RNA (shRNA) codiert. Diese Strategie wurde bereits für die Inaktivierung des Enzyms Arginase-1 angewendet (269). Dieses System würde es dadurch, dass ein DNA-Vektor eingesetzt wird, erlauben, eine Inhibierung über einen längeren Zeitraum aufrecht zu erhalten. Hierdurch würden sich Untersuchungen auch in dem Langzeitmodell der pulmonalen Kryptokokkose erlauben.

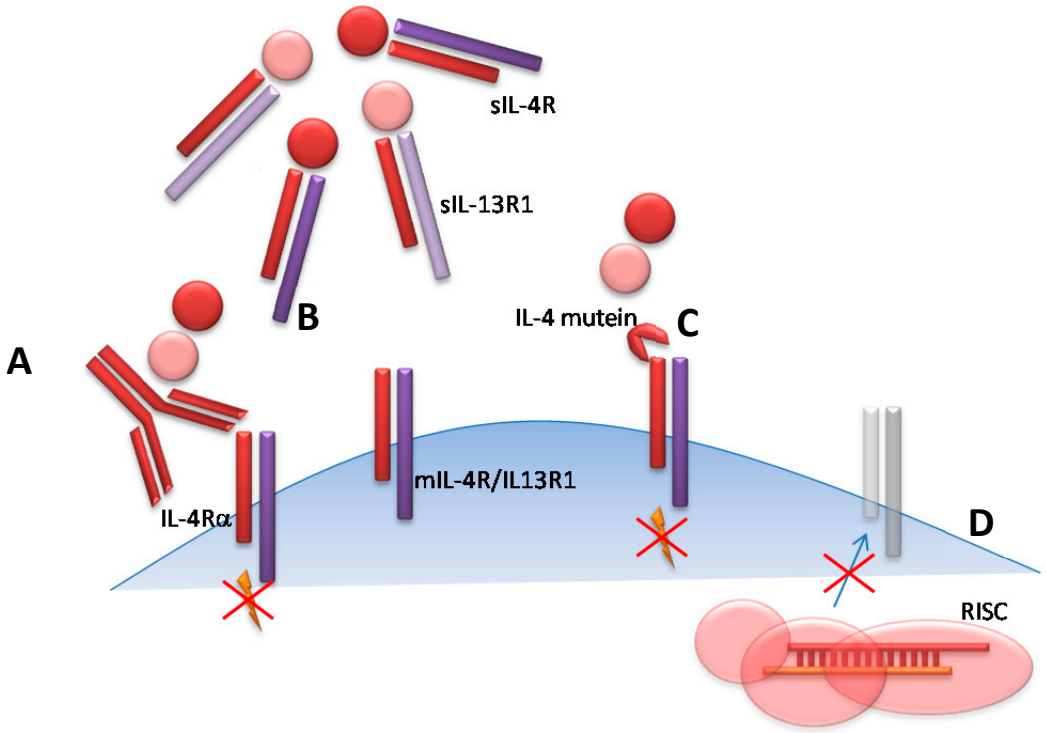

Abb. 4.2: Möglichkeiten zur Blockierung des IL-4R-Signals. (A) Die Blockade der Liganden-Rezeptorbindung durch Andocken IL-4Rα-spezifischer Antikörper an die Bindungsstelle der IL-4Rα-Kette verhindert die Aktivierung des Rezeptors und die Signaltransduktion zu STAT6. (B) Lösliche IL-4R (sIL-4R) und IL-13R (sIL-13R1) fangen die Zytokine IL-4 bzw. IL-13 und verhindern so die Bindung an die membranständigen IL-4R/IL-13R1 (mIL-4R/IL13R1). (C) Die Blockade der Liganden-Rezeptorbindung durch einen modifizierten Liganden (IL-4-Mutein) inhibiert die Signaltransduktion(D). Durch Einbringen von siRNA oder shRNA wird die IL-4Rα-mRNA durch den RISC-Komplex gespalten und somit inaktiviert. Die Bildung von IL-4Rα wird dadurch verhindert und IL-4R und IL-13R1 nicht auf der Zelloberfläche exprimiert. ● IL-4, ● IL-13.

4.8 Zelluläre Beteiligung an der Immunpathologie der pulmonalen Kryptokokkose

Die vorliegende Arbeit hat wichtige Erkenntnisse hinsichtlich der wichtigsten Zelltypen ergeben, die bei der Immunpathologie der pulmonalen Kryptokokkose eine Rolle spielen. Die IL-4Rα-Kette als Ziel einer Therapie gegen Kryptokokken ist vielversprechend, da der IL-4R-Komplex entscheidend in der Pathogenese der pulmonalen Kryptokokkose ist. Von besonderer Bedeutung für die Pathologie sind hierbei aber vor allem die beteiligten Zellen des Immunsystems. Eine wichtige Rolle als Effektorzelle in der Kryptokokkose spielt hierbei der bereits beschriebene alternativ aktivierte Makrophage. Andere Arbeitsgruppen konnten die Bedeutung der alternativen Aktivierung von Makrophagen in der pulmonalen Kryptokokkose bestätigen (124,184). Die Signaltransduktion über den IL-4R ist für die alternative Aktivierung und die damit einhergehende Immunreaktion essentiell. Insbesondere an der Induktion der Immunpathologie, über die pulmonale Kryptokokkose hinaus, hat der IL-4R einen bedeutenden Anteil.

4.8.1 Der alternativ aktivierte Makrophage

Es konnte in einem murinen Lungen-Allergie-Modell, wobei in Tieren eine Allergie gegen Hühnereiweiß induziert wurde, die besondere Bedeutung von Makrophagen für die Pathogenese gezeigt werden. So sind in IL-4R- und Lymphozyten-defizienten Mäusen, denen Th2-Zellen und IL-4R-exprimierende Makrophagen appliziert werden, eine deutlich erhöhte Eosinophilen-Rekrutierung gegenüber solchen Tieren nachweisbar, die IL-4R-defiziente Makrophagen erhielten. Die IL-4R-exprimierenden Makrophagen wiesen dabei Oberflächenmarker von alternativ aktivierten Makrophagen auf, wie z.B. YM-1. Weiterhin war die Expression von Chemokinen, die die Eosinophilen-Rekrutierung fördern (z.B. Eotaxin-1 und RANTES) erhöht. Diese Ergebnisse veranschaulichen die Bedeutung alternativ aktivierter Makrophagen in allergischen Reaktionen in der Lunge (270). Auch im Falle von Asthma bronchiale ist die Zahl dieser Zellen erhöht (271-273) und es konnte gezeigt werden, dass alternativ aktivierte Makrophagen Symptome des Asthma bronchiale induzieren können (274). Laut einer Studie in einem Mausmodell einer chronischen allergischen Lungenerkrankung können Makrophagen die Hyperreagibilität der Bronchien und die Schleimbildung direkt fördern (275,276). Hierbei werden Mäuse mit murinen Parainfluenza-Viren infiziert und entwickeln eine chronische Entzündung der Lunge. Die dabei entstehenden alternativ aktivierten Makrophagen sind in der Lage nach Stimulation durch NKT-Zellen in großen Mengen IL-13 zu bilden.

4.8.2 Die Th2-Zelle

Der Makrophage ist als Effektorzelle in der Kryptokokkose von zentraler Bedeutung (Abb. 4.1). Aber für die vollständige Aktivierung ist er auf andere Zellen angewiesen. Aus diesem Grunde war das Anliegen

der vorliegenden Arbeit, nachzuweisen, welcher Zelltyp die alternative Aktivierung induziert. Die alternative Aktivierung ist abhängig von Th2-Zytokinen. Hierbei lag der besondere Fokus auf den T-Helferzellen, die eine zentrale Rolle in der Orchestrierung der Art der Immunantwort gegen Erreger spielen und Th2-Zytokine sezernieren können. Durch Einsatz von T-Helferzell-spezifischen IL-4Rα-defizienten Mäusen sollte ermittelt werden, ob die T-Helferzellen eine zentrale Rolle in der Pathogenese der pulmonalen Kryptokokkose spielen.

In einem murinen *Leishmania*-Infektionsmodell konnte mit Hilfe dieser Mäuse gezeigt werden, dass die IL-4R-abhängige Th2-Zelldifferenzierung wichtig für die Immunpathologie ist und Th2-Zellen das Leishmanien-Wachstum nicht eindämmen können. Im Gegensatz zu T-Helferzell-spezifischen IL-4Rα-defizienten Mäusen sind IL-4Rα-defiziente Mäuse nicht vollständig vor einer Leishmanien-Infektion geschützt. Dieser Befund zeigt, dass die Wirkung von IL-4 und IL-13 auf nicht-Th-Zellen wichtig für die Kontrolle des Erregers ist (161).

4.9 Die polyfunktionale Th-Zelle

In der pulmonalen Kryptokokkose konnte im Rahmen der vorliegenden Arbeit beobachtet werden, dass sich eine IL-4/IL-13-Stimulation auf Th-Zellen und anderen Zellen, wie z.B. Makrophagen, gleichermaßen negativ auf die Erregerkontrolle auswirkt (241,242). Hierbei stellte sich heraus, dass die T-Helferzell-Makrophagen-Achse für die Pathogenese von entscheidender Bedeutung ist. Erste Hinweise auf die Bedeutung von Th-Zellen in der Immunpathologie der pulmonalen Kryptokokkose wurden durch Depletion von Th-Zellen gewonnen (122). Der zugrunde liegende Mechanismus war allerdings noch unklar. Die Induktion einer Th2-Antwort durch Th-Zellen ist dabei äußerst interessant und konnte mittels polychromatischer Durchflusszytometrie in der vorliegenden Arbeit aufgedeckt werden. Mit Hilfe der polychromatischen Durchflusszytometrie ist es möglich, die Bildung mehrerer Zytokine gleichzeitig in einer individuellen Zelle zu bestimmen. Wie sich heraus stellte, ist in der pulmonalen Kryptokokkose nicht nur die Quantität der Th2-Zellen von Bedeutung, sondern insbesondere die Qualität (243). Die Wichtigkeit der Qualität der Th-Zellen wurde, bisher ausschließlich in Th1-Immunantworten ermittelt (189,193,277). Der Begriff „Qualität“ beschreibt hierbei, das Vorhandensein sowohl von Th-Zellen, die individuell ein Zytokin bilden (monofunktional) als auch solchen, die zwei oder mehr Zytokine zeitgleich produzieren (bi-/tri- bzw. multi- oder polyfunktional). So konnte z.B. im Bereich der Th1-Antwort in Vakzinierungsmodellen ermittelt werden, dass vor allem solche Zellen, die polyfunktional sind und z.B. IL-2 und IFN-γ bilden, eher zu Gedächtniszellen werden (193,277), als solche, die ausschließlich IFN-γ sezernieren. In der vorliegenden Arbeit wurden Analysen an Th-Zellen aus *C. neoformans*-infizierten Lungen von Kontroll- und Th-zellspezifischen IL-4Rα-defizienten Mäusen vergleichend durchgeführt. Durch Wahl dieser Mausgruppen konnte die Bedeutung von polyfunktionalen antigenspezifischen Th2-Zellen, also solchen Zellen, die mehr als ein Th2-Zytokin (IL-4, IL-5, IL-13) gleichzeitig bilden und

spezifisch für *C. neoformans*-Antigene sind, bestimmt werden. Von besonderer Bedeutung ist hierbei, dass Th-Zellen auch ohne den IL-4R Th2-Immunreaktionen auslösen können und Th2-Zytokine bilden: Dies wurde auch durch Experimente anderer Arbeitsgruppen bestätigt (155). In den Versuchen der vorliegenden Arbeit zeigte sich, dass die Qualitäten der Th2-Zellen zwischen den Th-zellspezifischen IL-4Rα-defizienten Tieren und den Kontrollmäusen sehr unterschiedlich war. Während die Kontrollmäuse trifunktionale Th2-Zellen (Zellen, die IL-4, IL-5 und IL-13 gleichzeitig bilden) aufwiesen, war diese Population in den defizienten Tieren stark reduziert. Dies ist ein Hinweis auf die besondere Bedeutung des IL-4R in der Immunpathologie der pulmonalen Kryptokokkose, wie man sie auch in anderen Lungenerkrankungen wie z.B. Asthma findet (278,279). Die Zahl der polyfunktionalen, insbesondere trifunktionalen Th2-Zellen ist zwar gering, die Zellen weisen aber, wie durchflusszytometrisch ermittelt werden konnte, eine hohe Expression der zu untersuchenden Th2-Zytokine auf. Die von den trifunktionalen Th2-Zellen sezernierten Zytokinmengen sind deutlich höher als die monofunktionaler Zellen. Somit kann die kleine Population der polyfunktionalen Th2-Zellen dennoch wichtige Impulse in der Pathologie der pulmonalen Kryptokokkose geben. Aufgrund der Korrelation der Pathologie (Eosinophilen-Rekrutierung, Becherzellhyperplasie, alternative Aktivierung von Makrophagen) mit dem Vorhandensein dieser polyfunktionalen Th2-Zellen, bietet die Untersuchung dieser Zellen in der pulmonalen Kryptokokkose ein diagnostisches Mittel, um prospektiv den Verlauf der Infektion zu ermitteln. Mit Hilfe der Befunde wird es zukünftig möglich sein, zu entscheiden, ob sich die Immunantwort eher zu einer schützenden Th1- oder zu einer pathologischen Th2-Antwort entwickelt.

In aktuellen Untersuchungen der Entwicklung des Immunrekonstitutions-Entzündungssyndroms (IRIS) in HIV-Patienten mit einer antiretroviralen Therapie, konnte gezeigt werden, dass polyfunktionale antigenspezifische Th-Zellen die Pathologie des IRIS hervorrufen (280). Im Falle des Kryptokokken-IRIS bei HIV-Patienten kommt es aufgrund einer hochaktiven retroviralen Therapie zu einer Rekonstitution der T-Helferzellen. Diese T-Helferzellen schütten allerdings aufgrund des Vorhandenseins von Kryptokokkenantigen große Mengen an Zytokinen aus, ein sogenannter Zytokinsturm, der ernsthafte pathologische Effekte für den Patienten hat. Diese Pathologien können bis hin zu einer Meningitis führen. Da solche polyfunktionalen antigenspezifischen Th-Zellen auch im Menschen vorkommen, könnte man ggf. auch die polyfunktionalen Th2-Zellen als diagnostischen Marker für die klinische Entwicklung in immungeschwächten Menschen mit einer Kryptokokkose nutzen. Darüber hinaus wäre auch ein klinisches *Monitoring* von Patienten über einen längeren Zeitraum denkbar. Neben der Beurteilung der Aussichten in der Kryptokokkose wäre eine polychromatische Durchflusszytometrie diagnostisch, aufgrund der gezeigten ähnlichen Verhältnisse auch im Asthma-Geschehen denkbar.

4.10 Abschließende Betrachtungen zur pulmonalen Kryptokokkose

Die schwerwiegenden durch eine Kryptokokkose hervorgerufenen Pathologien, die in immungeschwächten Menschen auftreten, sind in immunkompetenten Individuen nicht vorhanden. Allerdings können auch immunkompetente Menschen persistent infiziert sein (5). Eine Hypothese besagt, dass eine durch *C. neoformans* hervorgerufene schwache Th2-Antwort in der Lunge (124) infizierter Individuen zu einer allergischen Reaktion bis hin zu Asthma führen könnte. Untersuchungen im Rattenmodell legen diese Vermutung nahe (123). In den Ratten wurden erhöhte Chitinase-Werte während einer pulmonalen Kryptokokkose ermittelt (223). Die gleiche Arbeitsgruppe hat ähnliche Befunde bei asthmatischen Kindern gefunden, welche pilzspezifische Antikörper in der bronchoalveolären Lavage zeigen (281).

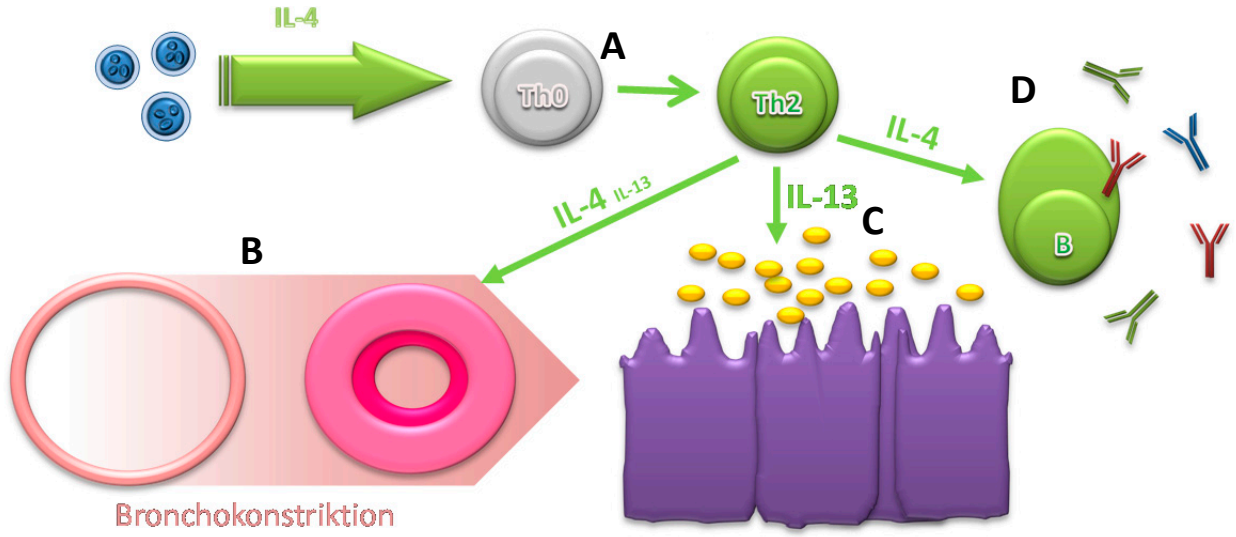

Abb. 4.3: Gemeinsamkeiten zwischen einer Kryptokokken-Infektion und allergischen Symptomen. Durch die Infektion wird die Bildung von IL-4 induziert (Quelle bisher unbekannt) und aus naiven Th-Zellen werden Th2-Zellen (A). Die aktivierten Th2-Zellen sezernieren IL-4 und IL-13 und sind für diverse Symptome der Allergie verantwortlich. IL-4 sensibilisiert die glatte Muskulatur, so dass auf die Stimulation durch einen muskarinergen Agonisten hin eine Bronchokonstriktion induziert wird (B). Durch die Bildung von IL-13 können Becherzellen in der Schleimhaut der Lunge aktiviert und zur Bildung von Schleim angeregt werden (C). Durch IL-4 können B-Zellen zur Bildung von IgE angeregt werden. Die Funktion des IgE in der pulmonalen Kryptokokkose ist noch nicht abschließend geklärt (D).

Diese Immunpathologie-assoziierte Mykose, von einigen englischsprachigen Autoren auch *allergic bronchopulmonary mycosis* (allergische bronchopulmonale Mykose) genannt (122,184,185), ist gekennzeichnet durch die Stimulation von Becherzellen durch IL-13 und Bronchokonstriktionen auf muskarinerge Agonisten, die z.T. IL-4- und IL-4R-abhängig sind (282). Diese Mechanismen sind auch in dem hier verwendeten Mausmodell der pulmonalen Kryptokokkose präsent. IL-13- und insbesondere IL-4R-defiziente Mäuse zeigen in der pulmonalen Kryptokokkose (238,241) keine oder nur eine geringe

Reaktion auf muskarinerge Agonisten, eine fehlende Mukusbildung und geringe oder fehlende IgE-Immunglobulinspiegel (Abb. 4.3). Immunglobulin E ist ein Marker für eine ablaufende Th2-Antwort auf ein Allergen oder eine Infektion mit extrazellulären Erregern, insbesondere Helminthen. Amerikanische Studien weisen auf die Bedeutung einer Kryptokokkeninfektion hinsichtlich des Asthmarisikos in Menschen hin (123,281). Eine Schlussfolgerung könnte sein, dass die Infektion mit Kryptokokken mit einer erhöhten Asthma-Entwicklung einhergehen könnte.

Eine weitere Studie dieser Arbeitsgruppe zeigt, dass die Bevölkerung in der Stadt einem höheren Erregerdruck ausgesetzt ist als die Landbevölkerung. Dieses könnte auch mit der höheren Dichte an Vektoren (Stadttauben) in Innenstädten zusammenhängen (283).In der Studie wurde zudem ermittelt, dass in städtischen Ballungsräumen nahezu jedes zweijährige Kind Antikörper gegen *C. neoformans* aufweist (284) und gerade in der Gruppe asthmatischer Kinder war der Spiegel Kryptokokken-spezifischer IgA-Immunglobuline höher als in der nicht-asthmatischen Kontrollgruppe. In den asthmatischen Kindern war hierbei ein erhöhter Wert für Chitinase messbar (281). Inwieweit diese Befunde auch für Mitteleuropa zutreffen, ist noch nicht untersucht worden.

In den empfänglichen Maus-Genotypen, die im Rahmen der vorliegenden Arbeit untersucht wurden, zeigt sich eine starke Hyperreagibilität der Lungenfunktion und auch die IgE-Spiegel sind deutlich erhöht (238). Über die Bedeutung der IgE-Spiegel in der pulmonalen Kryptokokkose ist allerdings noch wenig bekannt. So ist noch unklar, ob die Erhöhung des IgE-Spiegels nur eine Begleiterscheinung der Th2-Antwort ist oder ob IgE auch als Pathogenesefaktor gelten kann. Diese Frage versuchen wir momentan zu klären. Untersuchungen aus dem Institut für Immunologie belegen, dass der größte Teil der IgE-Immunglobuline Kryptokokken-unspezifisch ist (239). Sollte es sich herausstellen, dass IgE die Persistenz des Erregers unterstützt, welche die Pathologie verschlimmert, wäre eine anti-IgE-Therapie denkbar. So existieren anti-IgE-Antikörper für die Behandlung von Allergien im Menschen (Omalizumab).

Die vorliegenden Publikationen beleuchten die pathologischen Vorgänge der pulmonalen Kryptokokkose unter besonderer Berücksichtigung der Th2-Antwort und der damit zusammenhängenden Faktoren. Das hier verwendete murine Infektionsmodell zeigt gut die Interaktion der Immunzellen während der Th2-abhängigen Pathogenese der Kryptokokkose auf. Insbesondere die polyfunktionalen Th2-Zellen sind hierbei zu erwähnen. Interessant wäre in diesem Zusammenhang die Bedeutung von polyfunktionalen Th2-Zellen im Kontext von Asthma und Allergien zu untersuchen. Denn wie erwähnt, gibt es viele Ähnlichkeiten in der Immunantwort gegen eine pulmonale Kryptokokkose und Asthma.

4.11 Limitationen des *C. neoformans*-Infektionsmodells

Das hier verwendete intranasale murine Infektionsmodell hat natürlich auch Limitationen in seiner Übertragung auf den Menschen. So verhalten sich Wildtyp-Mäuse mit einem vollständig intakten Immunsystem fast wie immungeschwächte Menschen (238). Demzufolge sind die gemachten

Beobachtungen nur zum Teil auf immunkompetente Individuen übertragbar. Modelle, die der menschlichen Immunreaktion gegen Kryptokokken näher kommen, sind in Ratten etabliert. Außerdem unterscheidet sich die Immunabwehr gegen Kryptokokken zwischen Mensch und Maus. Während Mäuse gegen Kryptokokken vor allem reaktive Stickstoffintermediate sezernieren und damit den Erreger töten können (285), bildet der Mensch so gut wie keine Stickstoffverbindungen zur Erregerabwehr. Weiterhin werden für die Untersuchungen Inzuchtmäuse mit definiertem, einheitlichem Genpool verwendet, um die Beobachtungen auf das ausgeschaltete Gen beziehen zu können (Informationsgewinn durch Funktionsverlust), die zudem unter spezifiziert pathogenfreien Bedingungen gehalten werden. Da die Tiere sich noch nicht mit Krankheitserregern auseinandersetzen mussten, sind sie empfänglicher für Infektionen. Das trifft auf die menschliche Population natürlich nicht zu. Menschen setzen sich tagtäglich mit Umweltkeimen und vielfältigen Erregern auseinander und haben unterschiedliche genetische Ausstattungen. Die grundlegenden Infektionsrouten und Disseminationswege sind allerdings bei Mensch und Maus sehr ähnlich. So kann man sowohl bei immunkompetenten Menschen und resistenten Mauslinien (z.B. IL-4Rα-defiziente Mäuse) beobachten, dass die Infektion nicht steril eliminiert wird, sondern der Pilz im Wirt, vor allem in der Lunge persistiert (5).

Die hier dargestellten Studienergebnisse aus unserem murinen Modell belegen einen Zusammenhang zwischen der Art der Immunantwort und der Kontrolle des humanpathogenen Erregers *C. neoformans*. Die präsentierten Daten zeigen die Beteiligung von Immunfaktoren auf, die an der Pathologie beteiligt sind. Diese Moleküle und Zellen stellen gute Ziele für eine erfolgreiche Immuntherapie dar, die helfen könnte diese schwerwiegende Infektionskrankheit einzudämmen. Des Weiteren steht mit den polyfunktionalen Th2-Zellen ein diagnostisches Werkzeug zur Verfügung, um mittels durchflusszytometrischer Messungen eine Aussage zur Prognose des Krankheitsverlaufs machen zu können.

5 Zusammenfassung

Interleukin (IL)-4, IL-13 und der IL-4-Rezeptor – molekulare Schrittmacher der Immunpathologie in der Kryptokokkose

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C. neoformans ist ein pathogener einzelliger Pilz, der neben dem Menschen weitere Säugetiere schädigen kann, insbesondere, wenn diese immunsupprimiert sind. Aber auch im immunkompetenten Wirt setzt der Pilz immunpathologische Mechanismen in Gang, die es ihm erlauben, über Jahre hinweg im Wirt zu persistieren. In der vorliegenden Arbeit werden immunpathologische Mechanismen der pulmonalen und zerebralen Kryptokokkose mit Hilfe von transgenen Mausmodellen untersucht und es werden wichtige Befunde erhoben, die dazu beitragen könnten, neuartige molekulare Therapien zu entwickeln, um den Pilz zu kontrollieren. Möglicherweise wären diese Therapieansätze auch in der Lage Krankheiten mit ähnlichen Immunpathologien wie Asthma und Allergien zu behandeln.
Während eine durch T-Helfer (Th) 1-Zellen induzierte zelluläre Immunantwort mit zytotoxischen T-Zellen und klassisch aktivierten Makrophagen schützend gegen eine pulmonale Kryptokokkose ist, führt eine Th2-Zell-vermittelte humorale Immunreaktion zu einer Pathologie mit schwerwiegenden Folgen für den Wirt. Die Anfälligkeit der pulmonalen Kryptokokkose teilt Merkmale mit allergischem Asthma (z.B. Becherzell-vermittelte Schleimbildung und Hyperreagibilität der Bronchen). Mit Hilfe von Interleukin (IL)-4- bzw. IL-13-defizienten Mäusen, die defizient für die Th2-Zytokine IL-4 oder IL-13 sind, konnte gezeigt werden, dass sowohl IL-4 also auch IL-13 an der Immunpathologie während einer pulmonalen Infektion mit Kryptokokken beteiligt sind. Mäuse, die defizient für IL-4 oder IL-13 sind, zeigen in der pulmonalen Kryptokokkose eine signifikant reduzierte Sterberate, einhergehend mit verminderter Organlast der Lunge, reduzierter Becherzell-Hyperplasie und daraus resultierender Schleimproduktion. Die verstärkte Resistenz dieser Mäuse gegenüber suszeptiblen Wildtypmäusen weist auf die Bedeutung dieser Botenstoffe für die Pathologie-Induktion hin. Durch die Infektion mit *C. neoformans* ist die Hyperreagibilität der Bronchien deutlich erhöht. Dies ist ein Zeichen dafür, dass IL-4/IL-13-abhängige pathologische Mechanismen induziert werden, die die Bekämpfung des Keims in der Lunge erschweren. Die Bedeutung der Funktion von IL-13 konnte durch die Verwendung von IL-13-überexprimierenden Mäusen gezeigt werden. Diese Mäuse sind hochsuszeptibel und signifikant stärker betroffen als Wildtyp-Kontrollen.

Für die Induktion einer Th2-Antwort ist die Bildung von IL-4 von besonderer Bedeutung. Aus diesem Grunde wurden IL-4-produzierende Zelltypen durchflusszytometrisch u.a. mit der Hilfe von IL-4-Reporter-Mäusen charakterisiert. Hierbei fiel auf, dass erst spät ab Tag 42 nach Infektion eine IL-4-Bildung nachgewiesen werden konnte. Die IL-4-Bildung geht einher mit einem Anstieg an IgE-Antikörpern, der alternativen Aktivierung von Makrophagen und der Ausbreitung des Pilzes im Körper. Es stellte sich heraus, dass neben den IL-4R-Hauptproduzenten, den T-Helferzellen, nur noch Eosinophile in der Mauslunge IL-4 produzieren. Mittels Eosinophilen-defizienten Mäusen konnte gezeigt werden, an welchen pathologischen Mechanismen diese Zellen beteiligt sind. Es stellte sich heraus, dass Eosinophile die Rekrutierung von Th-Zellen während der pulmonalen Infektion fördern. Diese Zellen verstärken dadurch den Th2-Effekt (u.a. die IgE-Bildung).

Die Signaltransduktion von IL-4 und IL-13 erfolgt über die IL-4-Rezeptoren (IL-4R) vom Typ I und II, die eine identische alpha-Kette nutzen. Durch Ausschalten der IL-4Rα-Kette in einem Mausstamm wurden Tiere generiert, die nicht auf IL-4 und IL-13 reagieren können. IL-4Rα-defiziente Mäuse erwiesen sich gegenüber einer pulmonalen Kryptokokkose als hoch resistent und können durch Induktion einer Th1-Antwort klassisch aktivierte Makrophagen zur Erregerabwehr stimulieren. Die ausgeprägte Resistenz dieser Gruppe ließ die Frage aufkommen, inwieweit auch heterozygote Tiere, also IL4Rα$^{+/-}$, im Gegensatz zu den Wildtypmäusen eine gewisse Resistenz aufweisen. Durch diese Experimente konnte zum ersten Mal eine Gendosis-Beziehung des IL-4R nachgewiesen werden. Phänotypiche Veränderungen in der Immunantwort gegen Kryptokokken fiel zwischen den Wildtyptieren und IL-4Rα$^{+/-}$ Mäusen auf. IL-4R$^{+/-}$ Tiere weisen im Vergleich zu IL-4R$^{+/+}$ Tieren eine verminderte IL-4R-Expression auf. Daraus resultiert eine erhöhte Überlebensrate, reduzierte Organlasten, verminderte IgE-Bildung und eine Reduktion der Zahl alternativ aktivierter Makrophagen.

Wie sich bei den weiterführenden Untersuchungen zur Bedeutung des IL-4R in der pulmonalen Kryptokokkose gezeigt hat, nehmen die durch IL-4/IL-13-induzierten alternativ aktivierten Makrophagen Pilze auf, können diese aber nicht abbauen. Zudem legen unsere Untersuchungen nahe, dass alternativ aktivierte Makrophagen für die Verbreitung der Pilze in das Gehirn des Wirts von Bedeutung sind, und zu zerebraler Kryptokokkose führen. Im Gehirn kommt es zur Ausbildung von Foci mit Pilzmaterial, umgeben von alternativ aktivierten Makrophagen, die in der Lage sind, Entzündungen im Gehirn zu induzieren. Mikrogliazellen hingegen werden durch *C. neoformans*-Antigen nicht aktiviert und beinhalten auch kein Kryptokokken-Antigen. Die Befunde der Mikrogliazellen weisen darauf hin, dass die pilztragenden Makrophagen („*shuttle*“) aus der Peripherie kommen und dort mit Antigen beladen wurden. Alternativ aktivierte Makrophagen sind ein klares Zeichen für Suszeptibilität, denn in resistenten Mäusen, wie z.B. den IL-4- oder IL-4R-defizienten Tieren waren gegenüber naiven Tieren keine erhöhten Zahlen dieser Zellen während der Infektion im Gehirn zu finden. Daneben ist die Zahl an Kryptokokken im ZNS in IL-4-, IL-13- und IL-4R-defizienten Mäusen gegenüber den suszeptiblen Wildtyp-

Kontrollen deutlich reduziert. Dieses geht einher mit erhöhter Überlebensrate und verminderten Entzündungsreaktionen der Meningen.

Aufgrund dieser Untersuchungen ergibt sich ein recht klares Bild über die Abläufe während der Pathogenese der pulmonalen und zerebralen Kryptokokkose, die zu einem Großteil IL-4-, IL-13- und IL-4R-abhängig sind. In Bezug auf den IL-4R war unklar, welche IL-4R-exprimierende Zelle entscheidend für die Ausprägung einer Immunpathologie ist. Hierzu wurden in einem weiteren Versuchsansatz Tiere verwendet, die mittels der Cre/lox-Technik generiert wurden und spezifisch IL-4R-defizient auf Th-Zellen sind. Die Untersuchung der Tiere ergab, dass diese Zellen essentiell für die Ausbildung einer Pathologie während der pulmonalen Kryptokokkose sind, denn Th-zellspezifische IL-4R-defiziente Tiere zeigen geringere Organlasten einhergehend mit erhöhtem Überleben als ihre nichttransgenen Wurfgeschwister. Die Restimulation der Lungenleukozyten mit spezifischem Antigen zeigte, dass in den resistenten Tieren, die keinen IL-4Rα auf Th-Zellen besitzen, im Gegensatz zu den nichttransgenen Wurfgeschwistern die Th2-Zytokine IL-4, IL-5 und IL-13 vermindert produziert werden. Um dieses Phänomen aufzuklären, wurden intrazelluläre durchflusszytometrische Analysen bzgl. der Botenstoffmuster auf Einzelzellebene durchgeführt. Hierbei fiel besonders auf, dass Mehrfach-Produzenten (sogenannte polyfunktionale Th-Zellen) der drei untersuchten Th2-Zytokine in den Th-zellspezifischen IL-4R-defizienten Mäusen reduziert sind. Im Gegensatz zu den nichttransgenen Wurfgeschwistern weisen die zellspezifischen IL-4R-defizienten Tiere in der Lunge nahezu keine Dreifachproduzenten für Th2-Zytokine auf. Während die Bedeutung von polyfunktionalen Th1-Zellen für die Resistenzentwicklung bereits in Infektions- und Immunisierungsstudien untersucht wurde, beschreibt die vorliegende Studie zum ersten Mal die Relevanz der Polyfunktionalität von Th2-Zellen für die Suszeptibilität in einer Infektion. Diese Befunde machen deutlich, dass für Th2-assoziierte Immunpathologien die Qualität (d.h. das Th2-Zytokinprofil) der Th2-Zellen ausschlaggebend für die Krankheitsentwicklung ist. Th2-Mehrfachproduzenten sind stark an diversen Pathogenese-Mechanismen, wie Eosinophilen-Rekrutierung, Becherzellhyperplasie und alternativer Aktivierung von Makrophagen, beteiligt. Mit Hilfe dieser Untersuchungen in zellspezifischen IL-4R-defizienten Tieren konnte zudem die zentrale Achse der Immunpathologie ermittelt werden: IL-4R-exprimierende Th-Zellen (Th2) sind hierbei die bedeutendsten regulatorischen Zellen, die IL-4R-exprimierende Makrophagen alternativ aktivieren. Alternativ aktivierte Makrophagen sind in diesem Kontext schädliche Effektorzellen, die zur Suszeptibilität des betroffenen Individuums führen (Daten hier nicht gezeigt, Manuskript in Vorbereitung). Tiere, die IL-4R-defizient für Th-Zellen oder Makrophagen (Daten hier nicht gezeigt) sind, erwiesen sich als hoch resistent gegenüber einer pulmonalen Kryptokokkose. Diese beiden Zelltypen agieren somit als Schlüssel-Zellen in der Pathogenese.

Zusammenfassend lässt sich sagen, dass die hier präsentierten Studien eine Reihe von molekularen und zellulären Zielen für neuartige Therapien gegen die Kryptokokkose und möglicherweise auch gegen

Asthma identifiziert haben. Ein gezielter Ansatz gegen IL-4/IL-13 oder gegen ihren gemeinsamen Rezeptor IL-4R auf Th-Zellen oder Makrophagen sollte für zukünftige Therapien in Th2-abhängigen Immunpathologien, wie z.B. Allergien, in Betracht gezogen werden.

6 Summary

Interleukin (IL)-4, IL-13, and the IL-4 receptor – molecular pacemakers of immunopathology in cryptococcosis

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Cryptococcus neoformans is a pathogenic fungus which can affect humans and other mammalian hosts, especially if they are immunocompromised. But even in immunocompetent hosts the fungus can induce immunopathological mechanisms. These mechanisms help the cryptococci to persist for years in an immunocompetent host. In the study presented here the immunopathological mechanisms of pulmonary and cerebral cryptococcosis were studied in murine transgenic models. The results achieved can help to develop novel molecular therapies to control the fungal infection and potentially also related immunopathologies such as asthma and allergy.

A T helper (Th) 1 cell-induced immune response accompanied by cytotoxic T cells and classically activated macrophages is protective against pulmonary cryptococcosis. In contrast, a Th2 cell-induced immune response leads to pathology with severe consequences for the infected host. Susceptibility in pulmonary cryptococcosis shares features with allergic asthma (e.g. goblet cell mucus production, bronchial hyperreactivity). By using mice deficient for the Th2 cytokines interleukin (IL)-4 or IL-13, it could be shown that IL-4 and also IL-13 participate in immunopathology in pulmonary cryptococcosis. Mice deficient for IL-4 or IL-13 demonstrate a significantly elevated survival rate in pulmonary cryptococcosis concomitant with reduced lung burden and goblet cell hyperplasia with diminished mucus production. The enhanced resistance of IL-4/IL-13-deficient mice in comparison to susceptible wild-type animals is an indication for the role of these cytokines in the induction of pathology. The hyperreactivity of the bronchi is elevated in *C. neoformans*-infected wild-type mice. This clearly shows that IL-4/IL-13-dependent pathological mechanisms are induced and aggravate the control of the pathogen. The role of IL-13 in pulmonary cryptococcosis could be further shown by using IL-13-overexpressing mice. These mice are highly susceptible and the signs of pathological alterations are even stronger than in wild type animals.

For pathological Th2 development during pulmonary cryptococcosis the production of IL-4 was found to be essential. Therefore, in this study IL-4-producing cell types were analyzed by flow cytometry using IL-4 reporter mice. The earliest IL-4 was detected surprisingly late (i.e. on day 42 post infection). IL-4 expression is tightly associated with elevated levels of IgE, alternative activation of macrophages and

the dissemination of the fungal pathogen to the brain. Besides Th2 cells which were shown to be the main IL-4 producer in the lung, only one more cell type was found to produce IL-4, the eosinophilic granulocyte. Using eosinophil-deficient mice the pathological mechanisms in which eosinophils are involved could be elucidated. It was shown that eosinophils substantially support the recruitment of Th cells to the lung during infection. Eosinophils strongly boost the Th2 effect (e.g. IgE production).

Signal transduction of IL-4 and IL-13 is mediated by IL-4 receptor (IL-4R) types I and II that share the same ligand-binding alpha chain. By depletion of the IL-4Rα chain in mice animals were generated unable to respond to IL-4 and IL-13. These IL-4Rα-deficient mice were found to be highly resistant against pulmonary cryptococcosis. IL-4Rα-deficient animals are able to induce a Th1 response enabling them to classically activate macrophages to kill the pathogen. Based on the outstanding resistance of IL-4Rα-deficient mice it was examined if also IL-4Rα heterozygous mice show signs of resistance in comparison to susceptible wild-type animals. These experiments revealed for the first time a gene dosage effect for IL-4R expression resulting in a gradual phenotypic alteration (i.e. elevated resistance of IL-4Rα$^{+/-}$ mice as compared with IL-4Rα$^{+/+}$ mice upon infection with *C. neoformans*). In detail, reduced IL-4R expression of IL-4Rα$^{+/-}$ mice was associated with enhanced survival, reduced fungal burden, significantly lower IgE production, and reduction of alternatively activated macrophages.

As was shown in subsequent studies of the role of IL-4R in pulmonary cryptococcosis, IL-4/IL-13-dependent alternatively activated macrophages take up cryptococci, but are unable to kill them. Furthermore, our studies suggest that alternatively activated macrophages play a critical role in dissemination of the pathogen to the brain inducing cerebral cryptococcosis. In the brain fungal foci surrounded by alternatively activated macrophages develop and are able to induce local inflammation. In contrast, resident microglial cells will not be activated by *C. neoformans* and do not take up cryptococci. These findings point to uptake of cryptococci by macrophages in the periphery (i.e. lung) and subsequent migration (“shuttle”) to the brain. Alternatively activated macrophages are indicative of susceptibility, since in resistant mice (e.g. IL-4- or IL-4R-deficient animals) numbers of leukocytes are in the brain during infection are not elevated in comparison to naïve mice. The number of cryptococci in the central nervous system of IL-13-, IL-4-, and IL-4R-deficient mice is significantly reduced in comparison to susceptible wild-type control animals. The reduced brain burden is associated with an elevated survival rate and reduced inflammation of the meninges.

On the basis of the forementioned studies, immunopathology during pulmonary and cerebral cryptococcosis was found to be linked to expression of IL-4, IL-13, and the IL-4R. For the IL-4R it remained open what the critical IL-4R-expressing cell type is leading to immunopathology. Therefore, in another study mice generated by the Cre/lox technique were used that are specifically deficient for IL-4R expression on Th cells. The results show that IL-4R-expressing Th cells are essential for the induction of pathology during pulmonary cryptococcosis. In the absence of IL-4R on Th cells mice display reduced

organ burdens with a higher survival rate in comparison to non-deficient littermates. Restimulation of lung leukocytes with cryptococcal antigen demonstrated that resistant animals lacking IL-4Rα on Th cells, in contrast to susceptible non-deficient littermates, produce less Th2 cytokines, i.e. IL-4, IL-5, and IL-13. To elucidate this phenomenon on a single-cell level, intracellular multicolor flow cytometry of the cytokine pattern was performed. Interestingly, Th2 cytokine multiproducers expressing IL-4, IL-5, and IL-13 simultaneously, so-called polyfunctional Th2 cells, could be detected in the presence of IL-4R on Th cells. In contrast, resistant Th cell-specific IL-4Rα-deficient mice show almost no triple Th2 cytokine producers in the lung. While previously the role of polyfunctional Th1 cells for resistance has been described in infection and vaccination studies, this study describes for the first time the relevance of polyfunctional Th2 cells for susceptibility during infection. Therefore, it is evident that for Th2-associated immunopathologies the quality (i.e. Th2 cytokine pattern) of Th2 cells is decisive for disease development. Th2 polyproducers are strongly involved in several immunopathological mechanisms during cryptococcosis (e.g. eosinophil recruitment, goblet cell hyperplasia, and alternative activation of macrophages). Using cell-specific IL-4R-deficient mice a central axis of immunopathology was revealed for pulmonary cryptococcosis: IL-4R-expressing Th2 cells are key regulatory cells inducing susceptibility by driving the development of IL-4R-expressing alternatively activated macrophages as fatal effector cells (data not shown, manuscript in preparation). Mice deficient for IL-4R expression on either Th cells or macrophages (data not shown) are highly resistant against pulmonary cryptococcosis. Both cell types have a key role in immunopathology during cryptococcosis.

In conclusion, these studies have identified several molecular and cellular targets for novel therapies against cryptococcosis and possibly also against asthma. Targeting IL-4/IL-13 or their common receptor IL-4R on Th cells or macrophages should be envisaged for future ways of intervention in Th2-dependent immunopathology such as allergy.

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