Neue regulatorische T-Zell Subtypen und ihre Funktion in der experimentellen Glomerulonephritis

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1. Einleitung

1.1. Glomerulonephritis

Glomerulonephritiden (GN) sind eine heterogene Gruppe immunvermittelter entzündlicher Nierenerkrankungen, bei denen die Nierenkörperchen (Glomeruli) zuerst und vorrangig betroffen sind. Die Glomeruli sind ein Teil des Nephrons und in einer gesunden Niere zuständig für die Filtration des Urins. Die Glomerulonephritis ist in Europa und den USA eine der häufigsten Ursachen für eine terminale Niereninsuffizienz, die mit einer hohen Mortalität und Morbidität einhergeht. Bei 10-15 % der Patienten mit terminaler Niereninsuffizienz liegt als Grunderkrankung eine Glomerulonephritis vor [1, 2].

Viele verschiedene Formen der Glomerulonephritis sind bekannt. Die meisten sind durch eine Hämaturie und/oder Proteinurie gekennzeichnet. Unter den verschiedenen Formen der Glomerulonephritiden hat die Gruppe der rapid-progressiven Glomerulonephritiden (RPGN oder halbmondförmige GN) die schlechteste Prognose und geht mit einem raschen Verlust der Nierenfunktion einher. Unbehandelt kann es innerhalb von Wochen bis Monaten zum terminalen Nierenversagen kommen. Typisch für die RPGN ist die ausgeprägte histologische Halbmondbildung in den Glomeruli. Sie kann in drei unterschiedliche pathophysiologische Subtypen klassifiziert werden: Typ I RPGN, auch als Antibasalmembran-RPGN bekannt, wird durch Antikörper gegen die glomeruläre Basalmembran (GBM) verursacht. Die renale Ablagerung von Immunkomplexen ist typisch für die Typ II RPGN. Zu dieser kommt es zum Beispiel im Rahmen systemischer Immunerkrankungen wie dem Lupus erythematodes (SLE). Hier bestimmt sie ganz wesentlich die Prognose. Die häufigste Form der RPGN ist die Typ III RPGN, welche bei den sogenannten ANCA-assoziierten Vaskulitiden auftritt. Sie wird auch als pauci-immune RPGN bezeichnet, da sie weder durch Immunkomplexablagerung noch durch anti-GBM Antikörper gekennzeichnet ist. Vermutlich wird sie durch die Aktivierung von Neutrophilen als Reaktion auf anti-neutrophile zytoplasmatische Antikörper (ANCA) verursacht [1].

Obwohl die verschiedenen Formen der RPGN durch unterschiedliche Stimuli induziert werden, sind sie alle durch infiltrierende Immunzellen gekennzeichnet, die

die Glomeruli schädigen und zur Entstehung sogenannter glomerulärer Halbmonde führen.

Die pathophysiologischen Grundlagen der Glomerulonephritiden sind bisher weitgehend unbekannt. Daher sind die therapeutischen Optionen leider unspezifisch, nebenwirkungsreich und häufig ineffektiv. Die weitere Aufklärung der Pathogenese von Glomerulonephritiden ist daher ein wichtiges Ziel nephrologischer Forschung, um entzündliche Nierenerkrankungen zukünftig rascher identifizieren und erfolgreicher therapieren zu können.

1.2. Immunzellen in der Glomerulonephritis

Die Infiltration von Entzündungszellen in das Nierengewebe ist ein charakteristisches Merkmal von Glomerulonephritiden [3]. Insbesondere infiltrierende T-Zellen haben eine zentrale pathophysiologische Bedeutung bei der Initiation und Progression dieser Erkrankungsgruppe. Wegweisende Studien von Mosmann und Coffman haben die Konzepte der T-Zellbiologie durch die Identifizierung funktionell verschiedener Subklassen von T-Helferzellen (Th) bereits vor mehr als 20 Jahren revolutioniert [4, 5]. Seitdem ist die Th-Zellfamilie stetig gewachsen und umfasst aktuell neben den damals entdeckten Th1 und Th2 Zellen auch T-follikuläre Helferzellen (Tfh) und Th17-Zellen [6, 7]. Grundsätzlich sind T-Helferzellen für den Schutz vor Pathogenen, insbesondere Bakterien und Pilzen, unerlässlich. Dennoch ist ihre Überaktivierung mit der Entwicklung von Krankheiten assoziiert. So sind die Th2-Zellen an der Vermittlung von Allergien beteiligt und Th1- und Th17-Zellen wesentliche Mediatoren von autoimmunen Prozessen [8, 9]. Die Differenzierung in unterschiedliche T-Helferzellsubtypen wird durch spezifische Transkriptionsfaktoren reguliert (Abbildung 1). So induzieren beispielsweise T-bet, Stat1 und Stat4 einen Th1-Phänotyp [10, 11], Gata3 hingegen Th2-polarisierte Zellen [12, 13]. Die durch ihre Interleukin (IL-)17 Sekretion charakterisierte Th17-Zelle wird durch die Transkriptionsfaktoren RORγt und Stat3 definiert [14-16]. Eine zentrale Bedeutung von T-Zellen und insbesondere Th1- und Th17-Zellen für die Entstehung und das Fortschreiten von immunvermittelten Nierenerkrankungen konnte in zahlreichen Studien unserer Abteilung etabliert werden [17-22].

Dieses breite Spektrum an Effektor-T-Zellen (Teff) erfordert eine engmaschige immunologische Kontrolle, vor allem unter dem Risiko sich entwickelnder Autoimmunität. Diese wichtige Funktion wird einer weiteren T-Zellsubklasse zugeschrieben, den regulatorischen T-Zellen (Treg) [23]. Die antiinflammatorischen Eigenschaften von Tregs werden im Wesentlichen durch ihren spezifischen Transkriptionsfaktor Foxp3 programmiert [24]. Sie sekretieren eine Vielzahl an antiinflammatorischen Zytokinen, wie IL-10 und IL-35, welche der Autoimmunität entgegen wirken. Bis vor kurzem hatte man diese Foxp3$^+$ Tregs für eine homogene Zellpopulation gehalten. Es gab in jüngster Zeit jedoch einige Hinweise darauf, dass es wohl in Analogie zu den Th-Effektorzelllinien auch verschieden polarisierte Treg-Subtypen mit ganz unterschiedlicher Spezialisierung gibt. Auch diese Treg-Subtypen scheinen durch spezifische Transkriptionsfaktoren programmiert zu werden [25, 26].

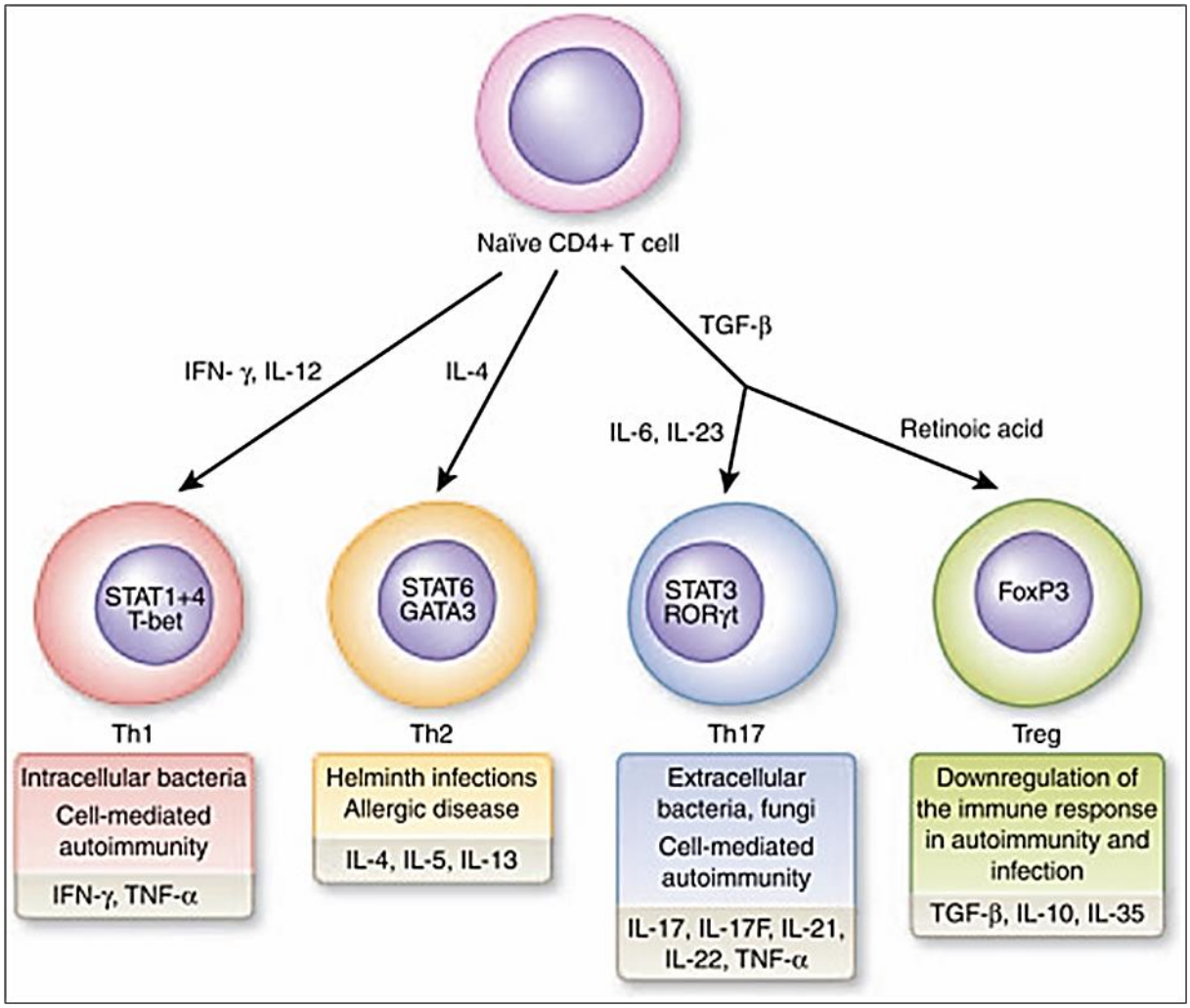

Abbildung 1: Aus naiven CD4$^+$ T-Helferzellen entwickeln sich hochspezialisierte T-Helferzellsubklassen, die sich durch ihre spezifischen Transkriptionsfaktoren sowie ihr charakteristisches Zytokinprofil definieren und voneinander unterscheiden (aus [22]).

1.3. Th1-Zellen

Die Heterogenität der T-Helferzellen wurde erstmals von Mosmann und Coffman im Jahr 1986 beschrieben [4, 5]. Sie unterschieden damals anhand der Zytokinproduktion zwei Subtypen – die Th1- und die Th2-Zellen. Th1-Zellen produzieren Interferon (IFN)-γ, IL-2, Tumornekrosefaktor und Lymphotoxin-α. Sie fördern die Aktivierung von Makrophagen und unterstützen B-Zellen bei der Antikörperproduktion um die Opsonisierung und Phagozytose zu vermitteln. Th1-Zellen sind für die Eliminierung intrazellulärer Pathogene, wie Viren, Listerien und Tuberkelbakterien wichtig [27].

Für die Induktion der Th1-Zellen sind IL-12 und ihr Haupteffektorzytokin IFNγ entscheidend. IFNγ erhöht dabei die Produktion von IL-12 aus Dendritischen Zellen und Makrophagen und in einem positiven Feedback Loop stimuliert IL-12 die IFNγ-Produktion in Th1-Zellen und fördert so das Th1-Profil [28]. IFNγ unterdrückt außerdem die Produktion anderer Zytokine, wie zum Beispiel des Th2-Zytokins IL-4. Es fördert also neben der Induktion zusätzlich auch den Erhalt der Th1-Antwort [29]. Dies ist in Abbildung 2 noch einmal schematisch dargestellt. IL-12 und IFNγ induzieren die Expression der wichtigsten Th1-Transkriptionsfaktoren signal transducer and activator of transcription 4 (STAT4) und T-box transcription factor TBX21 (T-bet), welche für die Entstehung des Th1-charakteristischen Phänotyps entscheidend sind [30].

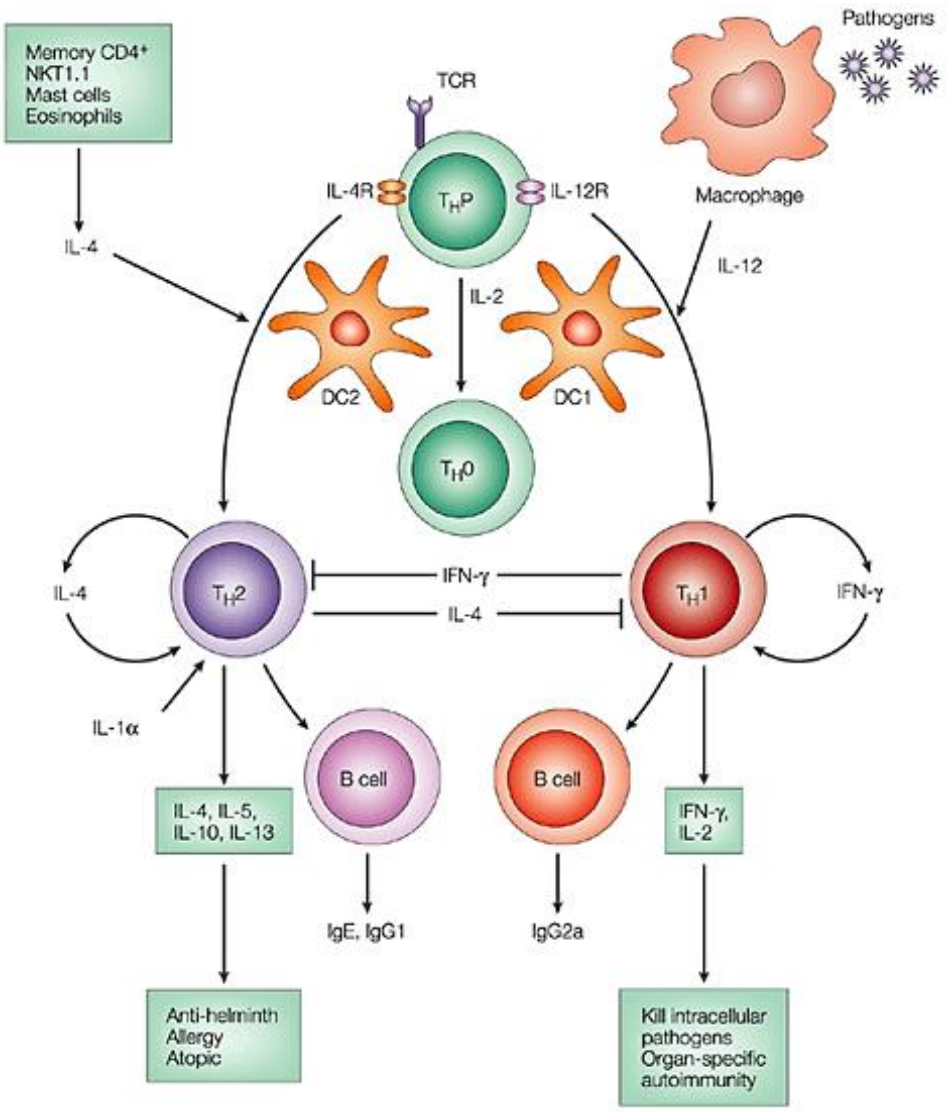

Abbildung 2: Induktion von Th1/Th2-Zelldifferenzierung: Aus naiven $CD4^+$ T-Helferzellen entwickeln sich in Abhängigkeit des Cytokinmilieus hochspezialisierte T-Helferzellsubklassen, hier dargestellt Th1- und Th2-Zellen. IL-12 fördert die Th1-Zellen, IL-4 hingegen die Differenzierung von Th2-Zellen. IFNγ und IL-4, die von Th1- bzw. Th2-Zellen gebildet werden, wirken auch als autokrine Wachstumsfaktoren und hemmen zusätzlich den jeweils anderen Th-Subtyp. Th1-Zellen verstärken die Synthese von IgG2a aus B-Zellen durch IFNγ, Th2-Zellen hingegen fördern die Bildung von IgE und IgG1 durch IL-4. Th1-Zellen vermitteln die Zerstörung von intrazellulären Pathogenen aber auch organspezifische Immunität. Th2-Zellen wirken gegen Parasiten und verstärken allergische Reaktionen (aus [31]).

1.4. Th17-Zellen

Im Jahr 2007 wurde ein weiterer T-Helferzellsubtyp entdeckt, der sich von den bis dahin bekannten Th1- und Th2-Zellen unterscheidet und eine wichtige Rolle in Autoimmunerkrankungen zu spielen scheint [32-34]. Diese Zellen rekrutieren neutrophile Granulozyten und produzieren hauptsächlich Interleukin (IL-)17A und

IL-17F. Daher wurden sie als Th17-Zellen benannt. Zudem produzieren sie aber auch IL-22, TNF-α und GM-CSF [35, 36].

Für die Differenzierung und Proliferation der Th17-Zellen sind verschiedene Schlüsselmoleküle verantwortlich. Es konnte gezeigt werden, dass IL-6, IL-1ß und Transforming growth factor beta (TGFβ) die Differenzierung von Th17-Zellen aus naiven T-Zellen induzieren [37-40]. Sie lösen hierfür die Expression der zentralen Transkriptionsfaktoren signal transducer and activator of transcription 3 (Stat3) und retinoic-acid-receptor-related orphan receptor gamma (RORγt) aus, welche für die Entstehung des Th17-charakteristischen Phänotyps entscheidend sind. Diese beiden Transkriptionsfaktoren vermitteln die Expression der Th17-spezifischen Genprodukte und Effektorzytokine [41-43]. Nach Aktivierung von Stat3 und RORγt beginnen diese intermediären Zellen dann IL-21 zu sekretieren, welches autokrin die Expansion der Th17-Zellen fördert. Schließlich ist IL-23 für die Stabilisierung, Aktivierung und abschließende Differenzierung, also die Induktion der charakteristischen Effektorzytokine wie IL-17A, IL-17F, IL-9, IL-21 und IL-22, und Traffickingrezeptoren der Th17-Zellen, nötig [44-46]. Die folgende Abbildung zeigt schematisch den Ablauf der Th17-Zelldifferenzierung.

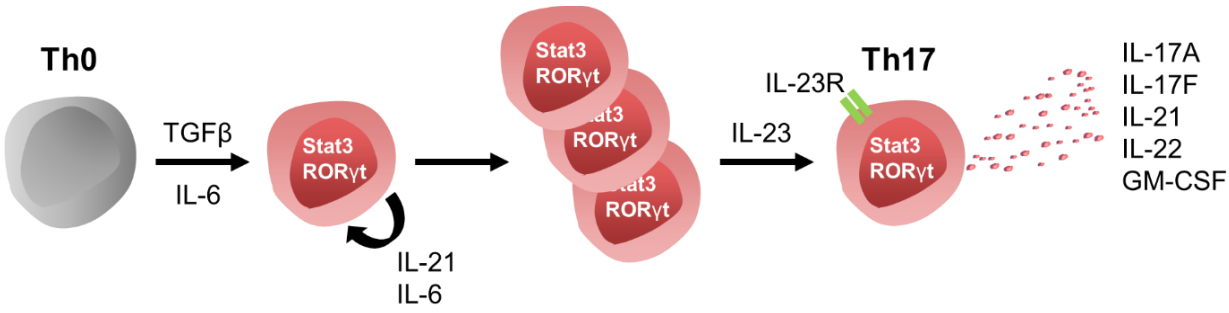

Abbildung 3: Th17-Zelldifferenzierung: IL-6 und TGFβ induzieren die Aktivierung der Transkriptionsfaktoren Stat3 und RORγt in naiven T-Helferzellen. Sekretion von IL-21 führt in einem autokrinen Feedback Loop zur Expansion. IL-23 stabilisiert und aktiviert die Th17-Zellen. Reife Th17-Zellen sekretieren IL-17A, IL-17F, IL-21, IL-22 und GM-CSF.

1.5. Regulatorische T-Zellen

Obwohl Effektor-T-Zellen für die Abwehr von Pathogenen essentiell sind, kann ihre fehlgerichtete Überaktivierung zur Entwicklung von Autoimmunität führen. Die

pathogene Rolle von Th1- und Th17-Antworten für den Verlauf immunvermittelter Nierenerkrankungen ist mittlerweile gut belegt. Die einzelnen T-Helferzellsubtypen sind dabei durch Zytokine und Wanderungsrezeptoren für ihre spezifische Aufgabe ausgestattet [20, 21, 47, 48]. Die Expression dieser charakteristischen Rezeptoren und Botenstoffe wird dabei durch linienspezifische Transkriptionsfaktoren kodiert [17]. Diese hochspezialisierten Effektorzellantworten bedürfen zum Schutz vor Dysfunktion und Autoimmunität einer engmaschigen und wirkungsvollen Kontrolle. Diese Funktion wird durch regulatorische T-Zellen vermittelt, deren Entwicklung bei Maus und Mensch wesentlich von ihrem Transkriptionsfaktor Foxp3 abhängt [23, 49, 50]. Bisher hatte man $Foxp3^+$ Tregs für eine singuläre Population gehalten. In Anbetracht der Vielfalt und molekularen Spezialisierung der proinflammatorischen T-Helferzellen erscheint es jedoch sehr unwahrscheinlich, dass eine einzige, pluripotente regulatorische T-Zelle effektiv das Immungleichgewicht aufrechterhalten kann.

In der Tat wurde in jüngster Zeit das Konzept der linienspezifischen Tregs vorgeschlagen, die hochspezialisiert ihrem korrespondierenden Effektorzellpartner gegenüberstehen [25]. Für die Nomenklatur dieser Zellen schlagen wir, in Anlehnung an ihre Th-Zellgegenspieler, die Bezeichnung Treg1-Zellen für die Tregs, die spezifisch Th1-Antworten kontrollieren, und Treg17-Zellen für die spezifisch Th17 regulierenden Tregs vor. Die folgende Abbildung veranschaulicht dieses hypothetische Konzept.

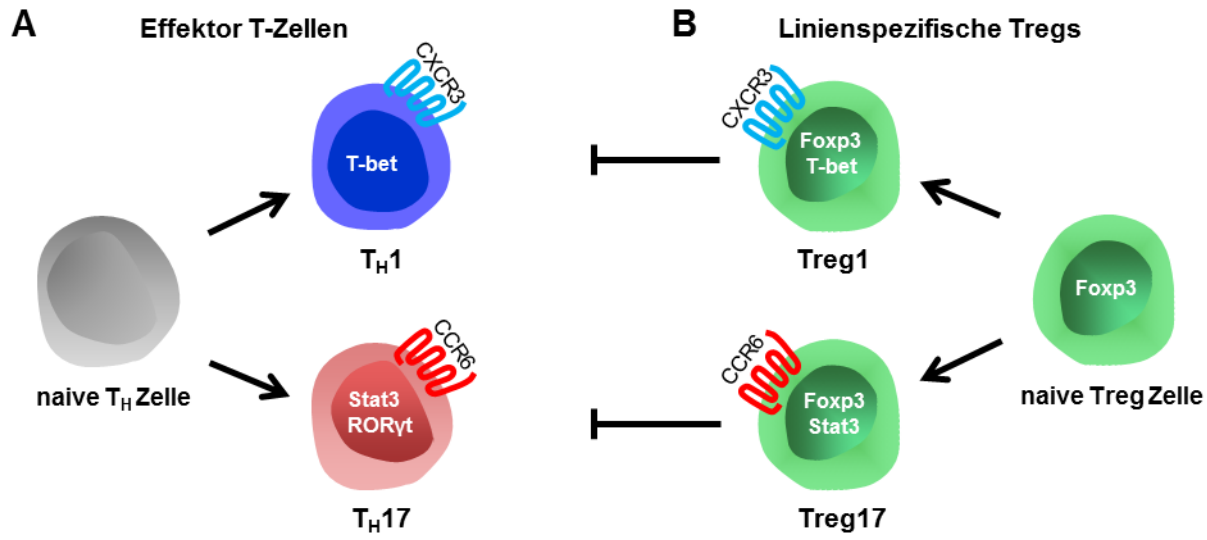

Abbildung 4: (A) Die Subklassenzugehörigkeit pathogener T-Effektorzellen wird durch spezifische Transkriptionsfaktoren definiert. Th1-Zellen werden durch T-bet, Th17-Zellen werden durch Stat3 und RORγt charakterisiert (adaptiert nach [51]). **(B)** Es kommt in Analogie zu den Effektor-T-Zellen zur linienspezifischen Differenzierung naiver regulatorischer T-Zellen. Th1-spezifische Treg1-Zellen sind von T-bet [52], Th17-spezifische Treg17-Zellen vom Transkriptionsfaktor Stat3 [53] abhängig.

1.5.1. Treg1-Zellen

Regulatorische T-Zellen, die spezifisch Th1-Antworten kontrollieren, wurden erstmals von Koch et al. beschrieben [52, 54]. Diese Treg1-Zellen hingen dabei nicht nur vom Treg-charakteristischen Transkriptionsfaktor Foxp3, sondern auch vom Transkriptionsfaktor T-bet ab, der eigentlich als wesentlicher Faktor der Entwicklung von Interferon-γ-produzierenden Th1-Zellen bekannt war. Es erscheint dabei durchaus logisch, dass die spezialisierte regulatorische T-Zelle zur effektiven und spezifischen Kontrolle der ihr gegenüberstehenden T-Effektorzelle eine gewisse Ähnlichkeit benötigt, um beispielsweise in dieselben entzündeten Gewebe rekrutiert oder durch ähnliche Stimuli aktiviert zu werden. Kolokalisation in entzündetem Gewebe könnte dabei durch gemeinsame Expression des Th1-charakteristischen Chemokinrezeptors CXCR3 erreicht werden [55]. Passend zu dieser Hypothese zeigten verschiedene Studien eine gleichzeitige Hochregulation von T-bet und CXCR3 auf Tregs während einer Typ 1 Entzündung durch eine Infektion, Malignom, Kontaktallergie, Autoimmunität oder Organtransplantation in Mäusen [56-59] und Menschen [60-63]. Die genaue funktionelle Rolle einer T-bet-Aktivierung in Tregs

bleibt jedoch weiterhin ungeklärt. Da es erst seit kurzem die Möglichkeit der Generierung einer Mauslinie gibt, der spezifisch in regulatorischen T-Zellen die Expression von T-bet fehlt (Foxp3^{Cre}xTbet$^{fl/fl}$), konnte dies in den bisherigen Studien nicht genauer untersucht werden. Während der Erstellung des zu dieser Dissertation gehörenden Manuskripts über Treg1-Zellen erschienen zwei Publikationen mit dieser Mauslinie. Yu et al. berichteten, dass der Treg-spezifische gemeinsame Verlust von T-bet und Gata3, einem weiteren Transkriptionsfaktor, zu spontaner Autoimmunität führt [64]. Die Rolle von T-bet^{+} Tregs für die Regulation von Th1-Antworten wurde jedoch nicht weiter untersucht. Das zweite Manuskript untersuchte das murine EAE-Modell der Multiplen Sklerose [65]. Erstaunlicherweise fanden die Autoren keine relevanten Effekte einer T-bet-Defizienz in Tregs für die Entstehung und Schwere der Entzündung des zentralen Nervensystems. Wie in der Studie von Yu et al. wurden aber auch hier die Effekte auf die Th1-Immunität nicht angesprochen. Es blieben also weiterhin viele Fragen T-bet^{+} Treg-Zellen betreffend offen, vor allem ihre funktionelle Rolle in der Th1-vermittelten Entzündung. Außerdem gab es diesbezüglich bisher für den gesamten Bereich der Nephrologie keinerlei Daten. Daher sollte eine der in dieser Dissertationsschrift angeführten Studien (Nosko et al.: T-Bet Enhances Regulatory T Cell Fitness and Directs Control of Th1 Responses in Crescentic GN [66]) die folgenden Fragen beleuchten:

1. Identifizierung und Charakterisierung von T-bet^{+} Treg-Zellen in der experimentellen akuten nekrotisierenden GN
2. Aufklärung der Rolle von T-bet für die Treg1 Generierung
3. Die Rolle der Treg1-Zellen für die Regulation der Th1-Zellen
4. Untersuchung der Rolle der Treg1-Zellen in der experimentellen akuten nekrotisierenden GN
5. Analyse des Wirkungsmechanismus der Treg1-Zellen

1.5.2. Treg17-Zellen

Wie vorstehend für die Th1-Antwort beschrieben, gab es Hinweise, dass ebenfalls eine Treg-Subpopulation existiert, welche spezifisch Th17-Zellen kontrolliert. Passend dazu konnte in einer Studie gezeigt werden, dass die Th17-Antwort durch spezialisierte Treg17-Zellen begrenzt wird. Diese Zellen waren dabei auf den Transkriptionsfaktor Stat3 angewiesen, der auch für die Programmierung von Th17-Zellen notwendig ist [15, 67]. Mäuse, denen Stat3 ausschließlich in $Foxp3^{+}$ Tregs fehlte, entwickelten eine spontane Kolitis aufgrund überschießender Th17-Antworten [53].

Wie auch bei den Treg1-Zellen, waren neben diesen „Proof-Of-Concept“ Untersuchungen die Funktion und Bedeutung von linienspezifischen Tregs weitgehend unklar. Dies galt insbesondere für ihre Untersuchung in Erkrankungsmodellen sowie ihr Vorkommen und ihre molekulare Regulation beim Menschen. Auch hier gab es für den Bereich der Nephrologie bisher keinerlei Daten. Unsere Arbeitsgruppe konnte kürzlich Stat3-abhängige Treg17-Zellen als wichtige Mediatoren des renalen Gewebeschutzes in einem Modell der akut nekrotisierenden Glomerulonephritis identifizieren [68]. Im Sinne der Hypothese fand sich der Verlauf der Erkrankung in Treg17-defizienten Tieren deutlich verschlechtert. Den regulatorischen T-Zellen der Treg17-defizienten $Foxp3^{Cre}xStat3^{fl/fl}$ Mäuse fehlte selektiv der Chemokinrezeptor CCR6. CCR6 ist auf den Th17-Effektorzellen als Wanderungsrezeptor zur Rekrutierung in entzündete Nierengewebe gut bekannt [51]. CCR6 ist nach unseren Erkenntnissen also nicht nur für die Th17-Infiltration, sondern auch für die Wanderung regulatorischer Treg17-Zellen in die entzündeten Nieren essentiell [68]. Dieser Mechanismus führt zur gezielten Kolokalisation der polarisierten Th17-Effektorzelle mit ihrem spezialisierten Gegenspieler.

Während diese Studie eine wichtige Rolle der Treg17-Zellen in der akuten GN zeigte, fehlen Daten zu chronischen Erkrankungen. Diese sind von besonderer klinischer Relevanz, da viele Patienten, die unter chronischer progressiver renaler Entzündung leiden, eine lebenslange Behandlung benötigen. Ebenfalls ist nichts über den Einfluss von Treg17-Zellen auf die Entstehung der Autoimmunität und den klinischen Verlauf beim systemischen Lupus erythematodes (SLE) bekannt. Daher wurde in der zweiten Publikation dieser Dissertationsarbeit (Kluger et al.: Treg17 cells are programmed by Stat3 to suppress Th17 responses in systemic lupus [69]) die Rolle

von Treg17-Zellen auch beim systemischen Lupus untersucht. Eine einmalige peritoneale Injektion des natürlich vorkommenden Öls Pristan (2,6,10,14-Tetramethylpentadecane) führt bei Mäusen zu einer chronischen Entzündung mit Entwicklung einer dem SLE sehr ähnlichen Autoimmunität. Diese beinhaltet insbesondere die Entstehung von SLE-typischen Antikörpern, sowie einer Immunkomplexnephritis und pulmonalen Vaskulitis, mit einer großen Ähnlichkeit zur humanen Erkrankung [70, 71]. Drei unabhängige Studien konnten erst kürzlich die Rolle der IL-17/Th17-Achse für diese Modellerkrankung beschreiben [72-74]. Die Bedeutung von Treg17-Zellen für die komplexe Pathogenese des SLE war bisher jedoch völlig unklar. Dies sollte in dieser Studie untersucht werden, mit besonderem Fokus auf die Entstehung von Autoimmunität und Endorganschaden.

1.6. RORγt^{+}Foxp3^{+} biTregs

Für den Th17-typischen Transkriptionsfaktor Stat3 konnte wie oben beschrieben eine essentielle Rolle während der Ausbildung eines Treg17-Phänotyps bei regulatorischen T-Zellen etabliert werden. Th17-Zellen werden jedoch nicht nur durch Stat3, sondern auch durch den Transkriptionsfaktor RORγt definiert. Es ergibt sich also die drängende Frage nach dem Vorkommen und der Funktion von RORγt in Foxp3^{+} Tregs. Eine Pionierarbeit auf diesem Feld wurde von der Arbeitsgruppe um Gérard Eberl vorgestellt. Die Autoren konnten zeigen, dass nicht nur Stat3, sondern auch der andere Th17-Haupttranskriptionsfaktor RORγt von einer Subpopulation Foxp3-positiver Zellen exprimiert wird [75].

Eberl und Kollegen beschrieben, dass RORγt^{+}Foxp3^{+} Zellen (im Folgenden biTregs genannt) *in vitro* potente suppressive Eigenschaften besitzen und große Mengen Interleukin 10 produzieren. In einer nachfolgenden Publikation fand eine unabhängige Arbeitsgruppe jedoch, dass biTregs unter entzündlichen Konditionen mit Simulation einer Pilzinfektion auch proinflammatorisches Interleukin 17 exprimieren [76]. Dieser Befund lässt also sowohl pro- als auch antiinflammatorische Eigenschaften von biTregs vermuten, wobei ihre physiologische Rolle weitgehend unklar blieb. RORγt^{+}Foxp3^{+} biTregs kommen auch regelhaft im Blut gesunder Menschen vor [77, 78]. Einige Studien verschiedener Arbeitsgruppen konnten zudem unabhängig voneinander IL-17-produzierende Tregs bei Patienten mit

unterschiedlichen entzündlichen Erkrankungen wie Colitis ulcerosa [79], Kolonkarzinomen [80], Psoriasis [81], Peridontitis [82] und Arthritis [83] nachweisen. Obwohl alle diese Studien eine Assoziation von biTregs mit Entzündung und Tumoren beschreiben, blieb ihre tatsächliche Funktion weitgehend unklar.

Eine erst kürzlich veröffentlichte Studie von Ohnmacht et al., welche RORγt^{+}Foxp3^{+} biTregs im Darm charakterisierte, unterstrich die biologische Relevanz von biTregs entscheidend . Die Gruppe konnte zeigen, dass die Expression von RORγt in biTregs vermutlich durch das Mikrobiom im Darm induziert wird. Überraschenderweise fanden sie eine übersteigerte intestinale Th2-Antwort in Mäusen mit selektiver RORγt Defizienz in biTregs [84]. Dieser Befund deutet daraufhin, dass biTregs in Abhängigkeit von RORγt Th2-Antworten unterdrücken können. Die zu Grunde liegenden Mechanismen sind jedoch derzeit vollkommen unklar.

Bisher war außerdem völlig unbekannt, ob biTregs eine stabile und eigenständige Zelllinie darstellen oder ob sie nicht vielmehr Treg/Th17-Intermediäre repräsentieren, die während Transdifferenzierungsprozessen entstehen (von der regulatorischen zur Th17-Zelle oder in der entgegengesetzten Richtung) [41, 85, 86]. Bei so vielen offenen Fragen ist es daher auch nicht verwunderlich, dass es keinerlei Erkenntnisse über die Rolle von RORγt^{+}Foxp3^{+} biTregs bei Nierenerkrankungen gab. Die Kontrolle von Glomerulonephritiden hängt jedoch ganz wesentlich von Foxp3^{+} regulatorischen T-Zellen ab, wobei ihre Depletion zu einer gravierenden Verschlechterung, ihre Verabreichung zu einem deutlich milderen Verlauf einer experimentellen Glomerulonephritis führt [87, 88]. Auf der anderen Seite sind RORγt und sein Effektorzytokin IL-17 ganz wesentliche proinflammatorische Mediatoren der Glomerulonephritis [17], so dass insgesamt eine Bedeutung von RORγt^{+}Foxp3^{+} biTregs für entzündliche Nierenerkrankungen hochwahrscheinlich ist. Dieser Aspekt bekommt weitere klinische Relevanz durch zahlreiche derzeit in Entwicklung befindliche RORγt-blockierende Medikamente, die perspektivisch schon bald zur Therapie entzündlicher Autoimmunerkrankungen bei Patienten eingesetzt werden sollen [89-92].

Aus diesem Grund wurden in der dritten in dieser Dissertationsschrift vorgestellten Studie RORγt^{+}Foxp3^{+} biTregs im Modell der akuten GN [93] mit der folgenden Zielsetzung untersucht:

1. Charakterisierung des Vorkommens und der Dynamik von RORγt^{+}Foxp3^{+} biTregs im Verlauf der akuten nekrotisierenden Glomerulonephritis

2. Klärung, ob RORγt^{+}Foxp3^{+} biTregs eine eigenständige Zellpopulation, Treg17-Zellen oder Transdifferenzierungsstadien zwischen Tregs und Th17-Zellen darstellen

3. Untersuchung der funktionellen Bedeutung von RORγt^{+}Foxp3^{+} biTregs bei der akuten nekrotisierenden Glomerulonephritis

4. Klärung der spezifischen Rolle von RORγt für die Funktion von biTregs

In der letzten zu dieser Dissertationsschrift gehörenden Studie (Kluger & Nosko *et al*.: RORγt expression in Tregs promotes systemic lupus erythematosus via IL-17 secretion, alteration of Treg phenotype and suppression of Th2 responses. [94]) wurden diese RORγt^{+}Foxp3^{+} biTregs, wie oben für die Treg17-Zellen beschrieben, ebenfalls im Modell der Pristan-induzierten murinen Lupuserkrankung untersucht. Vor allem die folgenden Aspekte sollten dabei beleuchtet werden:

1. Charakterisierung der Dynamik von biTregs in den verschiedenen, im Verlauf des SLE betroffenen Organen

2. Analyse der Rolle von RORγt in biTregs mit besonderem Fokus auf die IL-17-Sekretion und Regulation der Th2-Antwort

3. Untersuchung des Beitrags der biTregs zur Organpathologie im Pristan-induzierten SLE

2. Material & Methoden und Ergebnisse

Die folgenden vier Publikationen enthalten die verwendeten Materialien und Methoden, sowie die Ergebnisse zu den oben genannten Fragestellungen, die die Grundlage dieser kumulativen Dissertationsschrift bilden. Im Anhang sind die vollständigen Originalarbeiten enthalten.

“T-bet enhances regulatory T cell fitness and directs control of Th1 responses in crescentic glomerulonephritis.”

Nosko A, Kluger MA, Diefenhardt P, Melderis S, Wegscheid C, Tiegs G, Stahl RAK, Panzer U, Steinmetz OM

J Am Soc Nephrol, 2016. 28(1)

“Treg17 Cells are Programmed by Stat3 to Suppress Th17 Responses in Systemic Lupus.”

Kluger MA, Melderis S, Nosko A, Goerke B, Luig M, Meyer M, Turner JE, Meyer-Schwesinger C, Wegscheid C, Tiegs G, Stahl RAK, Panzer U, Steinmetz OM

Kidney International, 2016. 89(1)

“RORgammat+Foxp3+ Cells are an Independent Bifunctional Regulatory T Cell Lineage and Mediate Crescentic GN.”

Kluger MA, Meyer MC, Nosko A, Goerke B, Luig M, Wegscheid C, Tiegs G, Stahl RAK, Panzer U, Steinmetz OM

J Am Soc Nephrol, 2015. 27(2)

“RORγt expression in Tregs promotes systemic lupus erythematosus via IL-17 secretion, alteration of Treg phenotype and suppression of Th2 responses.”

Kluger MA[1], Nosko A[1], Ramcke T, Goerke B, Meyer MC, Wegscheid C, Luig M, Tiegs G, Stahl RAK, Steinmetz OM

Clin Exp Immunol. 2016. Epub ahead of print, doi: 10.1111/cei.12905.

[1] diese Autoren trugen gleichermaßen zur Publikation bei

3. Diskussion

Die Ursachen und die Pathogenese der meisten Formen der GN sind noch sehr wenig aufgeklärt [95, 96]. Daher bleibt die GN eine der Hauptursachen für terminales Nierenversagen. Da aktuelle Therapien unspezifisch immunsuppressiv sind, zeigen sie ein hohes Maß an Nebenwirkungen. Die Therapie von Patienten mit GN ist somit eine Herausforderung und verläuft häufig frustrierend. Die weitere Aufklärung der Rolle und Funktion der verschiedenen an der Entstehung und dem Verlauf der GN beteiligten Zelltypen ist deshalb von großer Bedeutung um gezieltere Therapien zu entwickeln.

Viele Studien der letzten Jahre haben gezeigt, dass Th1- und Th17-Zellen eine wichtige pathogene Rolle spielen bei der GN [17-22]. Die Untersuchung der Mechanismen, die diese Zellen regulieren ist daher von großer klinischer Relevanz. Für die Kontrolle von Effektor-T-Zellen sind vor allem regulatorische T-Zellen verantwortlich [23]. Diese hatte man bis vor kurzem für eine homogene Zellpopulation gehalten. Einige Arbeiten haben jedoch ein neues Konzept beschrieben, das Subtypen regulatorischer T-Zellen definiert, welche auf die Herunterregulation eines bestimmten T-Effektorzelltyps spezialisiert sind. Wie dies genau im Detail abläuft ist bisher allerdings weitgehend unklar gewesen. In den vier in dieser Dissertationsarbeit vorgestellten Studien haben wir uns daher mit der weiteren Aufklärung der Rollen und Funktionen verschiedener Treg-Subtypen beschäftigt, um diese eventuell für therapeutische Zwecke nutzen zu können.

3.1. Treg1-Zellen in der GN

Die Zellen der Th1-Antwort könnten ein vielversprechendes therapeutisches Ziel in der GN sein. Unsere und auch andere Gruppen konnten zeigen, dass Th1-Zellen bei der Initiierung und dem Verlauf der Nierenschädigung in der GN eine entscheidende Rolle spielen [19, 97-101]. Daher ist es von hoher klinischer Relevanz, die Mechanismen, die diese hoch nephritogenen Th1-Antworten regulieren, zu verstehen. Wir wollten daher in der ersten in dieser Dissertationsschrift enthaltenen Studie (Nosko *et al*.: T-Bet Enhances Regulatory T Cell Fitness and Directs Control of Th1 Responses in Crescentic GN [66]) das neue Konzept der Th1-spezifischen Treg-Zellen evaluieren. Vor kurzem wurde postuliert, dass diese Treg1-Zellen von

der Aktivierung des Transkriptionsfaktors T-bet abhängen, welchen sie mit ihren proinflammatorischen Gegenspielern teilen [52]. Da bisher noch nichts über ihre Funktion in renalen Entzündungsprozessen bekannt war, untersuchten wir die Treg1-Zellen im NTN-Modell der cGN.

Tatsächlich fanden wir, dass die T-bet^{+} Treg1-Zellen innerhalb von zehn Tagen nach Induktion der NTN in Milz und Niere expandierten. Anschließend sank ihr Anteil in der Milz wieder, während die renale Treg1-Population stetig weiter anstieg, was für bevorzugte Anreicherung in den Bereichen der Gewebeschädigung spricht. Interessanterweise ähnelte die Dynamik der Treg1-Zellen der der Th1-Antwort, was auf eine Rolle der Treg1-Zellen bei der Gegenregulation der Th1-Immunität hinweist.

Die Analyse der Expression verschiedener Treg-charakteristischer Oberflächenmoleküle auf Treg1-Zellen zeigte einen individuellen Phänotyp, mit reduzierten Leveln an CD25, ICOS und GITR. CD103, PD-1, CD44 und CD62L hingegen waren auf Treg1-Zellen im Vergleich zu T-bet^{-} Tregs sehr viel stärker exprimiert.

Aufgrund dieser Ergebnisse, die für eine individuelle und bedeutende Funktion der Treg1-Zellen sprechen, sollten als nächstes die funktionelle Relevanz der T-bet-Expression in Tregs beurteilt werden. Hierfür generierten wir eine Foxp3^{Cre}xT-bet$^{fl/fl}$ Mauslinie, welche spezifisch in regulatorischen T-Zellen eine T-bet-Defizienz aufweist. Passend zu einer, während der Erstellung unseres Manuskriptes erschienenen, Studie waren diese Tiere gesund, fertil und zeigten eine normale Lebenserwartung [64]. Jedoch zeigte die Analyse der Immunantwort in diesen Mäusen einen bisher unbekannten Phänotyp. Obwohl die grobe Leukozytenkomposition in Blut und Milz naiver Mäuse unverändert war, fanden wir eine spontan überschießende Th1-Immunität. Die Th17-Antwort und die Produktion verschiedener Th2- und Treg-assoziierter Zytokine waren nicht signifikant verändert.

Die höhere Zahl systemischer Th1-Zellen in Foxp3^{Cre}xT-bet$^{fl/fl}$ Mäusen hatte auch funktionellen Einfluss auf einen weiteren Arm des Immunsystems. Die Analyse der humoralen Immunantwort zeigte hochgradig reduzierte Level der Th2-assoziierten Antikörpersubklassen IgG1 und IgG3. Es ist bekannt, dass diese durch das Th1-Zytokin IFNγ supprimiert werden [102]. Da wir an der Rolle der Treg1-Zellen in der GN interessiert waren, untersuchten wir natürlich ebenfalls den renalen Phänotyp

naiver Foxp3^{Cre}xT-bet$^{fl/fl}$ Mäuse. Unsere Analysen zeigten jedoch gesunde Nieren ohne Anzeichen von spontaner Inflammation oder Funktionsstörungen. Daher befassten wir uns als nächstes mit der renalen und systemischen Immunantwort nach Induktion der NTN. Auch hier fanden wir wieder eine selektive Verschiebung der Immunität in Richtung Th1, sowohl in der Milz als auch in der Niere von Foxp3^{Cre}xT-bet$^{fl/fl}$ Mäusen. Übereinstimmend mit diesem hyper-Th1 Phänotyp, waren die Level der Th2-assoziierten Antikörpersubklassen, IgG1 und IgG3, gegen das nephritogene Antigen Schafglobulin signifikant reduziert. Darüber hinaus resultierte die Dysregulation der Th1-Antwort in einem verstärkten Nierenschaden mit vermehrter Halbmondbildung und mehr interstitiellem Schaden. Die genauere Analyse des Niereninfiltrates zeigte erhöhte Anzahlen an T-Zellen, Makrophagen und Neutrophilen in den Nieren der Treg1-defizienten Mäuse. Im Gegensatz zur Verschlechterung der Nephritis in Foxp3^{Cre}xT-bet$^{fl/fl}$ Mäusen, welchen selektiv Treg1-Zellen fehlen, wurde kürzlich eine Abmilderung der NTN bei T-bet-pan-knockout Mäusen gezeigt. Diesen fehlen nicht nur die Treg1-Zellen, sondern auch die Th1-Antwort [99]. Diese Beobachtung unterstreicht, dass Th1-Zellen tatsächlich das Hauptziel der Treg1-Zellen sind. Um den zugrundeliegenden Mechanismus der verstärkten Th1-Antwort und der daraus resultierenden Verschlechterung der cGN in Foxp3^{Cre}xT-bet$^{fl/fl}$ Mäusen zu identifizieren, analysierten wir die Suppressionsfunktion der Treg-Zellen. *In vitro* Suppressionsassays zeigten unveränderte suppressive Kapazität in der Reduktion von IL-2-Spiegeln in Kokultur mit Effektor-T-Zellen und ebenso uneingeschränkte Induktion des immunsuppressiven IL-10. Wesentlich war außerdem, dass die *in vitro* Suppression der IFNγ-Produktion intakt blieb. Das weist darauf hin, dass die T-bet-Defizienz nicht zu einem generellen Defekt von Tregs in der Kontrolle der Th1-Antworten führt. Zusätzlich zeigte die Quantifizierung der mRNA-Expression verschiedener Treg-assoziierter Effektorzytokine identische Level an IL-10, IL-35/EBI-3 und TGF-β1 in Treg-Zellen beider Gruppen. Letztlich zeigte die Analyse einer Reihe von Oberflächenmolekülen, die mit der Treg-Funktion assoziiert sind, keine grundlegenden, durch das Fehlen von T-bet verursachten, Unterschiede. Diese Resultate sind in Übereinstimmung mit einer vorherigen Arbeit von McPherson *et al.* [65], welche unveränderte Kapazität der T-bet-defizienten Tregs fanden, die Teff-Expansion *in vitro* zu supprimieren. Um diese Beobachtungen noch zu erweitern, analysierten wir zusätzlich die Treg-Suppressionseffekte *in vivo* in einem Transfermodell. Tatsächlich zeigten unsere Ergebnisse hier ähnliche Teff-

Proliferation und Aktivierung nach Kotransfer mit T-bet-intakten oder T-bet-defizienten Treg-Zellen. Allerdings könnte es sein, dass eine veränderte Treg-Homöostase und Fitness in unseren konstitutiven T-bet-defizienten $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ Mäusen durch kompensatorische Mechanismen und fehlende Konkurrenz mit Wildtyp-Tregs verdeckt sind. Um diese Frage zu untersuchen, führten wir kompetitive Transferassays durch. Hierbei injizierten wir Milzzellen von $CD45.2^{+}$ $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ Mäusen zusammen mit der gleichen Anzahl Zellen aus $CD45.1^{+}$ Wildtyptieren in lymphopenische $Rag1^{-/-}$ Empfänger. Nach Induktion der NTN hatten die Wildtyp-Tregs ihre T-bet-defizienten Gegenspieler in der Milz und in der Niere deutlich auskonkurriert. Außerdem waren die Expressionslevel des Foxp3-Proteins sehr viel niedriger in den transferierten Knockout-Treg-Zellen, was für reduzierte regulatorische Kapazität im Vergleich zu Wildtyp-Treg-Zellen spricht. T-bet-Aktivierung fördert also die Expansion der Treg-Population während der Inflammation, sowie deren generelle Fitness. Außerdem fanden wir reduzierte Niere/Milz-Verhältnisse unter den transferierten Treg-Zellen aus den $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ Mäusen. Diese Beobachtung spricht für gestörtes kompetitives renales Wandern T-bet-defizienter Tregs. Aufgrund dieser Beobachtung untersuchten wir als nächstes, ob gestörte renale Treg-Rekrutierung zum beobachteten Hyper-Th-1-Phänotyp in den $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ Mäusen führt. Unterschiedliche Analysen nephritischer Nieren ergaben hierbei reduzierte Prozentsätze an Tregs in Nieren von $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ Mäusen. Da die systemischen Treg-Zahlen unverändert waren, spricht diese Beobachtung tatsächlich für gestörtes renales Wandern der T-bet-defizienten Tregs. Da Chemokine die Hauptregulatoren der gerichteten T-Zellmigration sind [47, 103], untersuchten wir das Treg-Chemokinrezeptorprofil. In Übereinstimmung mit mehreren vorherigen Studien, auch aus der eigenen Gruppe, fanden wir robuste Expression des Th1-charakteristischen Rezeptors CXCR3 auf Tregs der Milz und der Niere. Interessanterweise war die Expression von CXCR3 jedoch auf Treg-Zellen aus $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ Mäusen vollständig verschwunden. Dies spricht für völlige Abhängigkeit von der Aktivierung T-bets. Um genauer zu untersuchen, ob das Fehlen von CXCR3 die Hauptursache für die Reduktion der renalen Treg-Frequenzen ist, verglichen wir unsere Ergebnisse mit Mäusen mit Treg-spezifischer CXCR3-Defizienz. Passend zu unserer Hypothese fanden wir in diesen $Foxp3^{Cre}xCXCR3^{fl/fl}$ Tieren eine gleich stark ausgeprägte Beeinträchtigung renaler Treg-Infiltration und außerdem auch eine identische

Erhöhung der renalen Th1-Immunität wie in Foxp3^{Cre}xT-bet$^{fl/fl}$ Mäusen. Zusammengenommen sprechen diese Beobachtungen dafür, dass tatsächlich das gestörte Wandern durch das Fehlen des CXCR3 der beobachteten Reduktion renaler Treg-Prozentsätze zugrunde liegt. Das Fehlen des CXCR3 auf Tregs beeinträchtig vermutlich die Kolokalisation mit CXCR3^{+} Th1-Zellen [104], was die selektiv verstärkten Th1-Antworten in Foxp3^{Cre}xT-bet$^{fl/fl}$ Mäusen erklären könnte.

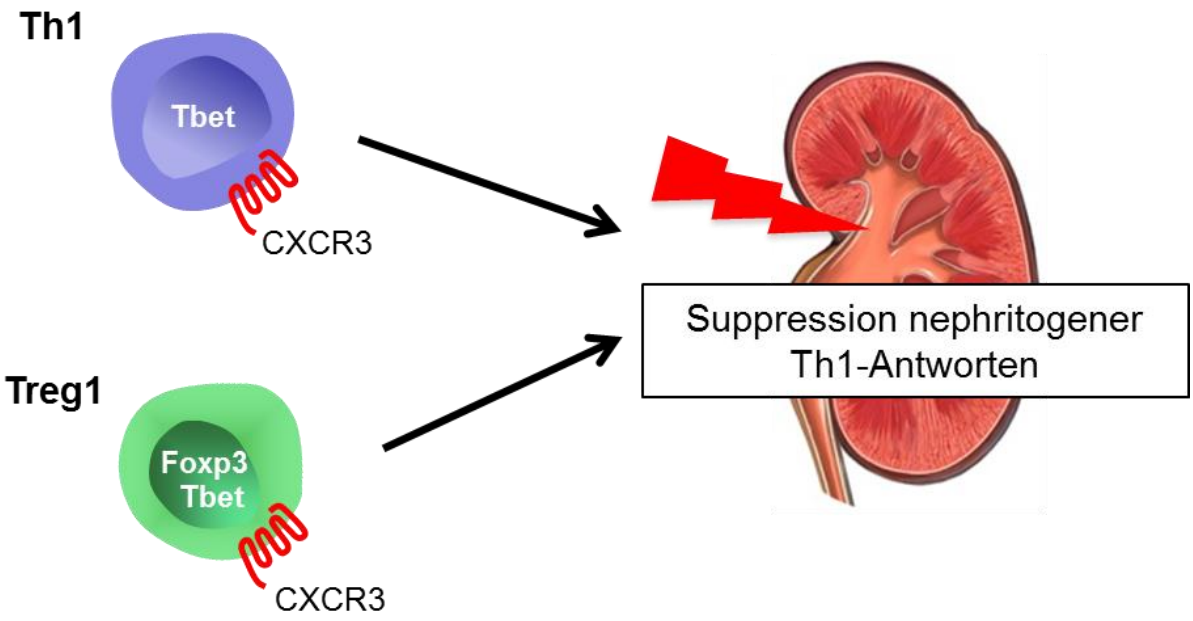

Abbildung 5: Treg1 Zellen exprimieren den Th1-charakteristischen Transkriptionsfaktor T-bet und in Abhängigkeit davon den Th1-charakteristischen Traffickingrezeptor CXCR3. Dadurch können die Treg1-Zellen in Areale der Th1-Inflammation wandern und diese dort spezifisch supprimieren.

Zusammenfassend konnten wir in der ersten hier vorgestellten Publikation erste Hinweise auf eine Rolle von T-bet^{+} Treg1-Zellen in der experimentellen cGN und damit generell in einem Modell induzierter Organinflammation finden. Es wurde gezeigt, dass die T-bet-Aktivierung zwei wichtige Treg-Eigenschaften moduliert. Zum einen war durch die Expression von T-bet die generelle Fitness der Tregs signifikant erhöht. Zum anderen induzierte die T-bet-Aktivierung die Expression des Chemokinrezeptors CXCR3. Dies ermöglicht optimale Kontrolle von Th1-Zellen durch Erleichterung des Wanderns der Treg1-Zellen in Areale der Th1-Inflammation (Abbildung 5). T-bet^{+} Treg1-Zellen wurden demnach als neue Regulatoren nephritogener Th1-Antworten identifiziert und verdienen damit die weiterführende Untersuchung als potentielles neues therapeutisches Ziel.

3.2. Treg17-Zellen bei Lupus

Die zweite in dieser Dissertationsarbeit enthaltene Studie hatte zum Ziel, die neu definierten Treg17-Zellen [53, 68] und ihre bisher unbekannte Rolle bei der Kontrolle von pathogenen Th17-Antworten im murinen SLE zu charakterisieren. Als Modellsystem wählten wir den Pristan-induzierten Lupus, da dieser der humanen Erkrankung sehr ähnelt [71]. Außerdem konnten zwei vorherige Studien anhand von IL-17-defizienten Mäusen zeigen, dass die IL-17/Th17-Achse eine wichtige Rolle bei der Entstehung der Autoimmunität und des Endorganschadens in diesem Modell spielt [72, 73]. Mit Pristan behandelte Mäuse entwickeln schnell eine Peritonitis mit Bildung von Ölgranulomen, welche in späteren Stadien zur Generierung einer Lupus-ähnlichen Autoimmunität führen. Wir untersuchten daher die peritoneale Immunantwort eine Woche nach Pristaninjektion in Treg17-defizienten $Foxp3^{Cre}xStat3^{fl/fl}$ Mäusen und $Foxp3^{Cre}$ Kontrollmäusen. Passend zu unserem Konzept beobachteten wir in Abwesenheit der Treg17-Zellen eine signifikante Verschiebung hin zur Th17-Antwort. Dies spricht für eine Kontrolle der Th17-Immunität durch Treg17-Zellen, bereits früh im Verlauf der Entzündung und deutlich vor der Etablierung der Autoimmunität. Als eine weitere frühe Manifestation der Pristan-induzierten Entzündung wurde gezeigt, dass Mäuse eine Nicht-Immunkomplex pulmonale Vaskulitis entwickeln [105]. Da wenig über diese nicht-autoimmune Manifestation durch Pristan bekannt ist, untersuchten wir zunächst die Lungen drei Wochen nach Behandlung mit Pristan in Wildtypmäusen. Unsere Ergebnisse zeigten eine schwere pulmonale Kapillaritis. Außerdem konnten wir Neutrophile als die vorherrschende Leukozytenpopulation identifizieren. Da Neutrophile die durch die Th17-Antwort hauptsächlich rekrutierten Zellen sind [22, 106], war es naheliegend, dass die Kapillaritis ebenfalls durch Treg17-Zellen beeinflusst wird. Wichtig für diese Studie und in Übereinstimmung mit einer vorherigen Studie unserer Gruppe [68], war das Überleben unbehandelter $Foxp3^{Cre}xStat3^{fl/fl}$ Mäuse in unserer Einrichtung normal. Im Gegensatz zu der Studie von Chaudry et al. [53] entwickelten unsere Mäuse keine Anzeichnen spontaner Autoimmunität und insbesondere keine Vaskulitis der Lunge. Wie jedoch erwartet, verursachte die Pristaninjektion eine frappierende Verschlechterung der pulmonalen Kapillaritis in der Abwesenheit von Treg17-Zellen. Dies führte zu einem dramatisch reduzierten Überleben der $Foxp3^{Cre}xStat3^{fl/fl}$ Mäuse. Detaillierte Untersuchungen der Lunge zeigten stark verschlimmerte pulmonale Hämorrhagie, Neutrophilie und

Gewebeschädigung in Foxp3^{Cre}xStat3$^{fl/fl}$ Mäusen. Als vermutliche Hauptursache fanden wir, dass die pulmonale Th17-Antwort in Abwesenheit der Treg17-Zellen selektiv erhöht war. Da die peritoneale und die pulmonale Entzündung beide früh nach Pristangabe auftreten und deutlich vor Beginn der Autoantikörperbildung, ist es naheliegend, dass die beobachteten Th17-Antworten keine adaptiven Antworten sind, sondern eher zu den kürzlich beschriebenen natürlichen Th17-Zellen gehören. Diese Zellen sekretieren nach Stimulation des TLR4 und des TLR7 sehr schnell antigenunspezifisch IL-17 [107]. Diese Annahme wird durch die frühere Beobachtung, dass Pristan-induzierte Entzündung vom TLR7 abhängt, unterstützt [108]. Es ist daher naheliegend zu spekulieren, dass Treg17-Zellen nicht nur adaptive Th17-Antworten kontrollieren, sondern auch die natürlichen Th17-Zellen, was einen wichtigen neuen Aspekt zu ihrer Natur hinzufügt.

Als nächstes untersuchten wir die Langzeitfolgen der Treg17-Defizienz auf die Entwicklung der Autoimmunität und den Endorganschaden im Pristan-induzierten SLE. Zunächst analysierten wir die systemische und renale Immunität nach vier Monaten, einem Zeitpunkt zu dem die Autoimmunität schon etabliert ist und sich die immunkomplexvermittelte Lupus-Nephritis gerade zu entwickeln beginnt. Auch hier fanden wir signifikant und selektiv erhöhte Th17-Antworten in Foxp3^{Cre}xStat3$^{fl/fl}$ Mäusen, sowohl systemisch in der Milz als auch lokal in der Niere.

Im nächsten Schritt sollte ermittelt werden, ob die erhöhten Th17-Level auch zu einem verstärkten Endorganschaden führen. Neun Monate nach der Pristaninjektion durchgeführte Analysen zeigten erneut verstärkte systemische und renale Th17-Immunität bei Fehlen der Treg17-Zellen. Die histologische Analyse der Nieren zeigte eine schwere Immunkomplex-Nephritis, welche passend zur gesteigerten renalen Th17-Infiltration in den Foxp3^{Cre}xStat3$^{fl/fl}$ Mäusen signifikant verschlechtert war. Um die Mechanismen, die zur beobachteten Aggravation der SLE-Manifestation in unseren Knockouts führten, genauer zu untersuchen, analysierten wir als nächstes die Tregs selbst. Im Gegensatz zu allen anderen untersuchten Leukozytensubpopulationen, war die Infiltration von Tregs in Foxp3^{Cre}xStat3$^{fl/fl}$ Mäusen signifikant verringert. Dies war nicht auf einen generellen Mangel an Tregs zurückzuführen, da die Frequenzen in der Milz in beiden Gruppen ähnlich waren. Auch konnten unsere und andere Gruppen kürzlich zeigen, dass die generelle *in vitro* Suppressionsaktivität von Tregs aus Foxp3^{Cre}xStat3$^{fl/fl}$ Mäusen nicht vermindert ist

[53, 68]. Unsere vorausgehenden Daten weisen darauf hin, dass ein Mechanismus der Th17-Spezifität von Treg17-Zellen die gemeinsame Expression des Traffickingrezeptors CCR6 ist, was ihre Kolokalisation erleichtert [68]. Tatsächlich zeigten unsere Analysen, dass nur Tregs aus den Kontrollmäusen eine robuste Expression des CCR6 hatten. Auf den Tregs aus Foxp3^{Cre}xStat3$^{fl/fl}$ Mäusen hingegen fehlte der CCR6 fast vollständig. Die renale Expression des einzigen bekannten CCR6 Liganden, CCL20, war ähnlich zwischen den Gruppen. Dies unterstreicht, dass wahrscheinlich eher die fehlende Rezeptorexpression als das Fehlen des Liganden die gestörte Akkumulation der Tregs in Foxp3^{Cre}xStat3$^{fl/fl}$ Mäusen verursacht. Das hebt noch einmal hervor, dass die Stat3-Aktivierung in Treg17-Zellen diese durch die Expression des Chemokinrezeptors CCR6 dazu befähigt, sich mit ihren Th17-Gegenspielern zu kolokalisieren (Abbildung 6). Das Prinzip ist also vermutlich dasselbe wie in der bereits erwähnten ersten Publikation für die Treg1-Zellen beschrieben, welche T-bet abhängig den CXCR3 exprimieren und so mit den Th1-Zellen kolokalisieren können [66].

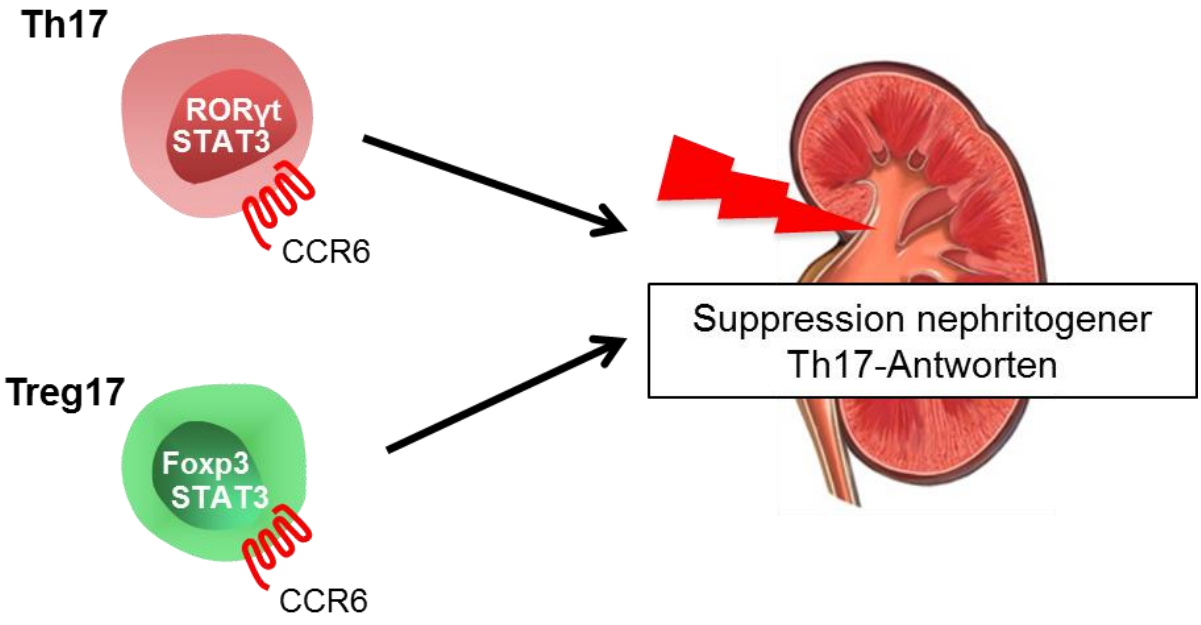

Abbildung 6: Treg17 Zellen exprimieren den Th17-charakteristischen Transkriptionsfaktor Stat3 und in Abhängigkeit davon den Th17-charakteristischen Traffickingrezeptor CCR6. Dadurch können die Treg17-Zellen in Areale der Th17-Inflammation wandern und diese dort spezifisch supprimieren.

Abschließend untersuchten wir noch, ob die humorale Autoimmunität durch das Fehlen der Treg17-Zellen auch beeinflusst war. Es wurde bereits gezeigt, dass Th17-Zellen die Antikörperproduktion von B-Zellen im SLE positiv beeinflussen [109].

Vorherige Studien aus unseren Reihen zeigten eine gestörte anti-ds-DNA- und U1-RNP-Autoantikörperproduktion bei Fehlen von IL-17 [72]. Jedoch waren in unseren Experimenten die Level der meisten analysierten Autoantikörper unverändert, IgG2b und IgG2c anti-ds-DNA-Antikörper waren sogar leicht reduziert in den Treg-17-defizienten Mäusen. Passend dazu waren die renalen Ablagerungen von Komplement-C3 und IgG ähnlich in den beiden Gruppen. Also scheinen Treg17-Zellen vornehmlich die zelluläre Immunität zu kontrollieren und in der Entstehung der humoralen Autoimmunität eine weniger wichtige Rolle zu spielen. Insgesamt lassen unsere Daten also darauf schließen, dass die unkontrollierten, pathogenen Th17-Antworten die Verschlimmerung des Pristan-induzierten Gewebeschadens in Treg17-defizienten Mäusen bedingen. Eine endgültige kausale Verbindung zwischen der überschießenden Th17-Immunität und der Krankheitsverschlimmerung muss jedoch noch etabliert werden.

Zusammengenommen belegt unsere Studie erstmals eine Rolle der Treg17-Zellen für die Kontrolle der Th17-Antwort und den Gewebeschutz während der akut entzündlichen und der chronisch autoimmun-vermittelten Stadien des Pristan-induzierten SLE. Unsere Daten geben also Anlass zur weiteren Untersuchung der Mechanismen, welche die T-Helferzellen regulieren, um potentielle therapeutische Ziele für die Behandlung des SLE zu identifizieren und zu beurteilen.

3.3. biTregs in GN und Lupus

Die eben beschriebenen Treg1- und Treg17-Zellen werden durch die Expression eines Hauptranskriptionsfaktors ihres proinflammatorischen Gegenspielers dazu befähigt, eben diesen spezifisch zu supprimieren. Bei den Treg17-Zellen handelt es sich dabei um den Transkriptionsfaktor Stat3. Th17-Zellen werden jedoch nicht nur durch Stat3, sondern auch durch den Transkriptionsfaktor RORγt definiert. Wie sich kürzlich herausstellte, gibt es ebenfalls Tregs, welche RORγt mit Foxp3 koexprimieren [75]. Die dritte hier enthaltene Publikation hatte daher zum Ziel die Biologie dieser RORγt^{+}Foxp3^{+} Tregs, von uns mit biTregs benannt, genauer zu charakterisieren und ihre Rolle in der akuten cGN zu untersuchen. Das ist von besonderer Relevanz, da sich der Th17-definierende Transkriptionsfaktor RORγt als potenter proinflammatorischer Mediator während der Glomerulonephritis erwiesen

hat [17] und bereits verschiedene Blocker entwickelt wurden und auf klinische Prüfung warten [89, 90, 92, 110]. Unsere Analysen zeigten das Vorhandensein von biTregs in Milz und Niere gesunder Mäuse. Interessanterweise beobachteten wir eine schnelle und massive Expansion der biTregs früh im Verlauf der NTN, welche parallel zu den Th17-Zellen verlief. Konventionelle RORγt negative Tregs (cTregs) hingegen zeigten eine andere, verzögerte Dynamik. Die Proliferationsraten der biTregs waren sehr hoch, was darauf schließen lässt, dass sie sich eher durch Teilung vermehren und nicht durch Rekrutierung oder Transdifferenzierung aus anderen Zelllinien. Die weitere Charakterisierung von biTregs zeigte ein bifunktionelles, pro- und antiinflammatorisches Profil. Sie exprimieren hohe Level an IL-17 und dem Th17-charakteristischen Chemokinrezeptor CCR6. Zur selben Zeit sekretieren biTregs jedoch auch große Mengen der antiinflammatorischen Zytokine IL-10 und IL-35. biTregs zeigten außerdem ein hohes Aktivierungslevel, was auf ihre funktionelle Relevanz hindeutet. Detaillierte Analysen der Oberflächenexpression von immunmodulatorischen Molekülen zeigte eine einzigartige Signatur, die sich sowohl von Th17-Zellen als auch von cTregs unterscheidet. Als ein Hauptkennzeichen fanden wir erhöhte Level von ICOS auf biTregs, was in Übereinstimmung zu zwei vorherigen Berichten steht [75, 111]. Interessanterweise wurde auch berichtet, dass die Expression von ICOS mit der Produktion von IL-17 assoziiert ist [112]. Außerdem zeigten biTregs eine besonders starke Expression von CTLA-4 und auch des Integrins CD103, welches kürzlich als Marker für regulatorische T-Zellen mit höchster suppressiver Kapazität beschrieben wurde [113]. Die Expression von CD25 und GITR war hoch und ähnlich zu cTregs, die Expression von PD-1 und Helios hingegen war signifikant reduziert. Weitere Analysen zeigten ebenfalls eine einzigartige Transkriptionsfaktorsignatur, welche sich deutlich von Th17-Zellen und cTregs unterscheidet. biTregs fehlte die mRNA-Expression von PZLF, welches kürzlich als charakteristisch für natürlich vorkommende nTh17-Zellen beschrieben wurde [107]. Auch Blimp-1, ein Transkriptionsfaktor der mit den Treg-Effektorfunktionen zusammenhängt [114], war in biTregs nur schwach exprimiert. Insgesamt lassen diese Ergebnisse darauf schließen, dass biTregs eine einzigartige und unabhängige Zelllinie darstellen, welche sowohl suppressive als auch proinflammatorische Eigenschaften zur gleichen Zeit aufweist.

In einem nächsten Schritt untersuchten wir den Entwicklungsursprung von biTregs. Wie oben beschrieben wurde kürzlich eine Treg-Subpopulation beschrieben, welche

darauf spezialisiert ist, Th17-Antworten herunterzuregulieren. Diese Treg17-Zellen hängen von der Expression des Transkriptionsfaktors Stat3 ab [53, 68]. Da Stat3 als Induktor von RORγt bekannt ist, wollten wir ermitteln, ob biTregs zu diesem neu identifizierten Treg-Subtyp gehören könnten. Jedoch waren biTregs in normalen Prozentzahlen in Mäusen, denen die Stat3-Aktivierung in Tregs fehlt, vorhanden. Dies deutet darauf hin, dass biTregs eine eigenständige, sich von Treg17-Zellen abgrenzende Zellpopulation darstellen. Unsere nächste Frage war, ob biTregs im Thymus oder in der Peripherie induziert werden. Analysen von Thymi zeigten keinerlei biTregs, was für eine periphere Induktion spricht. Wir wollten daher untersuchen ob biTregs in der Peripherie durch Transdifferenzierung aus RORγt einzelpositiven Th17-Zellen oder aus Foxp3 einzelpositiven cTregs entstehen. Zu diesem Zweck führten wir „fate reporter“ und Zelltransferstudien durch. Hoch aufgereinigte RORγt^{+}Foxp3^{-} Th17-Zellen und Foxp3^{+}RORγt^{-} cTregs wurden in Empfängermäuse transferiert und ihr Schicksal wurde in Milzen und Nieren sechs Tage nach NTN-Induktion analysiert, ein Zeitpunkt, zu dem die biTregs in diesem Modell vollständig expandiert sind. Die Ergebnisse zeigten, dass keine der Th17-Zellen Foxp3 hochreguliert hatte und ebenso keine der cTregs anfing, RORγt zu exprimieren. Wir konnten also daraus schließen, dass biTregs nicht aus Th17-Zellen oder cTregs entstehen. Unsere Erkenntnisse unterstützen also eher das von Hori [115] vorgeschlagene Modell der Foxp3-Linienheterogenität, als das Konzept der Foxp3/Th17-Linienplastizität. Es wurde jedoch in der Vergangenheit ebenfalls postuliert, dass Foxp3 transient während ihrer Aktivierung in nicht-Treg CD4^{+} Zellen induziert werden könnte [116-119]. Daher verfolgten wir das Schicksal von Foxp3^{+} Zellen kontinuierlich im Verlauf der NTN mittels „fate reporter“-Mäusen. Die Analysen zeigten, dass nahezu alle Foxp3^{+} Zellen in naiven Mäusen die Foxp3-Aktivierung ihr Leben lang beibehalten. Ebenso detektierten wir keinen Verlust der Foxp3 Expression in den ersten sieben Tagen der NTN, was unspezifische oder zeitweise Hochregulation von Foxp3 während der Effektor-T-Zell-Aktivierung ausschließt. Während späterer Phasen der NTN zeigte jedoch eine wachsende Fraktion Foxp3 „fate“-positiver Zellen den Verlust von Foxp3. Diese ex-Foxp3 Zellen wurden zu einem Zeitpunkt generiert (12 Tage nach NTN), an dem die Zahl der cTregs stabil ist, während die biTreg Zahlen rapide zurückgehen. Es stellte sich daher die Frage, ob der Grund für die beobachtete Abnahme der biTregs, Verlust von Foxp3 und/oder Transdifferenzierung in Th17-Zellen ist. Um diese Frage zu beantworten

transferierten wir hochreine biTregs aus Tieren, die den kongenen Marker CD45.2 auf ihren Leukozyten tragen, in Tiere, die den Marker CD45.1 exprimieren. An Tag zehn nach NTN Induktion in den Empfängertieren fanden wir deutlich vergrößerte Milzen, welche hohe Zahlen an CD45.2 Spender-Zellen enthielten, die die Anzahl der zuvor transferierten Zellen um das 10-fache überschritten. Passend dazu zeigten die CD45.2 Spenderzellen eine hohe proliferative Aktivität, welche die der CD45.1 Empfängerzellen weit überschritt. Interessanterweise zeigte die Analyse der transferierten RORγt^{+}Foxp3^{+} Spenderzellen, dass die große Mehrheit der Zellen sowohl Foxp3 als auch RORγt herunterreguliert hatte. Zwei kleine Fraktionen hatten entweder Foxp3 oder RORγt herunterreguliert, ohne Tendenz in der präferenziellen Transdifferenzierung zu RORγt^{+}Foxp3^{-} Th17 oder konventioneller Foxp3^{+}RORγt^{-} cTregs. Diese Beobachtungen unterstützen insgesamt ein Konzept, in dem Foxp3^{+}RORγt^{+} biTregs eine eigenständige Zelllinie darstellen, welche in der Inflammation rasch in der Peripherie aus einem in naiven Mäusen vorher vorhandenen Pool expandieren. Anschließend ziehen sich die biTregs wieder zurück und regulieren beide Transkriptionsfaktoren RORγt und Foxp3 herunter.

Nachdem wir die Dynamik und das Schicksal von biTregs aufgeklärt hatten, sollte ihre Funktion untersucht werden. Wir transferierten daher biTregs in Wildtyp-Empfängertiere und induzierten anschließend die NTN Nephritis. Passend zu ihrer Foxp3-Expression und zu den Beobachtungen von Lochner *et al.* [75], zeigten biTregs regulative Kapazität und schützten wirksam vor renaler Schädigung. Wesentlich war, dass der Schutz durch exogene biTregs lange Zeit anhielt und den renalen Schaden auch noch 30 Tage nach Krankheitsinduktion verbesserte. Weitere Transferstudien zeigten außerdem, dass biTregs einen ähnlichen Grad des Schutzes bei der GN vermitteln wie cTregs. Angesichts der auffälligen Unterschiede zwischen cTregs und biTregs auf verschiedenen Ebenen, wie Zytokinprofil, Oberflächenmolekülsignatur und Transkriptionsfaktorexpression, wollten wir untersuchen, ob sie auch funktionell unterschiedlich sind. Tatsächlich wiesen *in vitro* Suppressionsassays auf unterschiedliche Mechanismen der Immunsuppression hin. Während nur cTregs die IL-2 Produktion in Kokultur mit Effektor-T-Zellen ausreichend supprimierten, zeigten biTregs eine stark erhöhte Produktion des antiinflammatorischen IL-10. Diese Daten identifizieren biTregs als einzigartige regulatorische T-Zellen mit einem in der Summe antiinflammatorischen Effekt in der Nephritis. Wir vermuteten jedoch, dass biTregs auch pathogenes Potential haben

könnten. Besonders, da die Expression von RORγt stark mit proinflammatorischen Eigenschaften assoziiert ist [17] und wir robuste Sekretion von IL-17 aus biTregs beobachteten. Wir generierten daher Mäuse mit selektiver RORγt-Defizienz in $Foxp3^+$ Tregs. Da unsere bisherigen Experimente zeigten, dass keine andere $Foxp3^+$ Population neben biTregs RORγt aktiviert, handelt es sich gewissermaßen um ein Knockout von RORγt in biTregs. Die Entwicklung der Mäuse mit RORγt-defizienten biTregs war normal und sie zeigten keine Anzeichen von spontaner Autoimmunität, was für eine erhaltene regulatorische Funktion von RORγt defizienten biTregs spricht. Die Analyse der Immunantworten zeigte jedoch das vollständige Fehlen der IL-17-Sekretion durch biTregs in den Knockouts. Es ist wichtig zu erwähnen, dass die Treg- und Th17-Antworten ansonsten unverändert waren, was den unabhängigen Charakter der biTregs noch weiter unterstreicht. Passend zum Fehlen der IL-17-Sekretion war die Schwere der NTN in den Knockoutmäusen stark abgeschwächt, was eine proinflammatorische Rolle von RORγt in den biTregs belegt. Um diese Beobachtungen in einem zweiten Modell zu validieren, untersuchten wir auch den Verlauf der akzelerierten NTN [17] und fanden auch hier Schutz vor renalem Schaden bei fehlender RORγt-Aktivierung in biTregs.

Unsere Daten führen also zu der Schlussfolgerung, dass biTregs potente antiinflammatorische, aber auch einige, durch RORγt vermittelte, proinflammatorische Eigenschaften besitzen. Diese bifunktionelle Natur mag zunächst unlogisch erscheinen. biTregs könnten jedoch evolutionär entstanden sein, um die wichtige Lücke zwischen Mediatoren der Wirtsverteidigung und des Gewebeschutzes zu füllen. Ihre einzigartigen Eigenschaften rüsten sie dazu aus, Pathogene zu bekämpfen, während sie uns gleichzeitig vor kollateralem Gewebeschaden und Entstehung von Autoimmunität schützen können. Interessanterweise können die proinflammatorischen Funktionen durch die Aufhebung des RORγt-Signalings blockiert werden, was biTregs zu einem vielversprechenden Ziel für RORγt-gerichtete Therapien inflammatorischer Krankheiten macht.

Nach den Untersuchungen im akuten Modell der NTN-Nephritis, wollten wir die $ROR\gamma t^+Foxp3^+$ biTregs im Modell des Pristan-induzierten SLE untersuchen. Dieses Modell ähnelt dem humanen SLE signifikant [71] und die klinische Relevanz der biTregs sollte so weiter bestätigt werden. Da auch im Rahmen des SLE nichts über

das Vorkommen und die Biologie dieser Zellen bekannt ist, begannen wir mit einer genauen Analyse der zeitlichen und organspezifischen biTreg Expansion. In den ersten zwei Wochen nach Pristaninjektion entwickeln die Mäuse eine sterile Peritonitis, welche vom angeborenen Immunsystem vermittelt wird [71]. Während dieser akuten, nicht-antigenvermittelten Inflammation, blieben die biTregs auf dem Basislevel. Eine weitere antigenunabhängige Manifestation des Pristan ist die akute pulmonale Vaskulitis mit diffuser alveolarer Hämorrhagie [120]. Ähnlich der Peritonitis, fanden wir auch hier keine nennenswerte Expansion der biTregs während der Phase der pulmonalen Inflammation durch die angeborene Immunität. Die Persistenz der Inflammation nach der Pristaninjektion führt jedoch zur progressiven Entwicklung einer chronischen Autoimmunität. Während dieser bilden sich zunächst peritoneale und pulmonale Lymphogranulome, welche ungefähr drei Wochen nach der Injektion sichtbar werden. Interessanterweise stiegen die biTreg Prozentzahlen in dieser Phase in beiden Organen signifikant an, was für eine Rolle bei der Etablierung der Granulome und der Autoimmunität spricht. Außerdem weisen diese Daten darauf hin, dass die Expansion der biTregs antigenabhängig abläuft, was in Übereinstimmung zu einem jüngsten Bericht ist [84]. Im Laufe der Zeit nach Pristaninjektion entstehen verschiedene Typen von Autoantikörpern [70, 121]. Diese lagern sich in der Niere ab und resultieren in progressiver Immunkomplex-abhängiger Lupus-Nephritis. Während dieses Prozesses zeigten die biTregs einen ganz individuellen und konzertierten zeitlichen Verlauf. Ihre Population begann früh während der renalen Inflammation zu expandieren und erreichte ein Maximum nach ungefähr fünf Monaten. Anschließend sanken die renalen biTreg Prozentzahlen langsam wieder und waren neun Monate nach Pristaninjektion nahezu auf dem Grundlevel. Im Gegensatz zur massiven Expansion der biTregs in Peritoneum, Lunge und Niere, stieg ihre Zahl in der Milz nur sehr gering. Das stimmt mit der Tatsache überein, dass die Pristaninjektion nur in einer milden Inflammation der Milz resultiert. Zusammengefasst scheinen biTregs also frühe Mediatoren der adaptiven Inflammation und der Gewebeschädigung während des Pristan-induzierten SLE zu sein. Wie bereits in der NTN beschrieben und von anderen bestätigt [77, 79, 122], produziert eine robuste Fraktion von biTregs in allen Organen und zu allen untersuchten Zeitpunkten IL-17. Aufgrund der beobachteten Expansion der biTregs in allen betroffenen Organen, vermuten wir, dass biTregs eine funktionelle Schlüsselrolle während der Entwicklung des SLE spielen könnten. Wie unsere eben

beschriebenen Daten aus der akuten Nephritis zeigten, scheint Aktivierung von RORγt die Pathogenität der biTregs zu vermitteln. Um dies auch im Pristan-Modell zu untersuchen, verwendeten wir ebenfalls die bereits beschriebene Foxp3^{Cre}xRORc$^{fl/fl}$ Mauslinie. Zunächst wollten wir sehen, ob diese Mäuse vor der pulmonalen Vaskulitis geschützt sind, welche eine seltene, aber lebensbedrohliche Komplikation des humanen SLE ist [123]. Analysen von Lungen zu frühen Zeitpunkten, vor Entstehung der Autoimmunität und Expansion der biTregs, zeigten einen ähnlichen Schadensgrad. Zu späteren Zeitpunkten hingegen waren Foxp3^{Cre}xRORc$^{fl/fl}$ Mäuse signifikant vor pulmonaler Vaskulitis geschützt und zeigten weniger Hämorrhagie, pulmonale Leukozyteninfiltration und histologischen Schaden. Außerdem waren die pulmonalen Granulome in Größe und Anzahl reduziert. Als nächstes wollten wir die Entwicklung der Lupus-Nephritis untersuchen, welche eine der schwersten Organmanifestationen im humanen SLE darstellt und mit einer schlechten Prognose einhergeht. Hierbei fanden wir, dass die Foxp3^{Cre}xRORc$^{fl/fl}$ Mäuse signifikant geschützt waren, belegt durch weniger histologischen Schaden und glomeruläre Zellproliferation. Auch funktionelle Parameter des Nierenschadens, wie die Harnstofflevel im Serum und die Albuminurie waren signifikant reduziert. Außerdem fanden wir stark verringerte proinflammatorische Infiltration von Leukozyten in die Niere bei den Mäusen, denen RORγt in biTregs fehlt. Um die Mechanismen zu untersuchen, durch welche die Aufhebung der RORγt-Aktivierung in biTregs vor der Organmanifestation des SLE schützt, wollten wir als nächstes die Entwicklung der pathogenen humoralen Autoimmunität beurteilen. Unsere Analysen zeigten jedoch unveränderte Level an Gesamt-IgG im Serum, sowie aller gemessenen Subklassen, in Foxp3^{Cre}xRORc$^{fl/fl}$ Mäusen. Ähnlich blieben auch die Level der SLE-typischen anti-dsDNA und anti-U1-RNP-Autoantikörper unverändert. Übereinstimmend mit unbeeinflussten Serumantikörpern fanden wir auch im selben Maße Komplement-C3 und IgG-Ablagerungen in den Nieren aus beiden Gruppen. In Kongruenz hierzu zeigten ebenfalls Analysen von B-Zellsubpopulationen, Plasmazellen und follikulären Helferzellen in der Milz keine Unterschiede.

Da wir in der humoralen Immunität keinen Unterschied finden konnten, untersuchten wir als nächstes die T-Zell-Immunantwort. Auffälligerweise und in Übereinstimmung mit der Bedeutung von RORγt für Th17-Zellen [17] war die Expression von proinflammatorischem IL-17 aus biTregs in den Foxp3^{Cre}xRORc$^{fl/fl}$ Mäusen vollständig verschwunden. Diese Beobachtung könnte zumindest teilweise die

Verbesserung des SLE-induzierten Gewebeschadens erklären. Um weitere potentielle Schutzmechanismen zu identifizieren, untersuchten wir, ob RORγt auch die Treg-Suppression und -aktivität beeinflusst. Ähnlich zu vorherigen Daten aus unserer Abteilung, bei denen Tregs aus RORc-pan-knock-out-Mäusen untersucht wurden, fanden wir unveränderte *in vitro* Suppressionskapazität. *In vivo* führte die Treg-selektive Deletion von RORγt jedoch zu erhöhten Leveln des Foxp3-Proteins, so wie erhöhte Expression von Treg-Aktivierungsmarkern. Diese Beobachtungen lassen vermuten, dass die RORγt-Expression in Tregs *in vivo* die regulatorische Funktion negativ beeinträchtigt.

Als nächstes wollten wir beurteilen, ob diese beobachteten Treg-Veränderungen zu relevanten Effekten in der systemischen Immunität führen. Interessanterweise fanden wir signifikant erhöhte antiinflammatorische Typ 2 Immunantworten in naiven und mit Pristan behandelten $Foxp3^{Cre}xRORc^{fl/fl}$ Mäusen, sowohl systemisch als auch im entzündeten Zielorgan. Die Th1- und Th17-Antworten waren hingegen unverändert. Diese Beobachtungen stimmen vollständig mit einer kürzlich, während unserer Arbeit an dieser Publikation, veröffentlichten Studie überein [84], welche biTregs als wesentliche Mediatoren zum Schutz vor Allergien identifizierte. Die Identifikation von biTregs als potente Regulatoren der Typ 2 Immunität ist ebenfalls bemerkenswert, da die Mechanismen welche die Th2-Antwort regulieren, bisher weitgehend unbekannt sind. Unsere Daten weisen jedoch nicht auf einen direkten suppressiven Effekt von biTregs auf Th2-Zellen hin, da die Generierung der Th2-Immunität in unseren in vitro Kokulturexperimenten von Teff mit RORγt-defizienten Tregs nicht verändert war. Vermutlich sind eher, wie von Ohnmacht *et al*. vorgeschlagen, indirekte Mechanismen, wie Interaktion zwischen Tregs und Dendritischen Zellen, entscheidend [84].

Zusammengenommen fanden wir auch im Pristanmodell, wie für das NTN-Modell bereits beschrieben, dass eine biTreg-selektive Deletion von RORγt in Verlust der IL-17-Sekretion und einer erhöhten Treg-Aktivierung resultiert. Zusätzlich fanden wir ebenfalls eine gesteigerte Th2-Immunität. Diese Veränderungen waren mit einem Schutz vor der pulmonalen Vaskulitis und der Lupus-Nephritis verbunden. Es bleibt jedoch weiterhin unklar in welchem Maß diese drei RORγt-abhängigen Veränderungen jeweils zur Krankheitsverbesserung beitragen. Wahrscheinlich ist, dass der Schutz aus einem gemischten Phänotyp resultiert, der alle drei

Mechanismen involviert. Mit diesem Aspekt sollten sich weitere Studien detaillierter befassen.

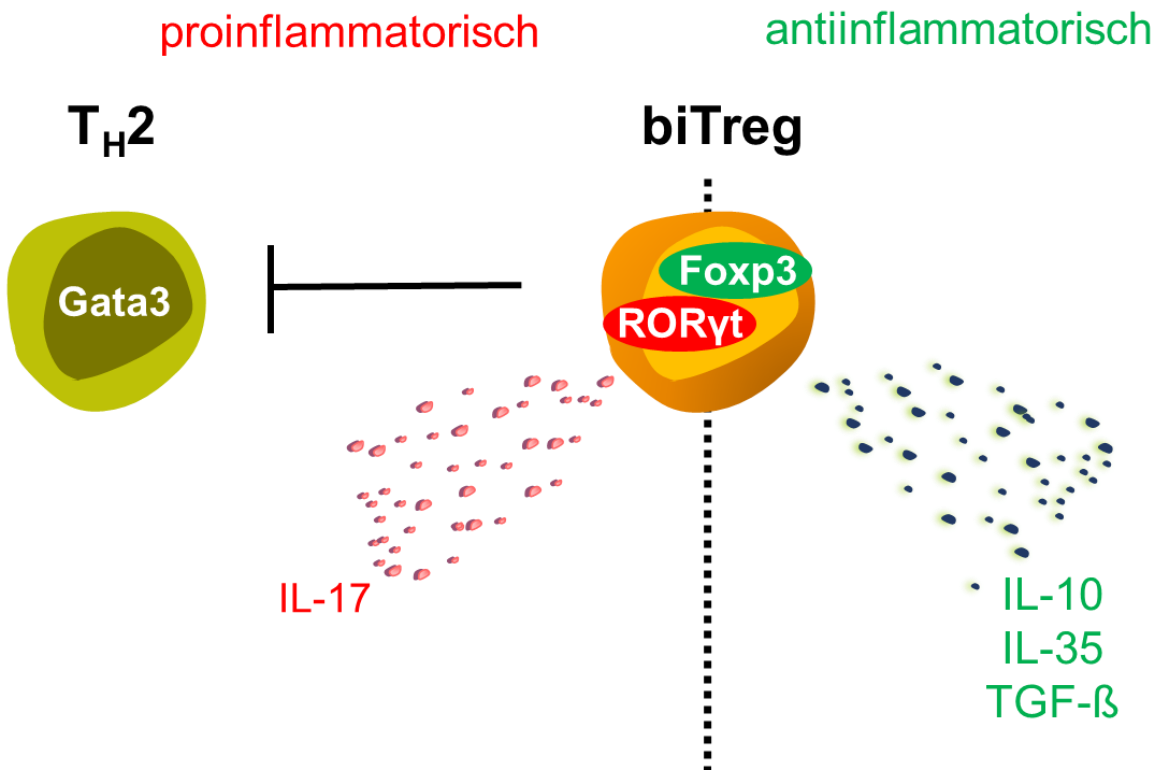

Abbildung 7: Unser aktuelles Verständnis der Bifunktionalität von biTregs: biTregs produzieren Foxp3-abhängig antiinflammatorische Zytokine wie IL-10, IL-35 und TGF-β. Diese protektive Seite der biTregs ist in der rechten Hälfte der Abbildung dargestellt. Auf der anderen Seite sind die durch RORγt vermittelten proinflammatorischen Eigenschaften gezeigt. Dazu gehören die Produktion von IL-17 und die Suppression der in der GN protektiven Th2-Immunität.

Zusammenfassend identifizieren unsere beiden Studien biTregs als neue Mediatoren der Glomerulonephritis, sowohl der akuten Nephritis als auch des chronischen SLE. biTregs stellen eine bis vor kurzem unbekannte, eigenständige und bifunktionelle regulatorische T-Zelllinie dar. Die obige Abbildung 7 soll unser aktuelles Verständnis der Bifunktionalität von biTregs noch einmal veranschaulichen. Insgesamt sind biTregs eine sehr interessante, neue Zelllinie mit großem Potential für zukünftige therapeutische Strategien.

3.4. Fazit

Die Bedeutung spezialisierter T-Zellen, insbesondere vom Th1- und Th17-Typ, für die Pathogenese von Glomerulonephritiden konnte über die letzten Jahre durch zahlreiche Arbeiten, unter anderem aus unserer Abteilung, etabliert werden. Die Aspekte ihrer Gegenregulation, insbesondere durch regulatorische T-Zellen, sind dabei nicht endgültig geklärt. Dies gilt insbesondere für die Frage nach der Existenz und der funktionellen Rolle von neuen Treg Subtypen, die auf die Kontrolle einer bestimmten Immunantwort spezialisiert sind.

Die vier zu dieser kumulativen Dissertationsschrift zusammengefassten Arbeiten beschäftigen sich daher mit dem Vorkommen und der Funktion neuer Subklassen regulatorischer T-Zellen bei immunvermittelten Nierenentzündungen. Die dargestellten Arbeiten haben dazu beigetragen, die Familie der regulatorischen T-Zellen, die man bisher für eine singuläre, pluripotente Population gehalten hatte, um einige spezialisierte Untergruppen wie Treg1, Treg17 und biTregs zu erweitern und sowohl ihre Herkunft als auch ihre spezifischen Funktionen weiter zu charakterisieren. Diese Erkenntnisse liefern wichtige Grundlagen zur Entwicklung neuartiger spezifischerer Therapien und haben perspektivisch somit auch erhebliche klinische Relevanz.

4. Zusammenfassung

Die Bedeutung spezialisierter T-Zellen, insbesondere vom Th1- und Th17-Typ, für die Pathogenese von Glomerulonephritiden (GN) konnte über die letzten Jahre durch zahlreiche Arbeiten, unter anderem aus unserer Abteilung, etabliert werden. Die Aspekte ihrer Gegenregulation, vor allem durch regulatorische T-Zellen (Treg), sind dabei bisher nicht endgültig geklärt. Dies gilt insbesondere für die Frage einer selektiven Spezialisierung von Tregs auf die Kontrolle einer bestimmten Immunantwort, für die es erste Hinweise gibt. Die vier zu dieser kumulativen Dissertationsschrift zusammengefassten Arbeiten beschäftigen sich mit dem Vorkommen und der Funktion neuer Subklassen regulatorischer T-Zellen bei immunvermittelten Nierenentzündungen.

In der ersten Arbeit wurde die Rolle von Th1-spezifischen regulatorischen T-Zellen (Treg1) untersucht. In einem spezifischen Knockout-Mausmodell konnte gezeigt werden, dass die Funktion dieser Zellen von dem Transkriptionsfaktor T-bet abhängig ist. Interessanterweise ist T-bet aber auch für die Generierung pathogener Th1-Zellen essentiell, die physiologisch über ihre lFNγ Sekretion zwar Pathogene abwehren, bei zahlreichen Erkrankungen jedoch mit Gewebeschädigung und der Entwicklung von Autoimmunität assoziiert werden konnten. Das Fehlen von Treg1-Zellen führte in unseren Analysen zu einer ausgeprägten Überhöhung der Th1-Antworten mit verschlechterter Nierenfunktion und massiv verstärktem Nierenschaden im Rahmen einer akut-nekrotisierenden Glomerulonephritis. Detaillierte Analysen von Treg-Zellen aus $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ Mäusen zeigten unveränderte Zytokinproduktion und Suppressionskapazität. In kompetitiven Transferexperimenten konnten die Wildtyp-Tregs ihre T-bet-defizienten Gegenspieler jedoch deutlich auskonkurrieren, was die Expansion und die Expressionslevel des Foxp3-Proteins betrifft. Das spricht dafür, dass T-bet für die generelle Fitness von Tregs wichitg ist. Außerdem konnte gezeigt werden, dass T-bet exklusiv für die Expression des Traffickingrezeptors CXCR3 auf Tregs zuständig ist und T-bet auf diesem Wege die Lokalisation von Tregs in der entzündeten Niere vermittelt. CXCR3 ist auch auf Th1-Zellen ein charakteristischer Chemokinrezeptor, so dass die Spezialisierung von Treg1-Zellen vermutlich im Wesentlichen durch die CXCR3-vermittelte Kolokalisation der spezialisierten Treg-Zelle mit ihrem Th1-Gegenspieler definiert wird. Zusammengefasst gelang die Identifikation und funktionelle

Charakterisierung einer neuartigen, auf die Abwehr von Th1-Zellen spezialisierten regulatorischen T-Zellsubpopulation bei entzündlichen Nierenerkrankungen im experimentellen Modell der akuten Glomerulonephritis.

Die zweite Arbeit untersucht die Rolle von Th17-spezifischen regulatorischen T-Zellen (Treg17) untersuchen. Im Modell der akuten GN konnte bereits in einer vorhergehenden Arbeit unserer Gruppe, in einem spezifischen Knockout-Mausmodell gezeigt werden, dass die Funktion dieser Zellen von dem Transkriptionsfaktor Stat3 abhängig ist. Dies folgt dem gleichen Prinzip wie für die Treg1-Zellen beschrieben. Auch Stat3 ist für die Generierung pathogener Th17-Zellen essentiell, die physiologisch über ihre IL-17 Sekretion Pathogene abwehren, aber ebenfalls bei zahlreichen Erkrankungen mit Autoimmunität assoziiert werden konnten. Auch das Fehlen von Treg17 Zellen führte zu einer Überhöhung der Th17-Antwort mit verschlechterter Nierenfunktion und massiv verstärktem Nierenschaden im Modell der akut-nekrotisierenden Glomerulonephritis. Wie bei den Treg1-Zellen für den CXCR3 gezeigt, scheint Stat3 ebenfalls exklusiv für die Expression eines Traffickingrezeptors auf Tregs zuständig zu sein und zwar bei den Treg17-Zellen für den CCR6. Genau wie der CXCR3 auf Th1-Zellen, ist auch der CCR6 auf Th17-Zellen ein charakteristischer Chemokinrezeptor, so dass die Spezialisierung von Treg17-Zellen vermutlich im Wesentlichen durch die CCR6-vermittelte Kolokalisation der spezialisierten spezialisierten Treg-Zelle mit ihrem Th17 Gegenspieler definiert wird. In der hier vorgestellten Arbeit sollte die Bedeutung von Treg17 Zellen beim systemischen Lupus erythematodes (SLE) als endogen autoimmun entstehender chronischer Systemerkrankung klären, wo auch Autoantikörper eine Rolle spielen. Auch während der durch Pristan-Öl induzierbaren systemischen Lupuserkrankung zeigte sich in den Treg17-defizienten Mäusen ein im Vergleich zur Kontrollgruppe deutlich aggravierter Verlauf mit verstärkten Th17-Antworten, stärker ausgeprägter pulmonaler Kleingefäß-Vaskulitis mit erhöhter Mortalität sowie verschlechterter Lupus-Nephritis im Langzeitverlauf über neun Monate. Interessanterweise waren die humoralen Immunantworten, also anti-RNP und anti-dsDNA-Autoantikörperproduktion sowie die renalen Komplement- und Immunglobulin-Depositionen, weitgehend unverändert. Dies betont noch einmal die Bedeutung von T-Zellen und insbesondere der Th17-Antworten in dieser Autoantikörper-vermittelten Erkrankung. Diese Arbeit erweitert die Erkenntnisse über Treg17-Zellen auf eine

weitere Klasse von Systemerkrankungen und verbessert unser Verständnis ihrer Pathogenese.

Die dritte Arbeit sollte anschließend die unbekannte Rolle des zweiten Th17-definierenden Transkriptionsfaktors, RORγt, in regulatorischen T-Zellen charakterisieren. Überraschend konnten wir diese RORγt^{+}Foxp3^{+} Zellen (biTregs) als gut abgrenzbare Population und in relevanter Frequenz bereits in unbehandelten Wildtypmäusen nachweisen. Die Dynamik von biTregs wurde dann in Kinetikstudien im Rahmen einer experimentellen akuten Glomerulonephritis untersucht. Sie zeigten dabei einen ganz anderen Zeitverlauf als konventionelle RORγt-negative Tregs mit massiver früher Expansion. Bemerkenswerterweise zeigten unsere Analysen, dass biTregs sowohl antiinflammatorische (IL-10, IL-35) als auch proinflammatorische (IL-17) Zytokine produzieren. Außerdem zeigten Expressoinsanalysen eine von Th17-Zellen und cTregs unterschiedliche Oberflächenmolekül- und Transkriptionsfaktorsignatur. Aus fluoreszenzmarkierten Doppelreportermäusen für RORγt und Foxp3 wurden daraufhin biTregs isoliert und weiter charakterisiert. Durch Transferstudien konnten wir belegen, dass biTregs weder aus konventionellen Tregs, noch aus Th17-Zellen entstehen. biTregs stellen also keinen Transdifferenzierungs-zustand dar, sondern repräsentieren eine eigenständige und unabhängige Zellpopulation. Der therapeutische Transfer von biTregs war in der Lage den Verlauf der Nephritis deutlich abzumildern, in einem ähnlichen Maße wie cTregs. Die genetische Ablation von RORγt in endogenen Tregs im gleichen Modell zeigte jedoch protektive Effekte. Diese scheinen durch die RORγt-abhängige IL-17-Sekretion der biTregs bedingt zu sein. Zusammengefasst identifiziert diese Arbeit RORγt^{+}Foxp3^{+} biTregs als eine neuartige, unabhängige, regulatorische T-Zell-Linie, die im Rahmen von Glomerulonephritiden sowohl pro- als auch antiinflammatorische Effekte vermitteln kann und eventuell ein neues therapeutisches Ziel darstellt.

Nach den Untersuchungen im akuten Modell der NTN-Nephritis, wollten wir die RORγt^{+}Foxp3^{+} biTregs im Modell des Pristan-induzierten SLE untersuchen. Dieses Modell ähnelt dem humanen SLE signifikant und es sollte so die klinische Relevanz der biTregs weiter bestätigt werden. Auch unsere Analysen im Pristanmodell zeigten eine charakteristische zeitliche und organspezifische biTreg Expansion, welche mit der Entwicklung der Autoimmunität und des Gewebeschadens zusammen fällt. Außerdem fanden wir auch hier, wie im NTN-Modell bereits beschrieben, dass eine

biTreg-selektive Deletion von RORγt in Verlust der IL-17-Sekretion und einer erhöhten Treg-Aktivierung resultiert. Zusätzlich fanden wir ebenfalls eine gesteigerte Th2-Immunität. Diese Veränderungen waren mit einem Schutz vor der pulmonalen Vaskulitis und der Lupus-Nephritis verbunden. Unsere Studie identifiziert biTregs also als neue Spieler im SLE und befürwortet RORγt gerichtete Interventionen als vielversprechende therapeutische Strategien.

5. Summary

Over the last years, the relevance of specialized T cells, especially Th1 and Th17 cells, for the pathogenesis of glomerulonephritides (GN) was established by many studies from different groups, including our own. However, the aspects of their counterregulation, particularly by regulatory T cells, are not yet fully understood. This is especially true for the selective specialization of Tregs to control a defined immune response, wherefore there is first evidence. The four publications that are combined to this cumulative dissertation deal with the appearance and function of new subclasses of regulatory T cells in immune mediated glomerular diseases.

In the first study the role of Th1-specific regulatory T cells (Treg1) was assessed. In a specific knockout mouse model we could show that the function of these cells depends on the transcription factor T-bet. Interestingly, T-bet is also essential for the generation of pathogenic Th1 cells, which physiologically fight pathogens by their secretion of IFNγ but are also associated with tissue injury and development of autoimmunity in many diseases. The lack of Treg1 cells led to a distinctive increase of Th1 responses with impaired renal function and massively increased renal damage in the nephrotoxic nephritis (NTN) model of crescentic GN (cGN). Deatiled analyses of Treg cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice revealed unaltered cytokine production and suppressive capacity. However, in competitive cotransfer experiments, wild-type Treg cells outcompeted T-bet-deficient Treg cells in terms of population expansion and expression levels of Foxp3, indicating that T-bet expression is crucial for general Treg fitness. Additionally, T-bet-deficient Treg cells lacked expression of the Th1-characteristic trafficing receptor CXCR3, which correlated with significant impairment of renal Treg infiltration. In summary, our data indicate a new subtype of Treg cells in cGN. These Treg1 cells are characterized by activation of the transcription factor T-bet, which enhances the overall fitness of these cells and optimizes their capacity to downregulate Th1 responses by inducing chemokine receptor CXCR3 expression.

The second study addressed the role of Th17-specific regulatory T cells (Treg17). In a previous study using a model of acute GN our group could already show with a specific knockout mouse model, that the function of these cells depends on the transcription factor Stat3. This follows the same principle as described for the Treg1 cells. Stat3 is also essential for the generation of pathogenic Th17 cells which physiologically put down pathogens by their secretion of IL-17 but are also

associated with tissue injury and development of autoimmunity in many diseases. Lack of Treg17 cells also led to a distinctive increase of Th17 responses with impaired renal function and massively increased renal damage in the nephrotoxic nephritis (NTN) model of cGN. As shown for the CXCR3 on Treg1 cells, Stat3 also seems to exclusively be responsible for the expression of a trafficking receptor. In case of the Treg17 cells this seems to be CCR6. Like the CXCR3 on Th1 cells CCR6 is also a typical chemokine receptor on Th17 cells. So the specialization of Treg17 cells seems to be basically defined threw CCR6-mediated colocalization with their Th17 counterpart. The study presented here investigated the meaning of Treg17 cells in systemic lupus erythematodes (SLE) as an autoimmune developing chronic systemic disease, where also autoantibodies play a role. Also during this pristane inducible systemic disease Treg17-deficient mice, compared to control group, showed a significantly aggravated course with enhanced Th17 responses, stronger developed pulmonary vasculitis and higher mortality. Also long-term lupus nephritis after 9 month was significantly worsened. Interestingly, humoral immune responses, as anti-RNP and anti-dsDNA autoantibodies as well as renal complement and immunoglobulin deposition, were largely unchanged. This again highlights the meaning of T cells and especially Th17 responses in this autoantibody mediated disease. Thus, Stat3-induced Treg17 cells are novel antiinflammatory mediators of SLE.

Subsequently, the third study was supposed to characterize the hitherto unknown role of the second Th17 defining transcription factor RORγt in regulatory T cells. Surprisingly, we were able to detect these RORγt^{+}Foxp3^{+} cells (biTregs) as a well definable population in a relevant frequency already in naïv wildtype mice. The dynamics of biTregs were assessed by kinetic studies in the nephrotoxic nephritis (NTN) model of acute cGN. They showed a time response completely different from that of conventional RORγt negative Tregs with a massive early expansion. Notably, analyses of the biTreg expression profile revealed production of both anti-inflammatory (IL-10, IL-35) and proinflammatory (IL-17) cytokines. Additionally, biTregs expressed a signature of surface molecules and transcription factors distinct from those of Th17 cells and conventional Tregs. We then isolatetd biTregs from RORγt Foxp3 double reporter mice and further characterized them. Via transfer studies we could show that biTregs do not derive from conventional Tregs, nor from Th17 cells. So biTregs are not a transdifferentiation state, but represent a discrete

and independent cell population. Therapeutic transfer of biTregs suppressed the development of nephritis to an extent similar to that observed with transferred cTregs. Intriguingely, endogenous biTregs displayed additional proinflammatory functions in NTN that were abrogated by cell-specific deletion of RORγt. This seems to be due to their IL-17 secretion. In summary, this study provides evidence that RORγt^{+}Foxp3^{+} biTregs are a novel and independent bifunctional regulatory T cell lineage distinct from cTregs, Treg17 cells, and Th17 cells. Furthermore, biTregs appear to contribute to crescentic GN and hence may be a novel therapeutic targets.

After our studies in the acute model of NTN-nephritis, we also wanted to investigate biTergs in the chronic model of pristane-induced SLE. This model significantly resembles human SLE and we thereby wanted to acknowledge their clinical relevance. Our analyses in the pristane-model also revealed expansion of IL-17 producing biTregs in a distinctive time-course and organ-specific pattern, coincident with the development of autoimmunity and tissue injury. As already shown in the NTN-model, we again found that IL-17 secretion by biTregs was abrogated completely in Foxp3^{Cre}xRorc$^{fl/fl}$ mice. Furthermore, Tregs showed a more activated phenotype after cell-specific inactivation of RORγt signaling. Finally, and remarkably, we found elevated Th2 immunity in the knockout mice, meaning biTregs seem to suppress antiinflammatory Th2 immunity in a RORγt-dependent manner. These modifications in Foxp3^{Cre}xRorc$^{fl/fl}$ mice were associated with significant amelioration of pristane-induced pulmonary vasculitis and lupus nephritis. Our study thus identifies biTregs as novel players in SLE and advocates RORγt-directed interventions as promising therapeutic strategies.

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7. Angaben zum individuellen Anteil

Alle im Rahmen dieser Dissertationsschrift beschriebenen Arbeiten sind das Resultat von Kollaborationen mit anderen Wissenschaftlern, deren Namen der jeweiligen Autorenliste zu entnehmen sind. Die Gewichtung der individuellen Leistungen wurde mit allen jeweiligen Koautoren abgesprochen und ist einvernehmlich geschehen. Zu den vorgestellten Arbeiten habe ich einen maßgeblichen Anteil beigetragen, den ich im Folgenden kurz erläutern möchte.

T-Bet Enhances Regulatory T Cell Fitness and Directs Control of Th1 Responses in Crescentic GN

Die Experimente zu dieser Publikation wurden gemeinsam mit Priv.-Doz. Dr. Oliver M. Steinmetz geplant. Den konkreteren Ablauf der Experimente habe ich selbstständig geplant und durchgeführt. Bei den aufwendigeren Experimenten wurde ich von Priv.-Doz. Dr. Malte A. Kluger, Paul Diefenhardt oder Dr. Simon Melderis unterstützt. Die erhaltenen Daten habe ich selbstständig ausgewertet und anschließend gemeinsam mit Priv.-Doz. Dr. Oliver M. Steinmetz kritisch bewertet und interpretiert. Das Manuskript wurde gemeinsam mit Priv.-Doz. Dr. Oliver M. Steinmetz verfasst.

Treg17 cells are programmed by Stat3 to suppress Th17 responses in systemic lupus

Für diese Arbeit wurden die Experimente hauptsächlich von Priv.-Doz. Dr. Malte A. Kluger in Zusammenarbeit mit Priv.-Doz. Dr. Oliver M. Steinmetz geplant. Ich habe Priv.-Doz. Dr. Malte A. Kluger bei der Durchführung einiger Experimente unterstützt und im späteren Studienverlauf einige abschließenden Experimente selbst durchgeführt und analysiert. Das Manuskript wurde von Priv.-Doz. Dr. Malte A. Kluger gemeinsam mit Priv.-Doz. Dr. Oliver M. Steinmetz verfasst. Ich habe einige finale Korrekturen vorgenommen.

RORγt^{+}Foxp3^{+} Cells are an Independent Bifunctional Regulatory T Cell Lineage and Mediate Crescentic GN

Für diese Arbeit wurden die Experimente hauptsächlich von Priv.-Doz. Dr. Malte A. Kluger in Zusammenarbeit mit Priv.-Doz. Dr. Oliver M. Steinmetz geplant, im späteren Studienverlauf habe ich mich an der Planung einiger Experimente beteiligt. Ich habe Priv.-Doz. Dr. Malte Kluger und Mathias C. Meyer bei der Durchführung einiger Experimente unterstützt und im späteren Studienverlauf einige abschließenden Experimente selbst durchgeführt und analysiert. Das Manuskript

wurde von Priv.-Doz. Dr. Malte A. Kluger und Matthias C. Meyer gemeinsam mit Priv.-Doz. Dr. Oliver M. Steinmetz verfasst. Ich habe einige finale Korrekturen vorgenommen.

RORγt expression in Tregs promotes systemic lupus erythematosus via IL-17 secretion, alteration of Treg phenotype and suppression of Th2 responses

Die Experimente zu dieser Publikation wurden von Priv.-Doz. Dr. Malte A. Kluger und mir gemeinsam mit Priv.-Doz. Dr. Oliver M. Steinmetz geplant. Den konkreteren Ablauf der Experimente haben Priv.-Doz. Dr. Malte A. Kluger und ich gemeinsam geplant und uns die Durchführung aufgeteilt. Bei den aufwendigeren Experimenten wurden wir teilweise von Torben Ramcke unterstützt. Die erhaltenen Daten habe ich selbstständig ausgewertet und anschließend gemeinsam mit Priv.-Doz. Dr. Malte A. Kluger und Priv.-Doz. Dr Oliver M. Steinmetz kritisch bewertet und interpretiert. Das Manuskript wurde gemeinsam mit Priv.-Doz. Dr. Malte A. Kluger verfasst, dabei wurden wir von Priv.-Doz. Dr. Oliver M. Steinmetz unterstützt.

Priv.-Doz. Dr. Oliver M. Steinmetz

8. Danksagung

Zuerst möchte ich mich bei Herrn Prof. Dr. Rolf A. K. Stahl für die Möglichkeit bedanken, meine Arbeit in seiner Abteilung durchzuführen.

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9. Publikationen und Konferenzen

Publikationen

Nosko A[1], Kluger MA[1], Ramcke T, Goerke B, Meyer MC, Wegscheid C, Luig M, Tiegs G, Stahl RAK, Steinmetz OM: RORγt expression in Tregs promotes systemic lupus erythematosus via IL-17 secretion, alteration of Treg phenotype and suppression of Th2 responses. Clin Exp Immunol. 2016. Epub ahead of print, doi: 10.1111/cei.12905.

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Lux M, Andrée B, Horvath T, Nosko A, Manikowski D, Hilfiker-Kleiner D, Haverich A, Hilfiker A: In vitro maturation of large-scale cardiac patches based on a perfusable starter matrix by ciclic mechanical stimulation. *Acta biomater*, 2016. 30: p. 177-87. (Masterarbeit)

Kluger MA, Melderis S, Nosko A, Goerke B, Luig M, Meyer M, Turner JE, Meyer-Schwesinger C, Wegscheid C, Tiegs G, Stahl RAK, Panzer U, Steinmetz OM: Treg17 Cells are Programmed by Stat3 to Suppress Th17 Responses in Systemic Lupus. *Kidney International*, 2016. 89(1): p. 158-66.

Kluger MA, Meyer MC, Nosko A, Goerke B, Luig M, Wegscheid C, Tiegs G, Stahl RA, Panzer U, Steinmetz OM: RORgammat+Foxp3+ Cells are an Independent Bifunctional Regulatory T Cell Lineage and Mediate Crescentic GN. *J Am Soc Nephrol*, 2015. 27(2): p. 454-65.

Luig M, Kluger MA, Goerke B, Meyer M, Nosko A, Yan I, Scheller J, Mittrucker HW, Rose-John S, Stahl RA, Panzer U, Steinmetz OM: Inflammation-Induced IL-6 Functions as a Natural Brake on Macrophages and Limits GN. *J Am Soc Nephrol*, 2015. 26(7): p. 1597-607.

Konferenzen

Nosko A, Diefenhardt P, Kluger MA, Melderis S, Torben Ramcke, Frederick Feindt Wegscheid C, Tiegs G, Stahl RA, Panzer U, Steinmetz OM: “IL-10 empowers Tregs to control Th17 immunity and protects from Glomerulonephritis”
DGfI Jahreskongress, Hamburg 2016, Vortrag

Nosko A, Kluger MA, Diefenhardt P, Melderis S, Wegscheid C, Tiegs G, Stahl RA, Panzer U, Steinmetz OM: “T-bet Enhances Treg Fitness and Directs Control of Th1 Responses in Crescentic GN”
American Society of Nephrology, ASN renal week, San Diego 2015, Vortrag

Nosko A, Kluger MA, Diefenhardt P, Melderis S, Wegscheid C, Tiegs G, Stahl RA, Panzer U, Steinmetz OM: “T-bet enhances regulatory T cell fitness and directs control of Th1 responses in crescentic glomerulonephritis”
DGfN Jahreskongress, Berlin 2014, Posterpräsentation (Posterpreis)

10. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbstständig und ohne fremde Hilfe verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, Februar 2017

Anna Nosko

11. Anhang

T-Bet Enhances Regulatory T Cell Fitness and Directs Control of Th1 Responses in Crescentic GN

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ABSTRACT

Th1 cells are central pathogenic mediators of crescentic GN (cGN). Mechanisms responsible for Th1 cell downregulation, however, remain widely unknown. Recently, it was proposed that activation of the Th1–characteristic transcription factor T-bet optimizes Foxp3$^+$ regulatory T (Treg) cells to counteract Th1-type inflammation. Because very little is known about the role of T-bet$^+$ Treg1 cells in inflammatory diseases, we studied the function of these cells in the nephrotoxic nephritis (NTN) model of cGN. The percentage of Treg1 cells progressively increased in kidneys of nephritic wild–type mice during the course of NTN, indicating their functional importance. Notably, naïve Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, lacking Treg1 cells, showed spontaneous skewing toward Th1 immunity. Furthermore, absence of Treg1 cells resulted in aggravated NTN with selectively dysregulated renal and systemic Th1 responses. Detailed analyses of Treg cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice revealed unaltered cytokine production and suppressive capacity. However, in competitive cotransfer experiments, wild–type Treg cells outcompeted T-bet–deficient Treg cells in terms of population expansion and expression levels of Foxp3, indicating that T-bet expression is crucial for general Treg fitness. Additionally, T-bet–deficient Treg cells lacked expression of the Th1–characteristic trafficking receptor CXCR3, which correlated with significant impairment of renal Treg infiltration. In summary, our data indicate a new subtype of Treg cells in cGN. These Treg1 cells are characterized by activation of the transcription factor T-bet, which enhances the overall fitness of these cells and optimizes their capacity to downregulate Th1 responses by inducing chemokine receptor CXCR3 expression.

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Multiple studies from the past have established key pathogenic roles for Th1 and Th17 responses in initiation and perpetuation of crescentic GN (cGN).[1–8] These observations have sparked growing interest in mechanisms counter-regulating nephritogenic Th1 and Th17 immunity. To this end, it has recently been proposed that specialized regulatory T (Treg) cell subtypes might exist, which are tailor made to downregulate distinct T helper cell lineages.[9–11] Interestingly, these lineage–specific Treg cells seem to achieve their specialization by activating some of the same transcription factors that are needed for programming of their proinflammatory counterparts. Cells specialized at downregulating Th17 responses, for example, coactivate Foxp3 together with the Th17–characteristic transcription factor Stat3. These Treg17 cells effectively control Th17 responses, and their absence leads to selectively overshooting Th17 immunity.[12] In a recent study, our group could also prove a protective role for Stat3–dependent Treg17 cells in acute cGN.[13] As one main mechanism enabling them to target Th17 responses, we identified expression of the chemokine receptor CCR6. This allows colocalization of Treg17 with Th17 cells, which also highly express the CCR6.[14,15] Interestingly,

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induction of a Treg17 phenotype is specifically achieved by Stat3 activation. In contrast, Foxp3$^+$ cells coexpressing the second Th17 master transcription factor RORγt constitute an independent and bifunctional T cell lineage, which we have recently characterized and termed biTreg cells.[16] With respect to regulation of Th1 responses, pioneering studies by Koch *et al.*[17,18] have suggested that Treg cell intrinsic expression of the master Th1 transcription factor T-bet optimizes them to target Th1 cells. Given their specialization for counter-regulation of Th1 responses, we will herein refer to T-bet–expressing Treg cells as Treg1 cells. In analogy to Treg17 cells, T-bet$^+$ Treg1 cells also seem to be equipped to traffic into the same areas as their proinflammatory Th1 counterparts. Close colocalization in inflamed tissues might be achieved by shared expression of the Th1–characteristic chemokine receptor CXCR3,[19] which has also been shown by us to mediate renal trafficking of Th1 cells in cGN.[20] In line with this hypothesis, various studies have shown coincidental upregulation of CXCR3 and T-bet on Treg cells during Th1-type inflammation caused by infection, cancer, contact hypersensitivity, autoimmunity, or organ transplantation in mice[21–24] and humans.[25–28] Although all of these studies point toward a role of T-bet in Treg cells for enhancing their immunosuppressive capacity specifically against Th1 responses, a recent study has reported opposing effects. Stimulation of Treg cells with the cytokine IL-12 from subjects with multiple sclerosis resulted in increased T-bet expression. However, *in vitro* suppressive capacity of these Th1–type T-bet$^+$ Treg cells was significantly reduced.[29] The functional role of T-bet activation in Treg cells thus remains elusive. In addition, because Foxp3^{Cre} and T-bet$^{fl/fl}$ mice have only recently become available, none of the above studies directly evaluated the role of Treg cell–expressed T-bet but rather, used adoptive transfer models or merely reported associations. To this end, two studies have been published recently during preparation of this manuscript. Yu *et al.*[30] reported that Treg cell–selective loss of T-bet did not result in development of spontaneous autoimmunity in mice. The potential role of T-bet$^+$ Treg cells for downregulation of Th1 responses at steady state or induced Th1–type inflammation, however, was not studied by Yu *et al.*[30] The second manuscript examined the murine EAE model of multiple sclerosis.[31] Surprisingly, the authors[31] did not find any relevant effects of T-bet deficiency in Treg cells for development and severity of central nervous system inflammation. As in the study by Yu *et al*,[30] effects of T-bet expression in Treg cells on Th1 immunity were not assessed, leaving this aspect unclear.

In summary, many questions regarding T-bet$^+$ Treg cells remain unanswered, including their functional relevance in Th1-mediated inflammation. This study, therefore, aimed to address the following aspects: (*1*) identify and characterize T-bet$^+$ Treg cells in experimental GN, (*2*) clarify the role of T-bet for Treg1 cell generation, (*3*) define the role of Treg1 cells for Th1 homeostasis, (*4*) study the role of T-bet$^+$ Treg1 cells in experimental GN, and (*5*) investigate Treg1 cell mechanisms of action.

RESULTS

A Functionally Distinct T-Bet$^+$ Treg Subset Expands during cGN

To investigate whether T-bet–expressing Treg1 cells might play a role in cGN, we studied spleens and kidneys of mice at different time points after induction of nephrotoxic nephritis (NTN). Indeed, our analyses showed robust T-bet activation in a subset of Foxp3$^+$ Treg cells in both nephritic kidneys (Figure 1A) and spleens. Interestingly, however, Treg1 cell dynamics showed organ-specific differences. Both renal Treg1 cells (Figure 1B) and T-bet$^-$ Treg cells (Supplemental Figure 1A) expanded continuously over the complete observation period up to 49 days after NTN induction. Importantly, however, Treg1 cell percentages steadily increased not only among CD4$^+$ T cells but also, within the Foxp3$^+$ Treg cell population (Figure 1B). The percentages of T-bet$^-$ Treg cells among Foxp3$^+$ cells, in contrast, decreased over time (Supplemental Figure 1B). Different from renal Treg cells, splenic Treg1 cells (Figure 1C) and T-bet$^-$ Treg cells (Supplemental Figure 1C) expanded to reach a maximum among CD4$^+$ T cells at around 10 days after NTN induction and subsequently retracted. Within the splenic Foxp3$^+$ population, Treg1 percentages also peaked at day 10 (Figure 1C), resulting in consecutively reduced percentages of T-bet$^-$ Treg cells at this time point (Supplemental Figure 1D). It is noteworthy that the dynamics of Treg1 cells paralleled those of Th1 cells in both organs (Figure 1, B and C). Detailed analyses of surface molecule expression associated with regulatory function and activation revealed a unique signature on Treg1 cells, highlighting their independent character. Although CD25, ICOS, and GITR were expressed at lower levels, Treg1 cells showed enhanced expression of CD103, PD-1, CD44, and CD62L in comparison with T-bet$^-$ Treg cells (Figure 1D).

Loss of T-Bet Expression in Treg Cells Results in a Spontaneous Hyper–Th1 Phenotype

To study the function of T-bet$^+$ Treg1 cells, we generated Foxp3^{Cre}xT-bet$^{fl/fl}$ mice. FACS (Becton Dickinson, San Diego, CA) analyses confirmed specific absence of T-bet in Foxp3$^+$ Treg cells in both spleens and blood (Figure 2, A and B). Treg1–deficient Foxp3^{Cre}xT-bet$^{fl/fl}$ mice were viable and fertile, and they did not reveal abnormalities in spleen cell numbers (Supplemental Figure 2A), CD3$^+$, CD4$^+$, or Foxp3$^+$ T cell subset composition in spleens or blood at steady state (Supplemental Figure 2, B and C). Interestingly, however, we found elevated percentages of T-bet$^+$ and IFNγ^+ Th1 cells in spleens (Figure 2C) and even more so in the blood (Figure 2D) of Treg1 cell–deficient mice. Th17 responses in contrast were unchanged as shown by similar frequencies of RORγt– and IL-17–expressing Th17 cells (Supplemental Figure 2, D and E). In line with enhanced Th1 responses, expression of the Th1–characteristic chemokine receptor CXCR3 was upregulated on blood T helper cells in Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, whereas Th17–characteristic CCR6 expression was reduced (Figure 2E). Splenocyte production of various cytokines, including Treg cell–associated IL-10 and TGF-β, was not different between

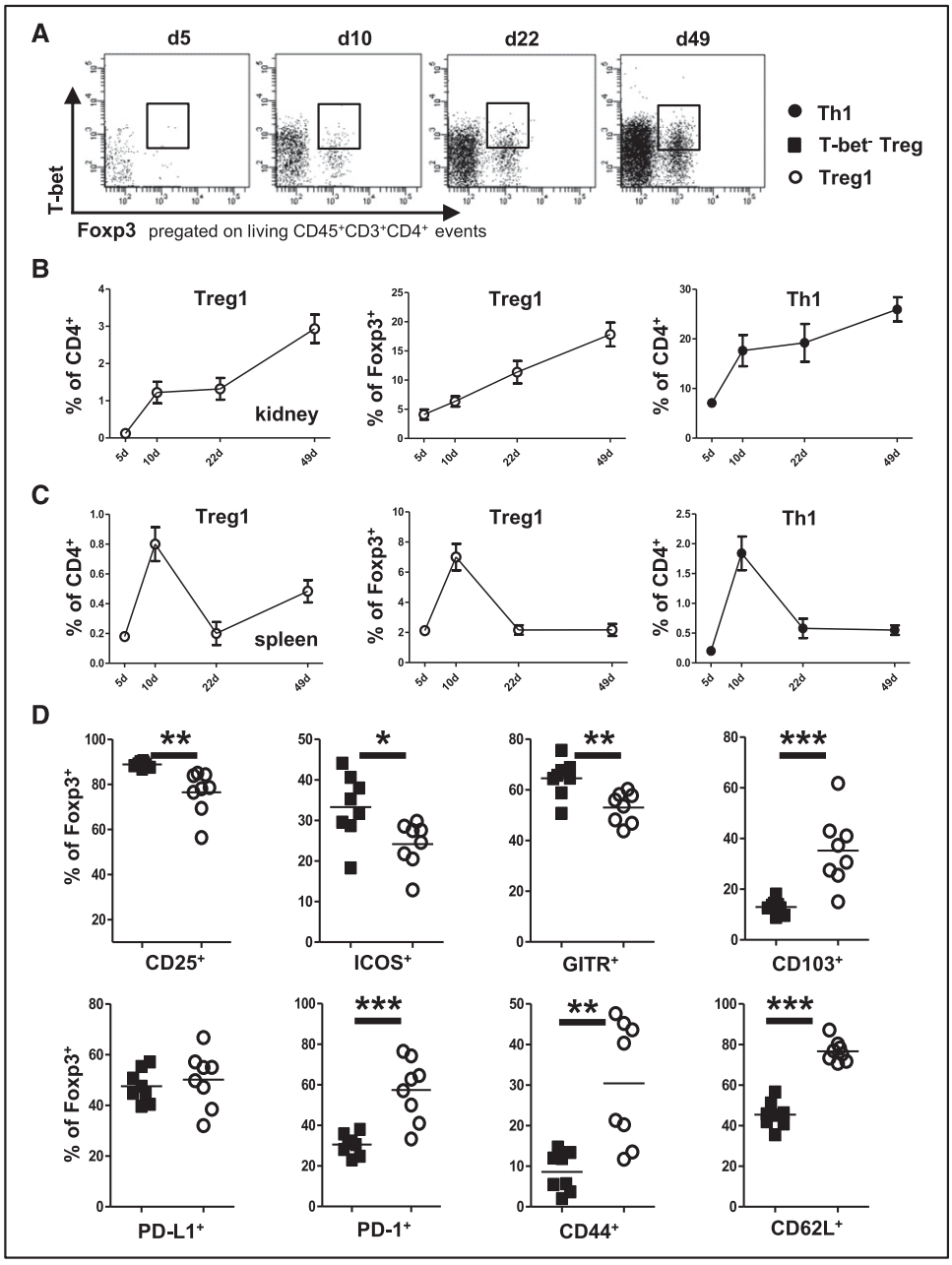

Figure 1. T-bet$^+$ Treg cells expand during cGN. (A) Representative FACS plots showing renal T-bet$^+$Foxp3$^+$ Treg1 cells at the indicated time points after induction of NTN. (B and C) Quantification of (B) renal and (C) splenic T-bet$^+$Foxp3$^+$ Treg1 and T-bet$^+$Foxp3$^-$ Th1 cells as percentages of CD4$^+$ or Foxp3$^+$ cells at the indicated time points after induction of NTN; $n \geq 5$ C57BL/6 mice were analyzed at each time point. (D) FACS analysis of surface molecule expression on the indicated splenic Treg cell populations at day 8 after induction of NTN; $n=8$ C57BL/6 mice were analyzed. Circles in D represent individual animals, and horizontal lines represent mean values. Error bars represent SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

the groups (Supplemental Figure 2F). However, similar to cellular immunity, humoral immune responses were also skewed toward Th1. Levels of total IgG were reduced in Foxp3^{Cre}xT-bet$^{fl/fl}$ mice (Figure 2F) as a result of drastically impaired production of the Th2–characteristic IgG1 and IgG3 subclasses (Figure 2G), which are known to be suppressed by Th1-derived IFNγ. Th1–dependent IgG2c antibody levels, in contrast, remained unchanged (Supplemental Figure 2G). Importantly, the observed hyper–Th1 phenotype did not result in spontaneous renal pathology as shown by normal renal histology (Supplemental Figure 2, H and I) and absence of albuminuria (Supplemental Figure 2J).

Nephritogenic Renal and Systemic Immunities Are Skewed toward Th1 in Treg1-Deficient Mice

Next, we induced NTN in Foxp3^{Cre}xT-bet$^{fl/fl}$ and Foxp3^{Cre} control mice. Analyses at day 15 revealed slightly reduced spleen cell

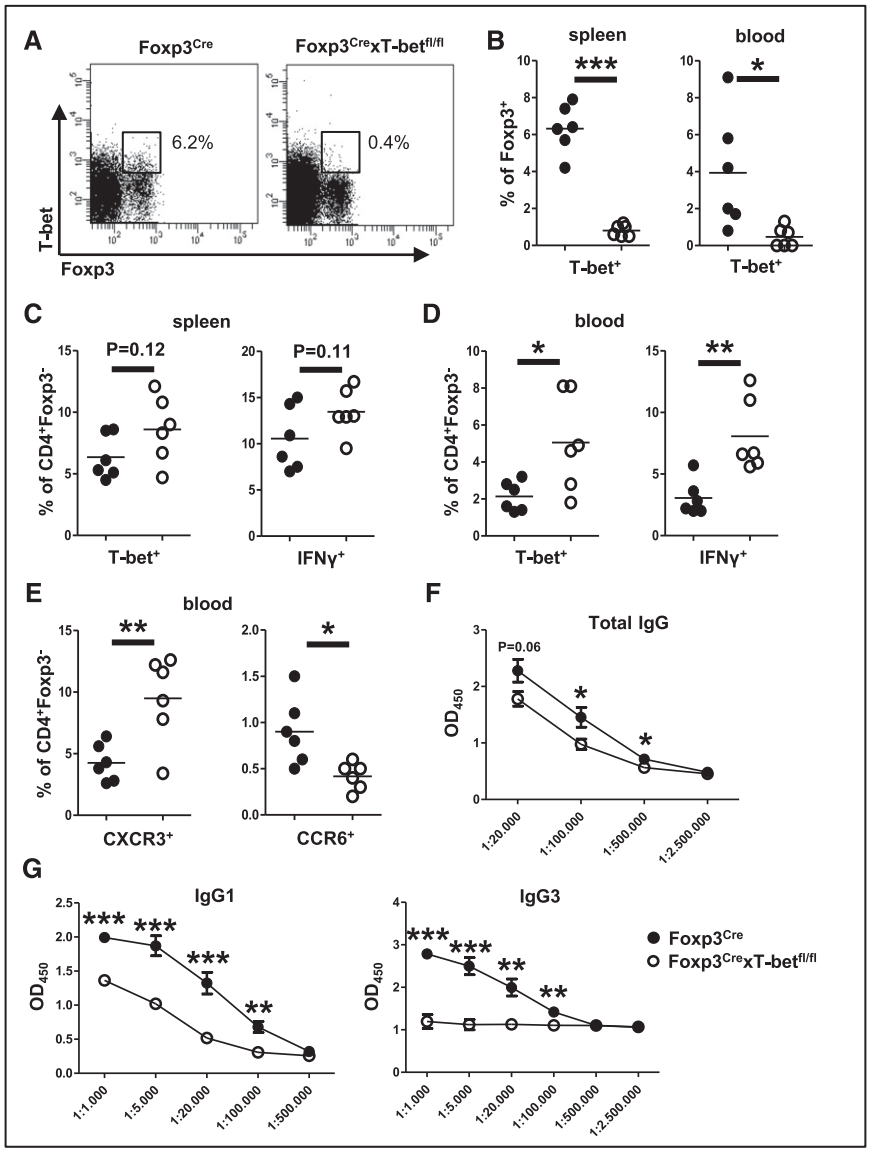

Figure 2. Spontaneous hyper–Th1 phenotype in Foxp3^{Cre}xT-bet$^{fl/fl}$ mice. (A) Representative FACS plots of splenic T-bet$^+$Foxp3$^+$ Treg1 cells from naïve mice of the indicated genotypes. (B) Quantification of T-bet$^+$Foxp3$^+$ Treg1 cells in spleens and blood of naïve mice. (C and D) Quantification of (C) splenic and (D) blood T-bet$^+$ or IFNγ^+ Foxp3$^-$ Th1 cells as indicated. (E) Quantification of chemokine receptor CXCR3 and CCR6 expression on Foxp3$^-$ T helper cells in the blood of naïve mice. (F and G) Levels of total IgG, IgG1, and IgG3 subclasses in the serum of naïve mice. ELISA data are shown as OD at 450 nm in serial dilutions as indicated. Numbers in FACS plots represent percentages of Foxp3$^+$ cells; n=6 versus six animals for all analyses. Circles in B–E represent individual animals, and horizontal lines represent mean values. Error bars represent SEM. *P<0.05; **P<0.01; ***P<0.001.

numbers in the absence of Treg1 cells (Figure 3A) with unchanged CD3$^+$ and CD4$^+$ T cell percentages (Supplemental Figure 3A). Importantly, both spleens (Figure 3B) and kidneys (Figure 3, C and D) showed significantly enhanced IFNγ^+ Th1 cell frequencies, whereas IL-17$^+$ Th17 cells remained unchanged. In line, renal infiltration with T helper cells expressing the Th1–associated chemokine receptor CXCR3 was increased, but percentages of T helper cells expressing the Th17-characteristic CCR6 were unchanged (Figure 3E).

Apart from elevated Th1 responses, systemic cellular immunity was similar between the groups as shown by spleen cell production of various other cytokines (Supplemental Figure 3B). In line with cellular immunity, analysis of sheep globulin–specific antibody production revealed skewing toward Th1-dependent subclasses as evidenced by profoundly impaired production of Th2–associated IgG1 and IgG3 isotypes (Figure 3F). Total IgG, IgG2c, and IgG2b anti–sheep globulin IgG levels remained unchanged (Supplemental Figure 3C).

NTN Is Aggravated in the Absence of Treg1 Cells

In line with overshooting renal and systemic Th1 responses, tissue damage was significantly aggravated at day 15 after NTN induction in kidneys of Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, with increased crescent formation and enhanced interstitial injury (Figure 4A). Likewise, renal function was worse in the absence of Treg1 cells as indicated by higher BUN levels (Supplemental Figure 4A). Albuminuria was not significantly different between the groups (Supplemental Figure 4B). Analysis of kidneys at a later time point (22 days after NTN induction) again showed significant aggravation of tissue damage (Figure 4B). In line with enhanced renal injury, analyses of the renal inflammatory cell infiltrate revealed increased numbers of T cells (Figure 4C) and macrophages (Figure 4D) as well as neutrophils (Figure 4E) in both glomeruli and the renal interstitial compartment of Foxp3^{Cre}xT-bet$^{fl/fl}$ mice at days 15 (Figure 4, C–E) and 22 (Supplemental Figure 4, C–E) after NTN induction.

Treg Cell Suppressive Function Is Maintained in the Absence of T-Bet Activation

Next, we wanted to assess whether T-bet deficiency affects Treg cell suppressive functions. We, thus, performed *in vitro* suppression assays by coculturing effector T cells (Teffs) with Treg cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ or Foxp3^{Cre} control mice. Our studies showed intact Treg function, including effective dose–dependent suppression of IL-2 production (Figure 5A), as well as induction of IL-10 secretion (Figure 5B). Importantly, also, suppression of IFNγ production remained unaffected by lack of T-bet in Treg cells, indicating unimpaired *in*

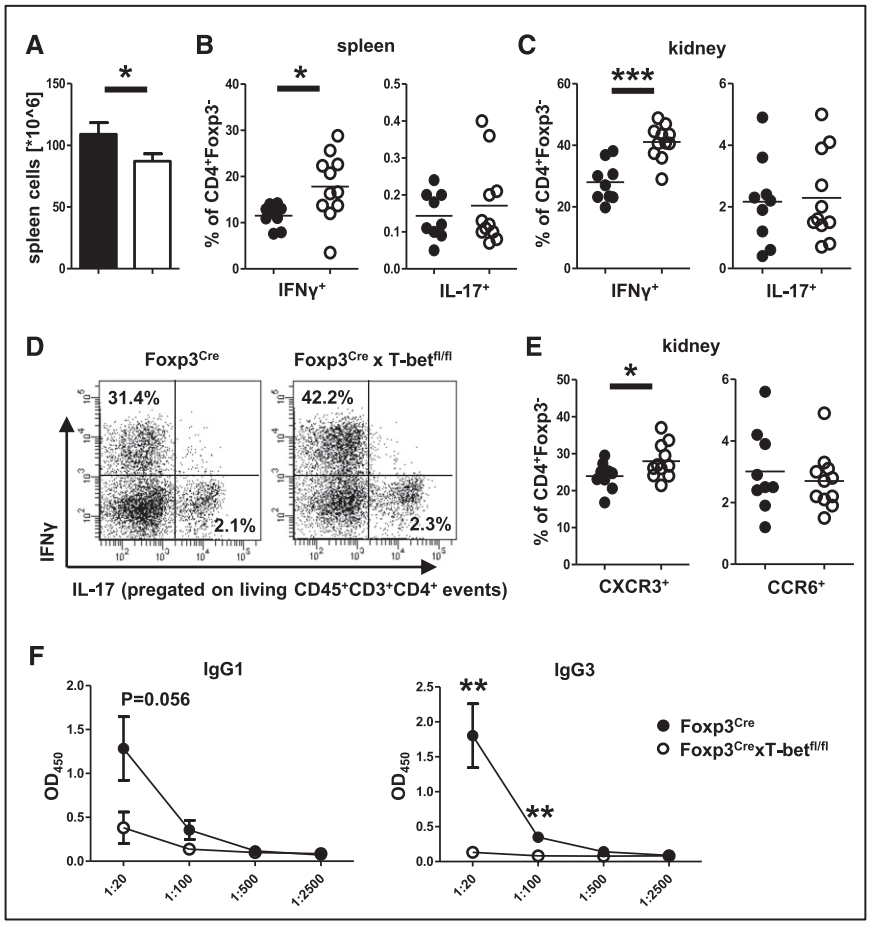

Figure 3. Skewing of renal and systemic immunity toward Th1 in Treg1 cell–deficient mice. (A) Quantification of spleen cell numbers. (B and C) Quantification of (B) splenic and (C) renal Foxp3$^-$ T helper cells expressing IFNγ and IL-17. (D) Representative FACS plots of renal T helper cells expressing the indicated cytokines. (E) Expression of the indicated chemokine receptors on renal Foxp3$^-$ T helper cells. Analyses in A–E were performed at day 15 after NTN induction. (F) Serum levels of IgG1 and IgG3 anti–sheep globulin antibodies at day 12 after sheep IgG immunization. ELISA data are shown as OD at 450 nm in serial dilutions as indicated. Numbers in FACS plots represent percentages of CD4$^+$ cells. Nine Foxp3^{Cre} versus 11 Foxp3^{Cre}xT-bet$^{fl/fl}$ mice were analyzed in A–E, and five Foxp3^{Cre} versus five Foxp3^{Cre}xT-bet$^{fl/fl}$ mice were analyzed in F. Circles in B, C, and E represent individual animals, and horizontal lines represent mean values. Error bars represent SEM. *P<0.05; **P<0.01; ***P<0.001.

vitro potential to suppress Th1 responses (Figure 5C). Furthermore, we isolated Treg cells from spleens of sheep IgG–immunized Foxp3^{Cre}xT-bet$^{fl/fl}$ or Foxp3^{Cre} control mice and analyzed expression of various Treg cell effector cytokines. No differences were detected with respect to IL-10, IL-35/EBI-3, and TGF-β1 mRNA levels (Figure 5D). In addition, analysis of a broad spectrum of surface markers revealed no major alterations of Treg cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, with the exception of slightly enhanced GITR expression (Supplemental Figure 5). Finally, we compared the effects of Treg cells on Teff proliferation and activation *in vivo*. Treg cells from Foxp3^{Cre} wild–type or Foxp3^{Cre}xT-bet$^{fl/fl}$ mice were cotransferred with wild–type CD4$^+$Foxp3$^-$ Teff into Rag1$^{-/-}$ recipients. Spleens of recipient mice were analyzed at day 8 after immunization with the nephritogenic antigen sheep globulin. Analyses revealed, that Treg cell percentages in both groups of recipients were similar. Furthermore, no T-bet expression was found in Treg cells from the knockout recipient group, confirming that no relevant *de novo* development of Treg cells had occurred (data not shown). Importantly, we found similar proliferation (Figure 5, E and F) and activation (Figure 5G) of Teff in both groups of recipients, which indicates similar suppressive capacity of wild–type and T-bet–deficient Treg cells.

T-Bet Expression in Treg Cells Is Required for Competitive Fitness

Next, we wanted to evaluate whether T-bet expression influences *in vivo* Treg fitness and thus, performed competitive transfer assays. Spleen cells from wild–type donor mice carrying the congenic marker CD45.1 were mixed at a 1:1 ratio with spleen cells from CD45.2$^+$ Foxp3^{Cre}xT-bet$^{fl/fl}$ mice and transferred into Rag1$^{-/-}$ recipients. Subsequently, NTN was induced, and Treg cells were analyzed in spleens and kidneys at day 14 (Figure 6A). In both organs, we found that wild–type Treg cells had significantly outcompeted T-bet–deficient Treg cells, because percentages of Treg cells among CD45.1$^+$ wild–type T cells were much higher than Treg cell percentages among CD45.2$^+$ T cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice (Figure 6B). Similarly, percentages of CD45.1$^+$ wild–type Treg cells were significantly higher than those of CD45.2$^+$ T-bet–deficient Treg cells among total Treg cells in both spleens and kidneys (Figure 6C). In line, expression intensity of Foxp3 protein was much lower in Treg cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, indicating reduced suppressive function (Figure 6, D and E). Finally, we noted decreased kidney-to-spleen ratios of Treg cells from CD45.2 Foxp3^{Cre}xT-bet$^{fl/fl}$ donor mice, which indicated impaired renal trafficking of T-bet–deficient Treg cells. Ratios of Teffs, as a control, were not different between knockout and wild–type donor populations, confirming that the trafficking defects were Treg cell specific (Figure 6F).

Renal Treg Cell Infiltration Is Impaired in Foxp3^{Cre}xT-bet$^{fl/fl}$ Mice Because of Absence of CXCR3

We next evaluated whether impairment of Treg cell trafficking might contribute to the observed aggravation of nephritis. To

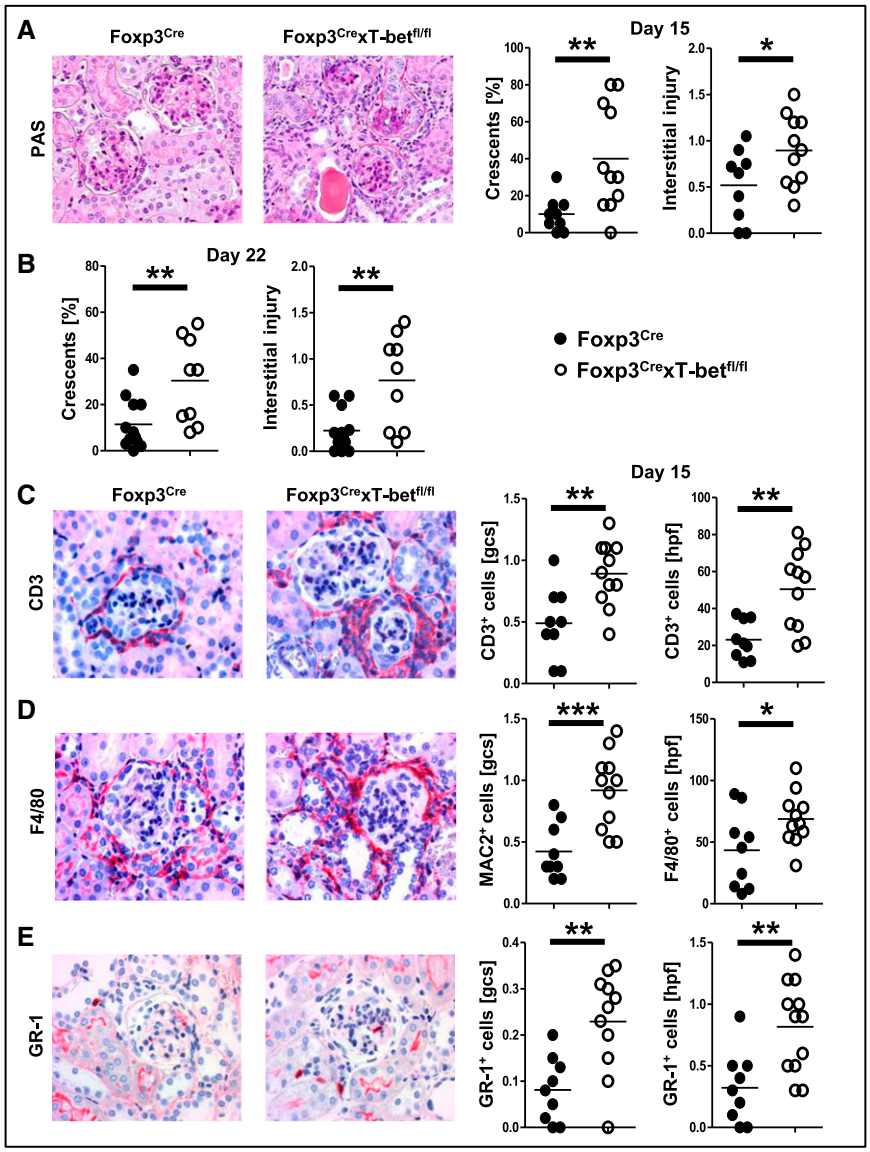

Figure 4. NTN is aggravated in the absence of Treg1 cells. (A) Representative photographs of periodic acid–Schiff (PAS) –stained kidney sections and quantification of crescents and interstitial injury at day 15 after NTN. Original magnification, ×200. (B) Quantification of crescents and interstitial injury at day 22 after NTN. (C) Representative photographs and quantification of glomerular and interstitial $CD3^+$ T cells. Original magnification, ×400. (D) Representative photographs of F4/80–stained renal macrophages and quantification of glomerular ($MAC\text{-}2^+$) and interstitial ($F4/80^+$) macrophages. Original magnification, ×400. (E) Representative photographs of GR-1–stained kidneys and quantification of glomerular and interstitial $GR\text{-}1^+$ neutrophils. Original magnification, ×400. One of two representative sets for A is shown. Nine $Foxp3^{Cre}$ versus 11 $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice were analyzed at day 15, and 12 $Foxp3^{Cre}$ versus nine $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice were analyzed at day 22. Circles represent individual animals, and horizontal lines represent mean values. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

this end, analyses showed reduced frequencies of Treg cells in kidneys from $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice by both immunohistochemistry (Figure 7A) and FACS (Figure 7B). Percentages of Treg cells among spleen cells, in contrast, remained unchanged (Figure 7C).

Because these findings indicated impaired Treg cell trafficking rather than reduced generation, we analyzed Treg cell chemokine receptor expression. Indeed, we found robust expression of the Th1–characteristic trafficking receptor CXCR3 on Treg cells from nephritic control mice, whereas expression on Treg cells from $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice was completely abrogated in both kidneys and spleens (Figure 7D). Conversely, expression of the Th17–characteristic chemokine receptor CCR6 was strongly upregulated (Supplemental Figure 6A). Expression of the chemokine receptor CCR5 on Treg cells remained unaffected by T-bet deficiency (not shown). To further evaluate whether lack of CXCR3 might, indeed, underlie the observed reduction of renal Treg cells, we studied $Foxp3^{Cre}xCXCR3^{fl/fl}$ mice with selective CXCR3 deficiency on Treg cells. In line with our concept, we found that $Foxp3^{Cre}xCXCR3^{fl/fl}$ and $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice showed identical reduction of renal Treg cell percentages in comparison with wild-type controls at 22 days after NTN induction (Figure 7E). Treg cell percentages in spleens of both strains were similar to wild-type mice, excluding systemic Treg cell deficiencies as causative for reduced renal Treg cells (Supplemental Figure 6B). Importantly, also, the hyper-Th1 phenotype was almost identical in kidneys of $Foxp3^{Cre}xCXCR3^{fl/fl}$ and $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice (Supplemental Figure 6C). This observation strongly supports the hypothesis that Treg1 cells use CXCR3, which is expressed under the control of T-bet, for trafficking into the kidney and control of renal Th1 immunity. Finally, we aimed to determine whether impaired Treg cell proliferation in the absence of T-bet might also contribute to the observed reduction of renal Treg cells. However, our analyses revealed similar renal and splenic Treg cell proliferative activity in $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ compared with wild-type mice (Figure 7F).

DISCUSSION

The etiology and pathogenesis of most forms of GN are still poorly understood.[7,32] As a consequence, GN remains a leading cause for ESRD. Because current therapeutic options are

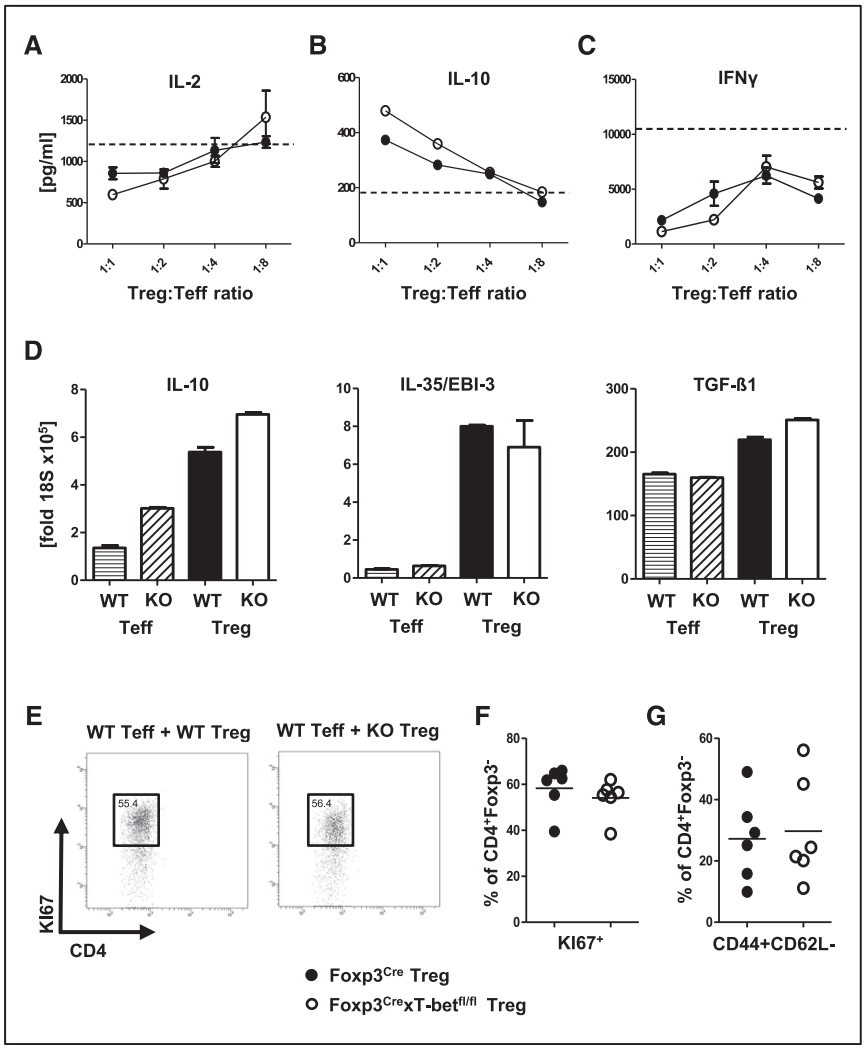

Figure 5. Intact Treg cell–suppressive function in the absence of T-bet activation. (A–C) *In vitro* suppression assays were performed by coculturing wild–type $CD4^+$ Teffs with Treg cells from $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice or $Foxp3^{Cre}$ controls at the indicated ratios (n=3 per group). Cytokine levels of (A) IL-2, (B) IL-10, and (C) IFNγ were analyzed in coculture supernatants as indicated. Dotted lines represent Teffs alone without Treg cells (n=3). (D) Quantification of IL-10, IL35/EBI-3, and TGF-β1 mRNA by quantitative RT-PCR from the indicated spleen cell populations FACS sorted at day 12 after immunization with sheep IgG. (E) Representative FACS plots and (F) quantification of $KI67^+$ proliferating $CD4^+Foxp3^-$ Teffs from spleens of immunized $Rag1^{-/-}$ recipients harboring Treg cells from the indicated mouse strains. (G) FACS analysis of splenic $CD4^+$ Teff activation from immunized $Rag1^{-/-}$ recipients harboring Treg cells of the indicated genotype; n=6 versus six mice were analyzed in E–G. Circles represent individual animals, and horizontal lines represent mean values. Error bars represent SEM. KO, $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice; WT, $Foxp3^{Cre}$ controls.

nonspecific and show a high degree of unwanted side effects, the therapy of patients with GN is challenging and often frustrating. Cells of the Th1 response, however, might represent a promising novel therapeutic target. We and others could show their key functions in initiation and progression of renal injury in crescentic forms of GN.[1–3,5,8,33] Thus, understanding the mechanisms that downregulate these highly nephritogenic Th1 responses is of great clinical importance. Our study, therefore, aimed to evaluate the new concept of Th1–specific Treg cells. These Treg1 cells have previously been postulated to depend on activation of the transcription factor T-bet, which they share with their proinflammatory Th1 counterpart.[17] Because to date, nothing is known about their function in renal inflammation, we studied the NTN model of cGN. Indeed, we found expansion of $T\text{-}bet^+$ Treg1 cells in spleens and kidneys within 10 days after induction of NTN. Subsequently, percentages in spleens decreased, whereas the renal Treg1 population steadily increased over time, suggesting preferential accumulation at sites of tissue injury. Interestingly, Treg1 cell dynamics paralleled those of Th1 responses, which is indicative of a role for Treg1 cells in counter-regulation of Th1 immunity. Analysis of Treg1 cell surface molecule expression revealed a distinct phenotype with reduced levels of CD25, ICOS, and GITR, whereas CD103, PD-1, CD44, and CD62L were expressed at much higher levels than on $T\text{-}bet^-$ Treg cells. Given these findings, which suggested unique and nonredundant functions of Treg1 cells, we next wanted to assess the functional relevance of T-bet expression in Treg cells. We, therefore, generated $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice with a Treg cell–selective T-bet deficiency. In line with a recent report, which was published during preparation of this manuscript, these animals were healthy and fertile, and they showed normal survival.[30] However, analysis of immune responses revealed a hitherto unknown phenotype. Although gross leukocyte composition of blood and spleens in naïve mice was unchanged, we found spontaneously overshooting Th1 immunity. In contrast, Th17 responses and production of various Th2– and Treg cell–associated cytokines were not significantly altered. Importantly, the higher systemic numbers of Th1 cells in $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice had a functional effect on another arm of the immune system. Analysis of humoral immunity revealed profoundly reduced levels of Th2–associated IgG1 and IgG3 antibody subclasses, which are known to be suppressed by the Th1 prototype cytokine IFNγ.[34] Because we were interested in studying the role of Treg1 cells in GN, we also evaluated the renal phenotype of naïve $Foxp3^{Cre}xT\text{-}bet^{fl/fl}$ mice. Our analyses, however,

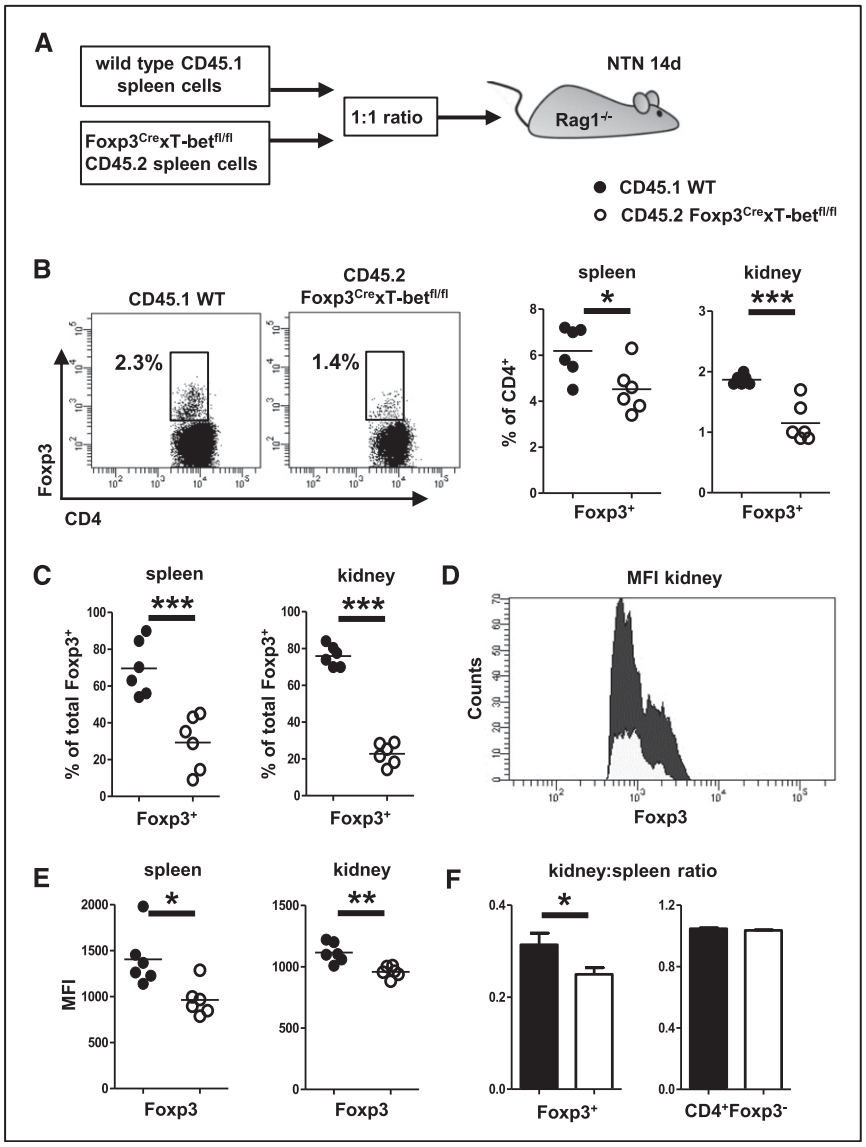

Figure 6. T-bet expression in Treg cells is required for competitive fitness. (A) CD45.1$^+$ wild–type and CD45.2$^+$ Foxp3^{Cre}xT-bet$^{fl/fl}$ splenocytes were transferred at a 1:1 ratio into Rag1$^{-/-}$ recipients, and NTN was induced. (B) Representative FACS plots of Foxp3$^+$ Treg cells from wild-type or Foxp3^{Cre}xT-bet$^{fl/fl}$ donors in kidneys of nephritic Rag1$^{-/-}$ recipients (left panel). Plots are pregated on living CD45.1$^+$ or CD45.2$^+$CD3$^+$ cells as indicated. Numbers in FACS plots indicate percentages of CD4$^+$ events. Quantification of Treg cell percentages among wild–type or Foxp3^{Cre}xT-bet$^{fl/fl}$ donor CD4$^+$ T cells in spleens and kidneys of nephritic recipient mice (right panel). (C) Quantification of CD45.1 and CD45.2 Treg cell percentages among total Treg cells in spleens and kidney as indicated. (D) Representative FACS plots of Foxp3 mean fluorescence intensity (MFI) in Treg cells from wild-type or Foxp3^{Cre}xT-bet$^{fl/fl}$ donors in kidneys of nephritic Rag1$^{-/-}$ recipients. Plots are pregated on living CD45.1$^+$ or CD45.2$^+$CD3$^+$CD4$^+$Foxp3$^+$ cells as adequate. (E) Quantification of Foxp3 MFIs in spleens and kidneys. (F) Kidney-to-spleen ratios of Foxp3$^+$ Treg cells (left panel) and CD4$^+$Foxp3$^-$ Teffs (right panel); n=6 recipient mice were analyzed in A–F. Circles represent individual animals, and horizontal lines represent mean values. Error bars represent SEM. *P<0.05; **P<0.01; ***P<0.001.

revealed healthy kidneys with no signs of inflammation or functional impairment. We, thus, went on to study renal and systemic immune responses after induction of NTN. Again, we found selective skewing of immunity toward Th1 in both spleens and kidneys of Foxp3^{Cre}xT-bet$^{fl/fl}$ mice. In line, antibody levels of the Th2–associated IgG1 and IgG3 subclasses against the nephritogenic antigen, sheep globulin, were significantly reduced. Even more importantly, dysregulation of Th1 responses resulted in enhanced kidney damage as evidenced by increased crescent formation and interstitial injury. Finally, evaluation of the renal inflammatory cell infiltrate showed increased numbers of T cells, macrophages, and neutrophils in kidneys of Treg1 cell–deficient mice. In contrast to aggravation of nephritis in Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, NTN was previously shown to be alleviated in T-bet pan knockout mice, which not only lack Treg1 cells but also, Th1 responses.[3] This observation highlights that, indeed, Th1 cells are the main target of Treg1 cells. To identify the mechanisms underlying enhanced Th1 immunity and subsequent aggravation of cGN in Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, we analyzed Treg cell–suppressive function. *In vitro* suppression assays revealed unchanged capacity to reduce IL-2 levels in coculture with Teffs as well as unchanged induction of immunosuppressive IL-10. Importantly, *in vitro* suppression of IFNγ production also remained intact, indicating that T-bet deficiency does not lead to a *per se* defective control of Th1 responses. Additionally, quantitation of mRNA expression of various Treg cell–associated effector cytokines showed identical levels of IL-10, IL-35/EBI-3, and TGF-β1 in Treg cells of both groups. Furthermore, analysis of a broad range of surface molecules associated with Treg cell function did not show major differences caused by absence of T-bet. These findings are in line with previous work by McPherson *et al.*,[31] which found unchanged capacity of T-bet–deficient Treg cells to suppress Teff expansion *in vitro*. To extend these observations, we analyzed *in vivo*–suppressive Treg cell effects. Indeed, our results showed similar Teff proliferation and activation in the presence of T-bet intact or T-bet–deficient Treg cells.

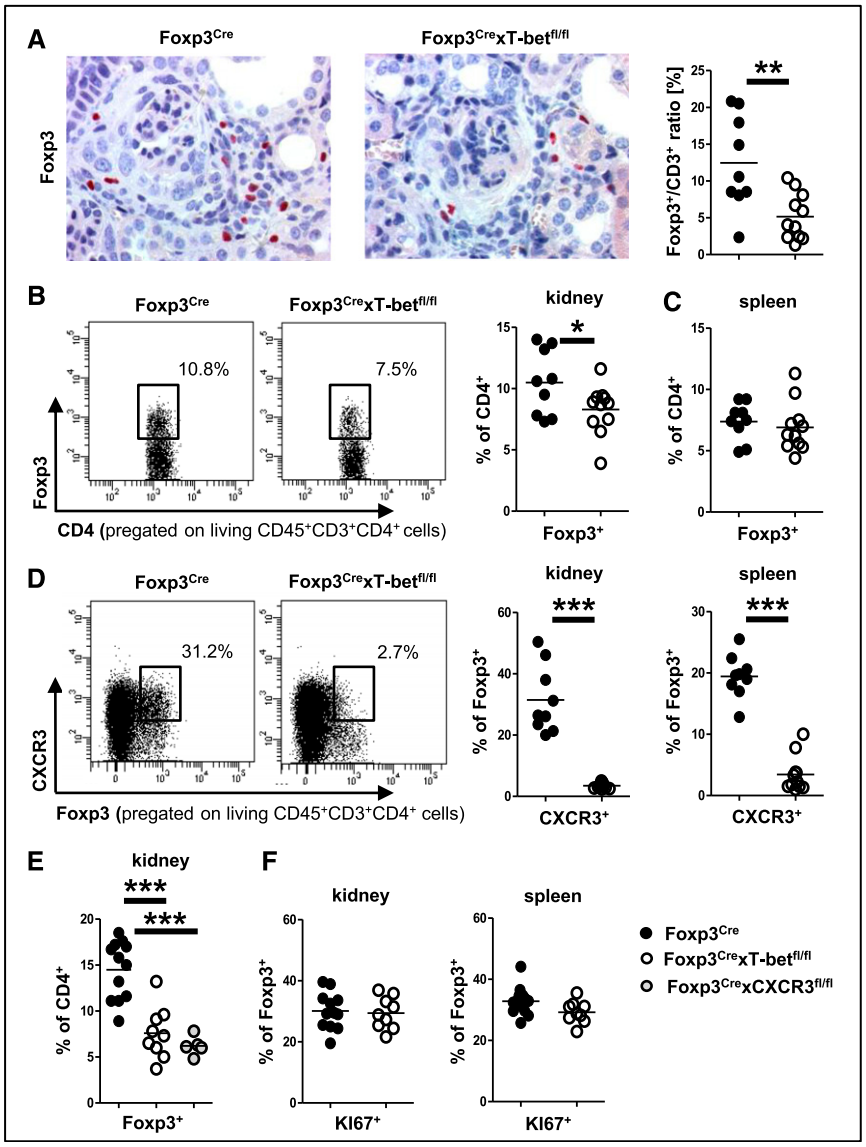

Figure 7. Impaired renal Treg cell infiltration in Foxp3^{Cre}xT-bet$^{fl/fl}$ mice caused by absence of CXCR3. (A) Representative photographs of Foxp3–stained kidney sections and quantification of renal Foxp3-to-CD3 ratios. (B) Representative FACS plots and quantification of renal Foxp3$^+$ Treg cells in mice of the indicated genotypes. Numbers in FACS plots indicate percentages of CD4$^+$ cells. (C) Quantification of splenic Foxp3$^+$ Treg cells by FACS. (D) Representative FACS plots of CXCR3$^+$ renal Treg cells (left panel). Numbers indicate percentages of Foxp3$^+$ events. Quantification of renal and splenic CXCR3$^+$ Treg cells (right panel). Analyses in A–D were performed at day 15 of NTN. (E) Quantification of renal Foxp3$^+$ Treg cells in mice of the indicated genotypes at day 22 after NTN by FACS. (F) Quantification of renal and splenic KI67$^+$ Foxp3$^+$ proliferating Treg cells in mice of the indicated genotypes at day 22 after NTN. Nine Foxp3^{Cre} versus 11 Foxp3^{Cre}xT-bet$^{fl/fl}$ mice were analyzed in A–D, 12 Foxp3^{Cre} versus nine Foxp3^{Cre}xT-bet$^{fl/fl}$ versus five Foxp3^{Cre}xCXCR3$^{fl/fl}$ mice were analyzed in E, and 12 Foxp3^{Cre} versus nine Foxp3^{Cre}xT-bet$^{fl/fl}$ were analyzed in F. Circles represent individual animals, and horizontal lines represent mean values. *P<0.05; **P<0.01; ***P<0.001.

However, alterations in Treg homeostasis and fitness might be masked in our constitutively T-bet–defective Foxp3^{Cre}xT-bet$^{fl/fl}$ mice because of compensatory mechanisms and lack of competition by wild–type Treg cells. To address this question, competitive transfer assays were performed by coinjecting CD45.2$^+$ spleen cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice with equal numbers of CD45.1$^+$ spleen cells from wild-type controls into lymphopenic Rag1$^{-/-}$ recipients. After induction of NTN, wild–type Treg cells had massively outcompeted their T-bet–deficient counterparts in spleens and nephritic kidneys. Furthermore, expression levels of Foxp3 protein were much lower in transferred knockout Treg cells, indicating reduced regulatory capacity in comparison with wild–type Treg cells. T-bet activation, therefore, enhances population expansion and general Treg cell fitness during inflammation. Additionally, we also found reduced kidney-to-spleen ratios among transferred Treg cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice. This observation indicated impaired competitive renal trafficking of T-bet–deficient Treg cells. Given this observation, we next wanted to study whether defective renal Treg cell recruitment might contribute to the observed Th1–type hyperinflammatory phenotype of Foxp3^{Cre}xT-bet$^{fl/fl}$ mice. In this respect, multimodal analyses of nephritic kidneys revealed significantly reduced percentages of Treg cells in kidneys of nephritic Foxp3^{Cre}xT-bet$^{fl/fl}$ mice. In light of unchanged systemic Treg cell percentages, this finding, indeed, indicated impairment of renal trafficking. Because chemokines are the main regulators of directional T cell migration,[35,36] we evaluated the Treg cell chemokine receptor profile. In line with several previous reports, including our own report,[17,21–28,37] we found robust expression of the Th1–characteristic receptor CXCR3 on splenic and renal Treg cells. Intriguingly, however, CXCR3 expression was completely abrogated on Treg cells from Foxp3^{Cre}xT-bet$^{fl/fl}$ mice, indicating absolute dependency on T-bet activation. To further investigate if lack of CXCR3 is the main reason for the observed reduction of renal Treg cell percentages, we performed comparisons with mice with Treg cell–selective CXCR3 deficiency. In line with our concept, we found equal impairment of renal Treg

cell infiltration and importantly, also, identical enhancement of renal Th1 immunity in Foxp3^{Cre}xT-bet$^{fl/fl}$ and Foxp3^{Cre}xCXCR3$^{fl/fl}$ mice. Furthermore, splenic and renal Treg cell proliferation was not altered by deletion of T-bet. Taken together, these findings indicate that, indeed, impaired trafficking caused by absence of CXCR3 and not diminished proliferative activity underlies the observed reduction in renal Treg cell percentages. Absence of CXCR3 on Treg cells is also likely to impair colocalization with CXCR3^{+} Th1 cells,[37] which could explain the selectively exacerbated Th1 responses in Foxp3^{Cre}xT-bet$^{fl/fl}$ mice.

In summary, we here provide the first evidence for a role of T-bet^{+} Treg1 cells in experimental cGN and thus, a model of induced organ inflammation in general. T-bet activation was found to modulate two important Treg cell properties. First, overall Treg cell fitness was significantly enhanced by T-bet expression. Second, T-bet activation induced expression of the chemokine receptor CXCR3. This allows for optimized control of Th1 responses by facilitating Treg1 cell trafficking into areas of Th1-type inflammation. T-bet^{+} Treg1 cells were, thus, identified as novel regulators of nephritogenic Th1 responses and therefore, warrant exploration as potential therapeutic targets.

CONCISE METHODS

Animals

loxP site–flanked Tbx21$^{fl/fl}$ mice[38] (referred to as T-bet$^{fl/fl}$) were provided by Steven L. Reiner (Columbia University, New York, NY). CXCR3$^{fl/fl}$ mice were generated as previously published.[37] Treg cell–specific deletion of T-bet or CXCR3 was achieved by crossbreeding with mice expressing a yellow fluorescent protein (YFP)-Cre recombinase fusion protein under the control of the Foxp3 locus[39] provided by Alexander Y. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY). Rag1$^{-/-}$ and CD45.1 mice initially derive from The Jackson Laboratory (Bar Harbor, ME). All mice are on a C57BL/6 background and were bred in our facility under specific pathogen–free conditions.

Animal Experiments and Functional Studies

Naïve mice were analyzed at 11–13 weeks of age. NTN was induced in 8- to 10-wk-old male Foxp3^{Cre}xT-bet$^{fl/fl}$, Foxp3^{Cre}xCXCR3$^{fl/fl}$, and Foxp3^{Cre}xT-bet$^{wt/wt}$ (referred to as Foxp3^{Cre}) littermate controls by intraperitoneal (ip) injection of nephrotoxic sheep serum.[40] Organs were harvested at the indicated time points between days 5 and 49 after injection. For immunization studies, mice were ip immunized with 0.5 mg sheep IgG, and organs were harvested at day 11. For comparison of *in vivo* Treg cell suppression, 4×10^5 FACS–sorted Treg cells from spleens of Foxp3^{Cre} or Foxp3^{Cre}xT-bet$^{fl/fl}$ mice were cotransferred together with 1.5×10^6 CD4^{+}Foxp3^{-} Teffs from spleens of Foxp3^{Cre} mice into two groups of Rag1$^{-/-}$ recipients. Subsequently, recipients were ip immunized with 0.5 mg sheep IgG, and spleens were analyzed at day 8. For competitive transfer assays, splenic leukocytes were isolated from either Foxp3^{Cre}xT-bet$^{fl/fl}$ (CD45.2^{+}) or wild–type C57BL/6 (CD45.1^{+}) donor mice. Viable cells were counted using a Bio-Rad TC21 Counter (Bio-Rad, Hercules, CA) after trypan blue staining and intravenously injected into Rag1$^{-/-}$ mice in a 1:1 ratio 1 day before induction of NTN. Renal and splenic leukocytes were isolated 14 days after NTN induction as described below and analyzed by FACS.[13] Urine samples were collected after housing the mice in metabolic cages. Albuminuria was determined by standard ELISA (Bethyl Laboratories). BUN and urinary creatinine were quantified using standard laboratory methods. Animal experiments were performed according to national and institutional animal care and ethical guidelines and approved by local committees (approval codes 37/11, 73/14, and 07/15).

Morphologic Studies

Crescent formation and glomerular necrosis were determined in a minimum of 50 glomeruli per mouse in 2-μm-thick periodic acid–Schiff–stained kidney sections in a blinded manner. Semiquantitative analysis of tubulointerstitial damage was performed using ten randomly selected cortical areas (×200) as described previously.[6] Paraffin-embedded sections were stained with antibodies directed against CD3 (A0452; Dako, Hamburg, Germany), F4/80 (BM8; BMA Biomedicals, Hiddenhausen, Germany), MAC2 (M3/38; Cedarlane Laboratories, Burlington, ON, Canada), GR-1 (NIMP-R14; Hycult Biotech, Uden, The Netherlands), or Foxp3 (FJK-16s; eBiosciences, San Diego, CA) and developed with a polymer–based secondary antibody-alkaline phosphatase kit (POLAP; Zytomed, Berlin, Germany). Fifty glomerular cross-sections and 30 tubulointerstitial high power fields (magnification of ×400) per kidney section were counted in a blinded fashion.[13,41]

Isolation of Leukocytes from Various Tissues

Spleens were harvested in HBSS and passed through 70-μm nylon meshes. After lysis of erythrocytes with ammonium chloride, cells were washed and passed over 40-μm meshes. Cells were then washed again and counted for either culture or FACS analysis. Kidneys were minced and incubated in digestion medium (RPMI 1640 medium [Gibco, Carlsbad, CA] containing 10% FCS, 1% HEPES, 1% penicillin/streptomycin, 8 μg/ml collagenase D, and 0.4 μg/ml DNase) at 37°C for 40 minutes. Tissues were then dissociated using the gentleMACS Dissociater (Miltenyi Biotec) to get a single-cell suspension and centrifuged at 300×*g* at 4°C for 8 minutes. To further purify the cells, Percoll gradient (37% Percoll; GE Healthcare, Waukesha, WI) centrifugation was performed at 500×*g* at room temperature for 20 minutes. Cells were washed and resuspended for staining and FACS analysis. Peripheral blood was drawn into EDTA-coated tubes, and red blood cell lysis was performed. Then, blood cells were washed and prepared for staining.[13]

Systemic Cellular and Humoral Immune Responses

Splenocytes (4×10^5 cells per milliliter) were cultured under standard conditions on plates precoated with anti-CD3 mAb (1 μg/ml; BD Biosciences, San Jose, CA), and supernatants were harvested after 72 hours. Commercially available ELISAs were used for detection of IFNγ (BioLegend, San Diego, CA), IL-4 (BioLegend), IL-6 (BioLegend), IL-10 (BioLegend), TGF-β1 (R&D Systems, Minneapolis, MN), and IL-2 (R&D Systems).[40] Circulating sheep globulin–specific serum

IgG titers were analyzed by ELISA using plates precoated with sIgG.[13,41] For analysis of total nonantigen–specific Igs, ELISA plates were precoated with anti–mouse total IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). ELISA plates were incubated with mouse serum, and the following secondary antibodies were used for detection: total IgG (Southern Biotech, Birmingham, AL), IgG1 (Southern Biotech), IgG2b (Invitrogen, Carlsbad, CA), IgG2c (Bethyl, Montgomery, TX), and IgG3 (Jackson ImmunoResearch Laboratories).

Flow Cytometry

Cells were surface stained for 30 minutes at 4°C with fluorochrome-labeled antibodies against CD45, CD45.1 CD45.2, CD4, CD44 (BD Bioscience), CD3, CD25, CD69, CD62L, CCR6, CXCR3, CCR5, ICOS, PD-1, CD103, PD-L1, and GITR (BioLegend) as previously described.[13,16] For intracellular and intranuclear staining, samples were processed using a commercial intranuclear staining kit (Foxp3 Staining Kit; eBiosciences). Fluorochrome-labeled antibodies against IL-17 (BioLegend), IFNγ (BioLegend), Foxp3 (eBiosciences), T-bet (eBiosciences), Gata3 (eBiosciences), KI67 (BD Biosciences), and RORγt (BD Biosciences) were used as recently published.[13,16] For intracellular cytokine staining, cells were activated with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO), ionomycin (1 μg/ml; Calbiochem, San Diego, CA), and brefeldin A (10 μg/ml; Sigma-Aldrich) for 3 hours. LIVE/DEAD Staining (Invitrogen Molecular Probes, Eugene, OR) was used to exclude dead cells during flow cytometry and ensure viability of the cells after the stimulation procedure. Experiments were performed on a BD LSRII Cytometer (Becton Dickinson). FACS sorting was performed from single-cell suspensions enriched for $CD4^+$ T cells by MACS sorting (T Cell Isolation Kit II; Miltenyi Biotec) from spleens of the indicated animal strains by the institutional HEXT FACS Sorting Core Facility using a BD ARIAIII Cytometer (Becton Dickinson) as previously described.[13]

Treg Cell Suppression Assay

$CD4^+$ spleen cells were enriched by using magnetic–activated cell sorting according to the manufacturer's protocol (MACS $CD4^+$ T Cell Kit II; Miltenyi Biotec). Treg cells and Teffs were then isolated by FACS sorting (performed on a BD ARIAIII Cytometer; Becton Dickinson). In total, 1×10^5 $CD45^+CD4^+YFP^-$ Teffs from $Foxp3^{Cre}$ mice were then cultured for 72 hours in anti-CD3 mAb (5 μg/ml; BD Biosciences) precoated 96–well plates either alone or in coculture with $CD45^+CD4^+YFP^+$ Treg cells from $Foxp3^{Cre}$ or $Foxp3^{Cre}$xT-$bet^{fl/fl}$ mice at the indicated ratios. Suppressive capacity was determined by cytokine ELISAs performed from the supernatants as recently published.[13,16]

Quantitative Real–Time PCR Analyses

Quantitative RT-PCR from RNA derived from FACS–sorted spleen cell populations was performed in a Stepone Plus Detector (Applied Biosystems, Foster City, CA) as described before.[42] For detection of IL-10, TGF-β1, and 18S, the sybr green method was used (primer sequences are available on request), whereas detection for EBI-3 was performed using Taqman probes (Applied Biosystems Assay ID: Mm00469294_m1). Samples were run in duplicates and normalized to 18S rRNA.[16]

Statistical Analyses

Results are expressed as means±SEMs. Groups were compared by *t* test, or in the case of three groups, ANOVA was used with *post hoc* analysis according to Tukey. A P value <0.05 was considered statistically significant.

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DISCLOSURES

None.

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Treg17 cells are programmed by Stat3 to suppress Th17 responses in systemic lupus

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Systemic lupus erythematosus (SLE) is a complex and potentially fatal autoimmune disorder. Although Th17 cells are thought to be central mediators of SLE, mechanisms underlying their counter regulation remain largely unknown. To help define this, we studied the function of the newly defined Stat3-dependent Th17-specific regulatory T cells (Treg17). Treg-specific deletion of Stat3 was achieved by generating Foxp3^{Cre} × Stat3$^{fl/fl}$ mice and SLE was induced by intraperitoneal injection of pristane. Lack of Treg17 cells in these mice caused selectively enhanced peritoneal Th17 inflammation. Importantly, Treg17 deficiency also resulted in aggravated pulmonary vasculitis with increased percentages of Th17 cells and significantly higher mortality. Similarly, 4 and 9 months after pristane injection, analysis of renal and systemic immunity showed overshooting Th17 responses in the absence of Treg17 cells, associated with the aggravation of lupus nephritis. Expression of the Th17 characteristic trafficking receptor CCR6 was strikingly reduced on Tregs of Foxp3^{Cre} × Stat3$^{fl/fl}$ mice, resulting in impaired renal Treg infiltration. Thus, Stat3-induced Treg17 cells are novel antiinflammatory mediators of SLE. One mechanism enabling Treg17 cells to target pathogenic Th17 responses is shared expression of the chemokine receptor CCR6.

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Systemic lupus erythematosus (SLE) is a common autoimmune disease causing high morbidity and mortality in a rather young collective of patients.[1–3] Multiple tissues can be affected including vital organ systems such as the central nervous system, lungs, and kidneys. In particular, development of lupus nephritis has been identified as a major risk factor for a poor prognosis.[4] Despite intensive research, the etiopathology of SLE remains widely elusive. As a consequence, therapeutic options are unspecific and insufficient, which highlights the need to identify novel therapeutic targets.[5–7]

One such promising target could be Th17 cells. Multiple studies have supported an important role for interleukin (IL)-17 and T helper (Th)17 cells in human SLE[8–10] and show their presence in lupus nephritis.[11] Moreover, in various experimental rodent models, the IL-17/Th17 axis has been demonstrated to be critical for the development of autoimmunity and renal pathology in SLE.[12–17] It is of note, however, that one recent study in two genetic mouse models of SLE did not find a vital contribution of IL-17 to the development of lupus nephritis.[18] This once more underlines the complex nature of SLE disease pathogenesis and highlights that a deeper understanding of Th17 biology is needed. In particular, the mechanisms responsible for effective downregulation of Th17 responses remain widely unknown to date. Generally, a T cell lineage with a highly regulatory phenotype termed regulatory T cells (Tregs) has been identified as crucial for counteracting pro-inflammatory Th cell responses. Tregs are commonly defined by expression of the transcription factor Foxp3[19–21] and until recently were regarded as a homogenous population. However, given the wide array of highly specialized and diverse T effector cell subpopulations, potent counter regulation by one singular anti-inflammatory Treg population seems unlikely.[22] In this respect, the intriguing concept of lineage-specific Tregs was proposed. According to this concept, Th1 responses are downregulated by Treg1 cells, whereas Th17 responses are under the control of Treg17 cells. Lineage specificity was suggested to be induced by sharing some of the same transcription factors with the respective effector cell population.[23–26] Recent studies have linked activation of the Th1-specific transcription factor T-bet in Foxp3^{+} Tregs to a Th1-specific Tregl phenotype.[23,24] Independently, another landmark study provided evidence for the presence of Treg17 cells that depend on the Th17 transcription factor Stat3.

A selective deletion of Stat3 in murine Tregs caused Treg17 deficiency, which resulted in overshooting Th17 responses.[25] In line, our group recently identified Stat3-dependent Treg17 cells as important mediators of renal tissue protection in a model of acute glomerulonephritis. In addition, we were able to identify Treg17 cells in kidney biopsies from human patients with antineutrophil cytoplasmic antibody (ANCA) associated vasculitis and to provide evidence that human Treg17 cells also depend on the activation of Stat3.[27] As one of the major mechanisms for specific suppression of Th17 cells, we could identify expression of the Th17 characteristic trafficking receptor CCR6 on Treg17 cells, which enables them to traffic into areas of Th17-mediated inflammation.

Although our study revealed an important role for Treg17 cells during acute renal inflammation, data with respect to chronic disease are completely lacking. This aspect is of special clinical importance, because many patients suffer from chronically progressive renal inflammation, requiring life-long treatment. Likewise, nothing is known about the impact of Treg17 cells on the complex development of autoimmunity and the clinical course of SLE. In order to address these important issues, we decided to study the murine model of pristane-induced lupus. Peritoneal injection of the naturally occurring hydrocarbon oil pristane (2,6,10,14-tetramethylpentadecane) leads to chronic inflammation with development of lupus-like autoimmunity. This particularly includes the formation of SLE characteristic antibodies as well as immune-complex nephritis and pulmonary vasculitis with a high degree of similarity to human SLE.[28,29] Importantly, we and others could recently show that the development of pristane-induced SLE crucially depends on the IL-17/Th17 axis, which makes a role for Treg17 cells quite likely.[15,16] In the present study, we therefore used this well-established model of murine SLE to define the role of Treg17 cells with special focus on the development of autoimmunity and end organ damage.

RESULTS

Peritoneal immunity is skewed towards Th17 responses in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice

Peritoneal immune responses were analyzed in Foxp3^{Cre} × Stat3$^{fl/fl}$ and Foxp3^{Cre} control mice at 1 week after pristane injection. Our studies showed enhanced peritoneal cell numbers in both strains of mice as compared with untreated controls (Figure 1a). Analysis of peritoneal leukocytes showed slightly higher frequencies of the CD4$^+$ Th subset in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice while all other populations were similar between the groups (Supplementary Figure S1A online). Importantly, we observed significant skewing towards Th17 responses in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice with selective enhancement of both RORγt$^+$ and IL-17$^+$ Th17 cells (Figure 1b). Conversely, infiltration of T-bet$^+$ Th1 cells was already observed in Foxp3^{Cre} controls but not yet in Th17-skewed Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. Interferon (IFN)γ production by Th1 cells was still at baseline levels in both strains of mice (Figure 1c). IL-17 production by the CD3$^+$CD4$^-$ T cell population, including CD4$^-$CD8$^-$ double negative and γδ T cells, was indistinguishable between the groups (Supplementary Figure S1B online) as were Th2 responses (Supplementary Figure 1C online).

Increased mortality from pulmonary vasculitis in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice

We next assessed the impact of Treg17 deficiency on the clinical course of pristane-induced SLE. Untreated Foxp3 × Stat3$^{fl/fl}$ control mice remained healthy over time and did not show any signs of diarrhea or wasting. Pristane treatment, however, resulted in excessive early mortality of Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (Figure 2a). Our search for the underlying cause of death revealed pristane-induced pulmonary hemorrhage with pronounced neutrophilia and leukocytoclastic capillaritis. Lungs of untreated Treg17-deficient Foxp3^{Cre} × Stat3$^{fl/fl}$ mice did not show any signs of inflammation (data not shown). After application of pristane, however, Foxp3^{Cre} × Stat3$^{fl/fl}$ mice developed severe pulmonary vasculitis, which was significantly aggravated in comparison with controls (Figure 2b). In line, pulmonary neutrophilia was strikingly increased (Figure 2c). As a potential underlying mechanism, we found overshooting pulmonary Th17 responses in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice, while percentages of Th1 and IL-17 producing CD3$^+$CD4$^-$ T cells were not significantly different (Figure 2d).

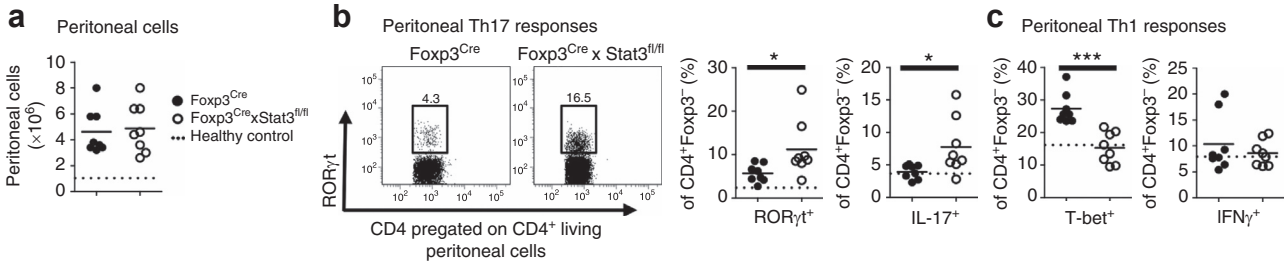

Figure 1 | Peritoneal immunity is skewed towards Th17 in Treg17-deficient mice. (a) Quantification of total peritoneal cells in Foxp3^{Cre} and Foxp3^{Cre} × Stat3$^{fl/fl}$ mice at day 7 after pristane administration (dotted line represents $n = 3$ untreated controls). **(b)** Representative FACS plots of peritoneal Th17 cells in Foxp3^{Cre} and Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (left, gating strategy as indicated). Flow cytometric quantification of peritoneal Th17 cells (right). **(c)** Flow cytometric quantification of peritoneal Th1 responses. Numbers in FACS plots indicate percentages of gated cells. Circles represent individual mice; horizontal lines indicate mean values. *$P < 0.05$, ***$P < 0.001$. FACS, fluorescence-activated cell sorting; Th, T helper cells; Treg, regulatory T cells.

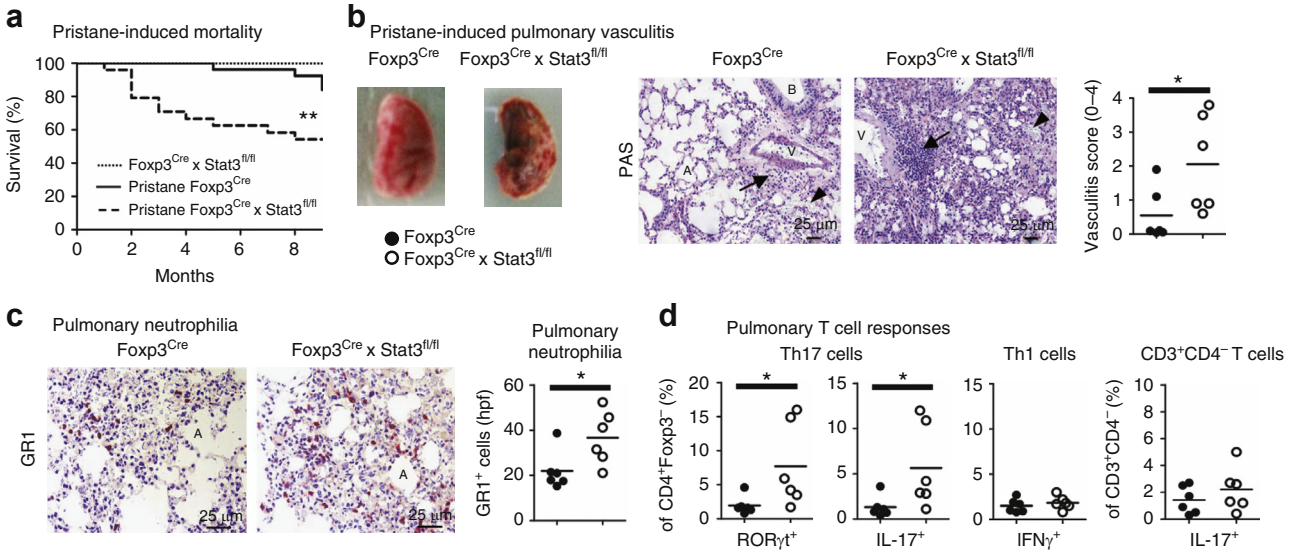

Figure 2 | Increased mortality from pulmonary vasculitis in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. (a) Kaplan–Meier plot of survival in untreated Foxp3^{Cre} × Stat3$^{fl/fl}$ ($n = 8$) and pristane-treated Foxp3^{Cre} ($n = 26$) and Foxp3^{Cre} × Stat3$^{fl/fl}$ ($n = 24$) mice. **(b)** Representative photographs of lungs at 3 weeks after pristane administration (left), showing severely aggravated alveolar hemorrhage in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice, as compared with Foxp3^{Cre} controls. Representative PAS-stained lung sections from Foxp3^{Cre} and Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (middle, magnification ×200) and quantification of pulmonary vasculitis (right). Arrows point to perivascular leukocyte infiltrates. Arrowheads indicate alveolar hemorrhage. A, alveolus; V, blood vessel; B, bronchiole. **(c)** Representative photographs of GR-1 (red) stained lung sections (magnification ×400) and quantification of pulmonary neutrophilia. A, alveolus. **(d)** Quantification of FACS-analyzed pulmonary T-cell infiltration of the indicated subtypes. Circles represent individual mice; horizontal lines indicate mean values. *$P < 0.05$, **$P < 0.01$. FACS, fluorescence-activated cell sorting; PAS, periodic acid–Schiff.

Enhanced renal and systemic Th17-immunity in Treg17-deficient mice

We next studied immune responses at later stages after pristane injection. Spleens and kidneys were analyzed at 4 months, a time point when systemic autoimmunity is already established and renal injury is just starting to develop. Th17 responses were markedly increased in spleens from Treg17-deficient mice when compared with Foxp3^{Cre} controls. Enhancement of renal Th17 infiltration was even more impressive (Figure 3a and e). Renal and splenic Th1 responses in contrast were similar in both groups (Figure 3b and e). At 9 months after pristane injection, a time point when full-blown lupus nephritis is established, analysis of splenic cytokine production again revealed increased Th17 cytokines IL-17 and TGF-ß1 in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (Figure 3c), whereas IFNγ (Figure 3d) as well as IL-4 and IL-13 (Supplementary Figure S2A online) secretion were unaltered. In line with systemic immunity, nephritic kidneys showed increased percentages of RORγt^{+} and IL-17^{+} Th17 cells in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (Figure 3c and e). Interestingly, at this later time point, we also found strikingly enhanced renal Th1 responses in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (Figure 3d and e). *In vitro* suppression assays, however, revealed intact regulation of Th1 responses by Stat3-deficient Tregs (Supplementary Figure S2B online), indicating an indirect cause for this observation. IL-17 production by the renal CD3^{+}CD4$^{−}$ non-Th17 T cell populations was not different between the groups at either time point (Supplementary Figure S2C online). Furthermore, comparison of splenocyte composition as well as systemic T cell activation (Supplementary Figure S2D and E online) did not reveal significant alterations.

Renal injury is aggravated in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice

We next sought to investigate the effect of overshooting Th17 responses on the degree of renal damage. At 9 months after pristane injection, kidney tissue showed severe proliferative glomerulonephritis. In line with enhanced Th17 immunity, renal injury was significantly aggravated in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (Figure 4a). Furthermore, glomerular cell proliferation was strikingly increased in the absence of Treg17 cells (Figure 4b). Finally, analysis of leukocyte infiltration revealed enhanced numbers of renal T cells (Figure 4c), neutrophils (Figure 4d), and macrophages (Figure 4e) in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice.

Renal Treg trafficking is impaired because of lack of CCR6

In striking contrast to the increased numbers of pro-inflammatory leukocytes, multi-modal analyzes of renal Tregs revealed significantly reduced Treg frequencies in kidneys of Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (Figure 5a). Reduction was not due to generally reduced Treg numbers because systemic Treg proportions remained unchanged (Figure 5b). Importantly, whereas the trafficking receptor CCR6 was abundantly expressed on renal and splenic Tregs in wild-type mice, Tregs from Foxp3^{Cre} × Stat3$^{fl/fl}$ mice almost completely lacked CCR6 on their surface (Figure 5c, left). CCR6 deficiency was specific for Tregs because expression was unaltered on Th17

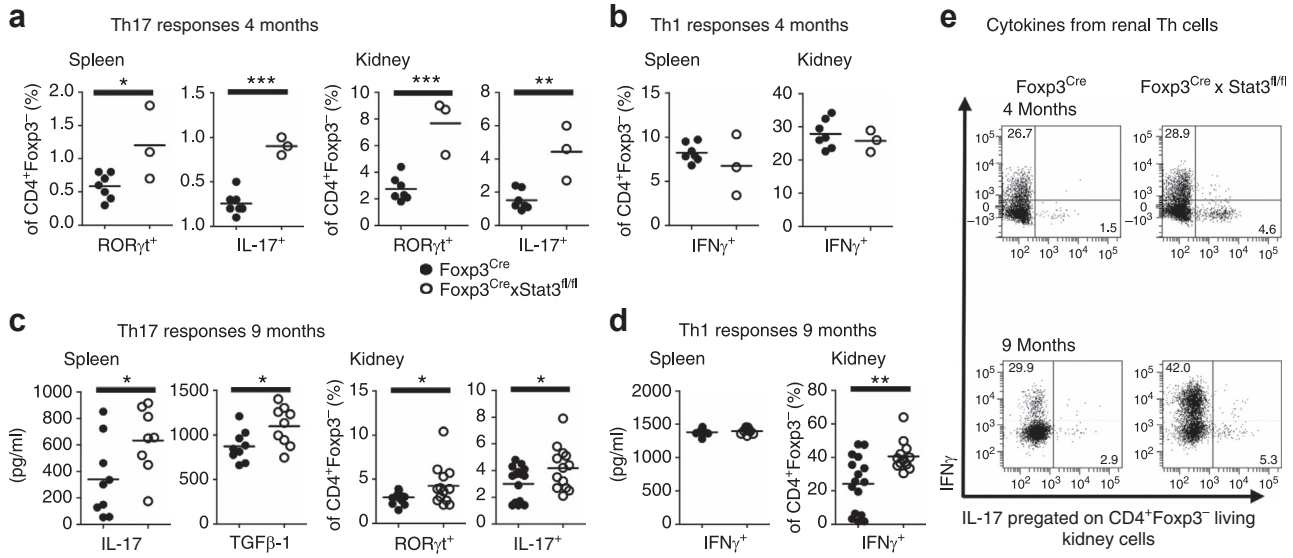

Figure 3| Enhanced renal and systemic Th17-immunity in Treg17-deficient mice. (a) Quantification of splenic (left) and renal (right) Th17 responses by flow cytometry at 4 months after pristane administration. **(b)** Quantification of splenic (left) and renal (right) Th1 responses. **(c)** Quantification of splenic (left) Th17-associated cytokines by ELISA and renal (right) Th17 responses by flow cytometry in $Foxp3^{Cre}$ and $Foxp3^{Cre} \times Stat3^{fl/fl}$ mice at 9 months after pristane administration. **(d)** Quantification of splenic (left) and renal (right) Th1 responses. **(e)** Representative FACS plots of renal Th1 and Th17 responses at 4 (upper row) and 9 months (lower row). Gating strategy as indicated; numbers indicate percentages of gated cells. Circles represent individual mice; horizontal lines indicate mean values. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. FACS, fluorescence-activated cell sorting; Th, T helper cells.

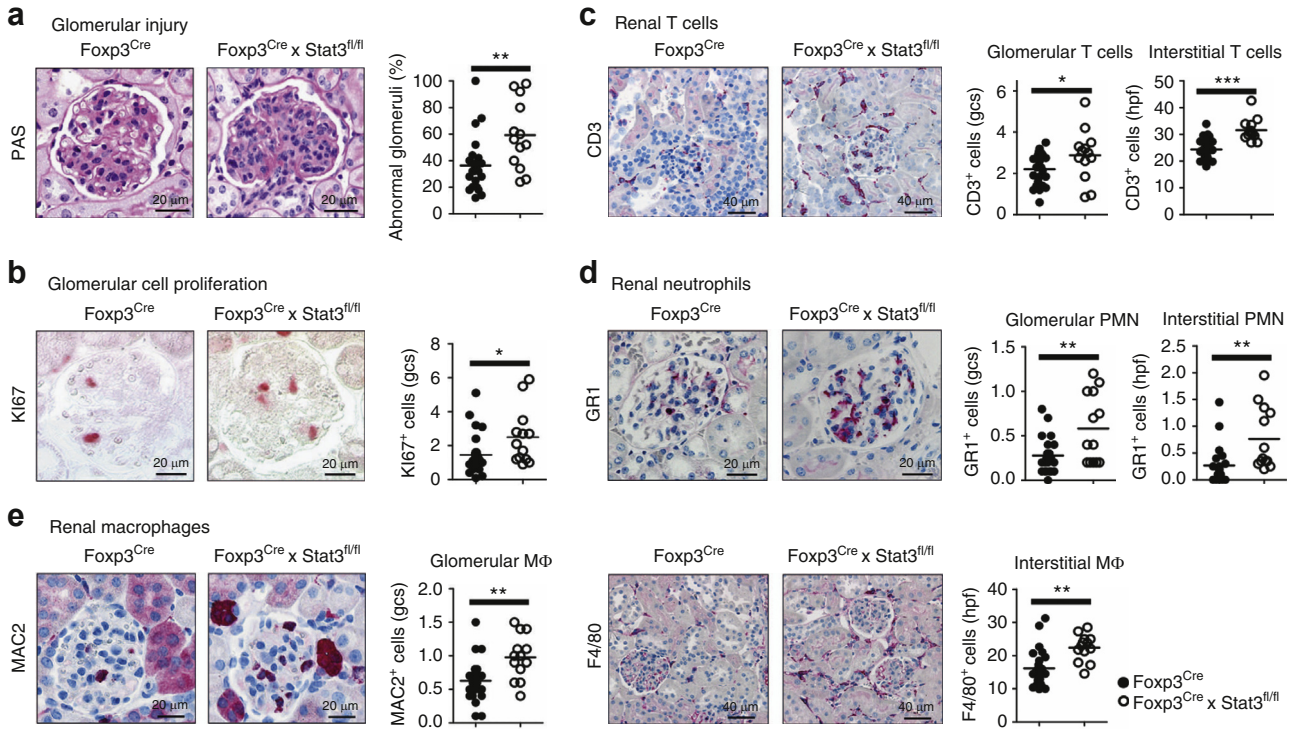

Figure 4| Renal injury is aggravated in $Foxp3^{Cre} \times Stat3^{fl/fl}$ mice. (a) Representative photographs of PAS-stained kidney sections and quantification of abnormal glomeruli (magnification ×400). **(b)** Representative photographs of KI67-stained kidney sections and quantification of positive cells (magnification ×400). **(c)** Representative photographs of CD3-stained kidney sections and quantification of glomerular and interstitial T cells (magnification ×400). **(d)** Representative photographs of GR-1–stained kidney sections and quantification of glomerular and interstitial neutrophils (magnification ×400). **(e)** Representative photographs of MAC2-stained (left) and F4/80-stained (right) kidney sections and quantification of glomerular ($MAC2^{+}$, left) and interstitial ($F4/80^{+}$, right) macrophages. All analyses were performed at 9 months after pristane injection. Circles represent individual mice, horizontal lines indicate mean values. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. PAS, periodic acid–Schiff.

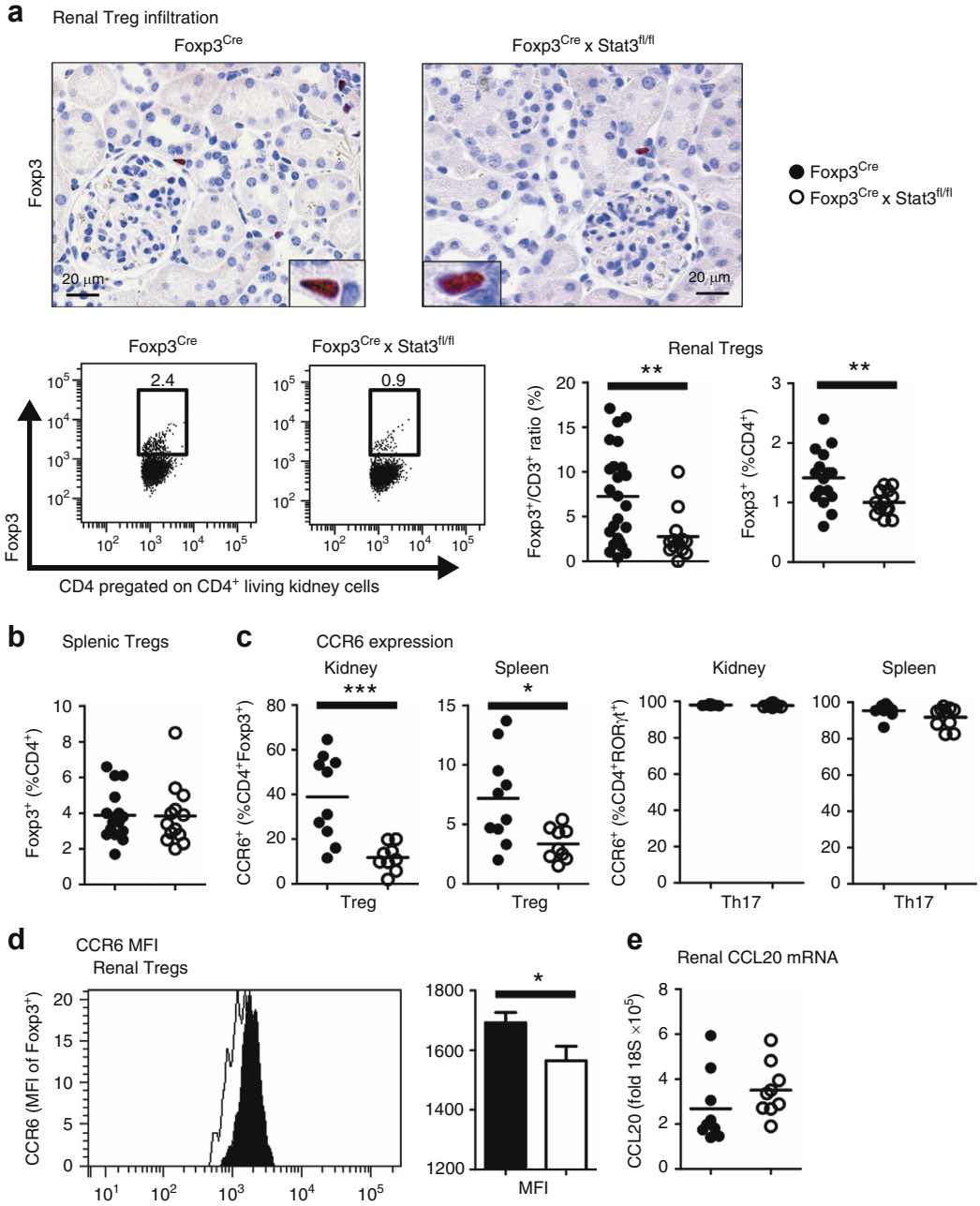

Figure 5 | Renal Treg trafficking is impaired owing to lack of CCR6. (a) Representative photographs of Foxp3-stained kidney sections at 9 months after pristane injection (upper row). Representative FACS plots of renal Tregs from the same experiment (lower row, left, gating as indicated). Quantification of renal Tregs via immunohistochemistry (lower row, middle) and FACS (lower row, right). **(b)** Flow cytometric quantification of splenic Tregs. **(c)** Frequencies of renal and splenic CCR6$^+$ Tregs (left) as well as renal and splenic CCR6$^+$ Th17 cells (right). **(d)** MFIs of CCR6 expression on renal Tregs as determined by FACS. **(e)** CCL20 expression from renal cortex was analyzed using qRT-PCR (right). Circles represent individual animals; horizontal lines represent mean values. Error bars represent standard errors of the mean. Numbers in FACS plots represent percentages of gated events. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; Th, T helper cells; Treg, regulatory T cells.

cells (Figure 5c, right). In line with reduced frequency of CCR6$^+$ Tregs, we also found significantly reduced CCR6 mean fluorescence intensity on Tregs from Foxp3^{Cre} × Stat3$^{fl/fl}$ mice (Figure 5d). Expression of CCL20, the single known CCR6 ligand, however, was similar in kidneys of both strains (Figure 5e).

Humoral autoimmunity in the absence of Treg17 cells

As renal injury depends on deposition of autoantibodies, we evaluated the effects of Treg17 deficiency on humoral immunity. Analysis of autoantibody production showed unchanged levels of total immunoglobulin (Ig)G anti–U1-RNP (Figure 6a) as well as IgG1, IgG2b, IgG2c, and IgG3

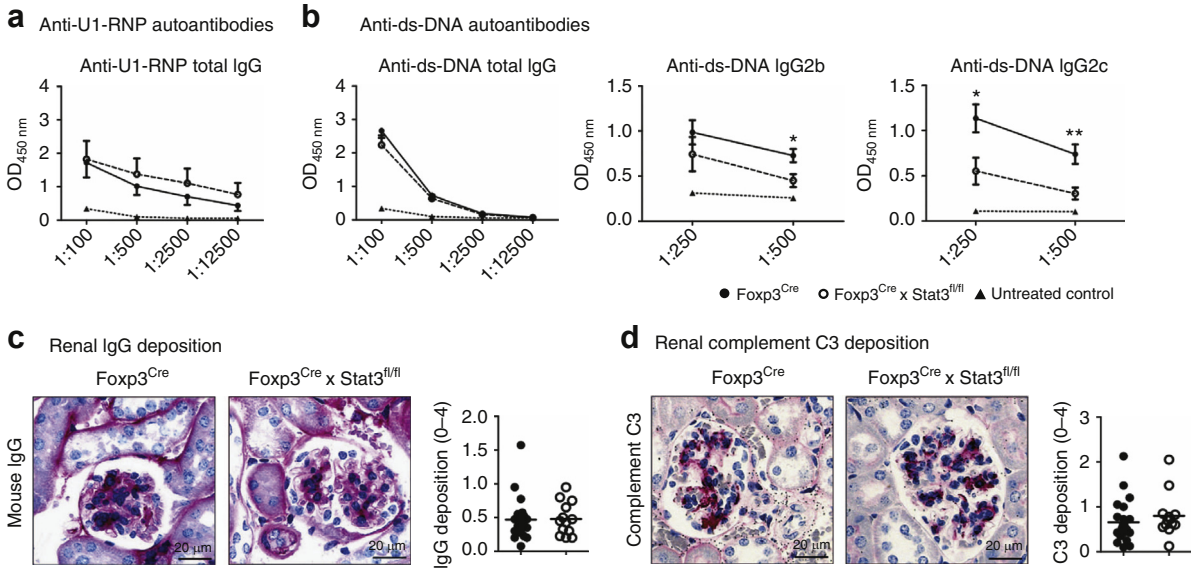

Figure 6 | Humoral autoimmunity in the absence of Treg17 cells. (a) Quantification of serum total IgG anti–U1-RNP auto-antibodies at the indicated dilutions. **(b)** Quantification of serum total IgG, IgG2b, and IgG2c anti–ds-DNA autoantibodies at the indicated dilutions. In **(a)** and **(b)**, serum of $n \geq 12$ Foxp3^{Cre} × Stat3$^{fl/fl}$, and $n = 24$ Foxp3^{Cre} control mice at 9 months after pristane application was analyzed by ELISA. **(c)** Representative photographs and quantification of glomerular mouse IgG (original magnification ×400) deposition. **(d)** Representative photographs and quantification of glomerular complement C3 deposition (original magnification ×400). Circles in **(c)** and **(d)** represent individual animals; horizontal lines represent mean values. Error bars represent standard error of the mean. *$P < 0.05$; **$P < 0.01$. Ig, immunoglobulin; Treg, regulatory T cells.

subclasses (Supplementary Figure S3A online). Similarly, total IgG anti–ds-DNA antibodies were unaltered in Treg17-deficient mice (Figure 6b). Levels of IgG2b and IgG2c anti–ds-DNA antibodies were slightly decreased (Figure 6b) while titers of IgG1 and IgG3 subclasses remained below detection level (Supplementary Figure S3B online). Autoantibodies of all investigated specificities and subclasses were undetectable in untreated controls (Figure 6a and b and Supplementary Figure S3A and B online). Finally, analysis of nephritic kidneys revealed that renal IgG (Figure 6c) and complement C3 (Figure 6d) deposition was similar between the groups.

DISCUSSION

Our study aimed to evaluate the hitherto unknown role of the newly defined Treg17 cells[25,27] for counter regulation of pathogenic Th17 responses in murine SLE. As a model system, we chose to study pristane-induced lupus as it closely resembles the human disease in many aspects.[29] Furthermore, two recent studies have highlighted the important role of the IL-17/Th17 axis for the development of autoimmunity and end organ damage in this model by showing protection of IL-17-deficient mice.[15,16] Pristane-injected mice rapidly develop peritoneal inflammation with oil granuloma formation, which at later stages results in the generation of lupus like autoimmunity. We thus assessed peritoneal immunity at 1 week after pristane administration in Treg17-deficient Foxp3^{Cre} × Stat3$^{fl/fl}$ versus Foxp3^{Cre} control mice. In line with our concept, we observed significant skewing towards Th17 responses in the absence of Treg17 cells. This indicates the control of Th17 responses by Treg17 cells already early on in the course of inflammation and well before autoimmunity is established. As a different early manifestation of pristane-induced inflammation, it has been reported that mice develop non-immune complex pulmonary vasculitis similar to human disease.[30] As little is known about this non-autoimmune manifestation of pristane, we analyzed lungs at 3 weeks after pristane injection. Our results revealed severe pulmonary small vessel capillaritis and identified neutrophils as the predominant leukocyte population. Given that neutrophils are the main effector arm of Th17 responses,[31,32] it was likely that capillaritis is also influenced by Treg17 mediation. Importantly for our study, and in line with our previous report,[27] the survival of untreated Foxp3^{Cre} × Stat3$^{fl/fl}$ mice was normal in our facility, and in contrast to the study by Chaudhry *et al.*,[25] our mice did not develop any signs of spontaneous autoimmunity and, in particular, no vasculitis of the lung was observed. As expected, however, pristane injection caused striking aggravation of pulmonary capillaritis in the absence of Treg17 cells, leading to dramatically reduced survival of Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. Detailed analyses of lungs showed much aggravated pulmonary hemorrhage, neutrophilia, and tissue injury in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. As the most likely underlying cause, we found pulmonary Th17 responses to be selectively dysregulated in the absence of Treg17 cells. As both peritoneal and pulmonary inflammation occur early after pristane administration and well before the onset of autoantibody formation, it is likely that the observed Th17 responses are not adaptive responses but rather belong to the recently defined natural Th17 cells. These cells rapidly secrete IL-17 in an antigen-unspecific manner upon TLR4

and TLR7 stimulation.[33] This assumption is supported by the previous observation that pristane-induced inflammation is highly dependent on TLR7.[34] It is thus tempting to speculate that Treg17 cells do not only control adaptive Th17 responses but also the innate like natural Th17 cells, which adds an important novel aspect to their nature.

Next, we wanted to study the long-term effects of Treg17 deficiency on the development of autoimmunity and renal end organ injury in pristane-induced SLE. We first analyzed systemic and renal immunity at 4 months, a time point when autoimmunity is already established and immune complex–mediated lupus nephritis is just starting to develop. Again, we found significantly and selectively increased Th17 responses in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice, both systemically in the spleens and also locally in the kidneys. In a next step, we sought to assess, whether the elevated Th17 responses were also associated with increased end organ injury. Analyses at 9 months after pristane injection once more revealed enhanced systemic and renal Th17 immunity in the absence of Treg17 cells. In line, histological analysis of kidneys showed severe immune complex nephritis, which we found to be significantly aggravated in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. Interestingly, although systemic Th1 immunity was unchanged, we observed much increased renal Th1 responses in the knockout group at this late time point. Analyses of *in vitro* Treg function, however, revealed fully intact capacity of Tregs from Foxp3^{Cre} × Stat3$^{fl/f}$ mice to suppress Th1 cells. It is thus unlikely, that the elevated renal Th1 responses are caused by a primary defect of Stat3-deficient Tregs, but rather develop secondarily, because of the enhanced tissue injury caused by excessive Th17 inflammation. The hypothesis that early Th17 dysregulation results in secondary development of elevated Th1 responses is also in line with our previous observations from another model of glomerulonephritis.[35]

In order to evaluate the mechanisms leading to the observed aggravation of SLE manifestations in our knockout animals, we next analyzed Tregs. In contrast to all other examined leukocyte subsets, we found renal Treg infiltration to be significantly decreased in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. This was not due to a general paucity of Tregs as frequencies in spleens were similar between the groups. Also, we and others could previously show that the general *in vitro* suppressive activity of Tregs from Foxp3^{Cre} × Stat3$^{fl/fl}$ mice is not impaired.[25,27] Our previous data, however, suggested that one mechanism of the Th17 specificity of Treg17 cells is shared expression of the trafficking receptor CCR6 by both T cell lineages, which facilitates their co-localization.[27] Indeed, our analyses showed, that only Tregs from control mice showed robust CCR6 expression, whereas the CCR6 was nearly absent on Tregs from Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. Renal expression of the only known CCR6 ligand, CCL20, was similar between the groups, underlining that lack of receptor expression rather than lack of the ligand caused impaired renal Treg accumulation in Foxp3^{Cre} × Stat3$^{fl/fl}$ mice. This emphasizes once more that Stat3 activation in Treg17 cells enables them to co-localize to their Th17 effector counterpart via expression of the chemokine receptor CCR6.

Finally, we wanted to evaluate whether humoral autoimmunity was also altered in the absence of Treg17 cells. Th17 cells have been shown to positively influence B cell production of autoantibodies in SLE[36] and our previous studies have shown impaired anti–ds-DNA and U1-RNP autoantibody production in the absence of IL-17.[15] However, levels of most analyzed autoantibodies were unchanged or, as in the case of IgG2b and IgG2c anti–ds-DNA antibodies, even slightly reduced in Treg17-deficient mice. In line, renal complement C3 and IgG deposition were similar throughout the groups. Thus, Treg17 cells predominantly control cellular immunity, whereas they seem to have a less important role in the development of humoral autoimmunity. Collectively, our data therefore suggest that indeed uncontrolled pathogenic Th17 responses account for the exacerbation of pristane-induced tissue injury in Treg17-deficient mice. However, a definite causal link between overshooting Th17 immunity and disease aggravation remains to be fully established.

Taken together, our study for the first time establishes a role of Treg17 cells for the control of Th17 responses and tissue protection during acute inflammatory and chronic autoimmune-mediated stages of pristane-induced SLE. Our data thus favor further investigation into the mechanisms mediating Th cell regulation to identify and evaluate potential therapeutic targets for the treatment of SLE.

MATERIALS AND METHODS

Animals

Stat3$^{fl/fl}$ mice were provided by Shizuo Akira, Osaka University, Japan.[37] Treg-specific deletion of Stat3 was achieved by crossbreeding with mice expressing a yellow fluorescent protein (YFP)-Cre recombinase fusion protein under the control of the Foxp3 locus,[38] which were kindly provided by Alexander Y. Rudensky, Memorial Sloan-Kettering Cancer Center, New York. All mice were bred in our facility. All animals used in this study were on a C57BL/6 background and were raised under specific pathogen-free conditions.

Animal experiments and functional studies

Pristane nephritis was induced in 8–10-week-old female Foxp3^{Cre} × Stat3$^{fl/fl}$ mice and Foxp3^{Cre} × Stat3$^{wt/wt}$ (referred to as Foxp3^{Cre}) littermate controls by single intraperitoneal injection of 500 μl of pristane oil (2,6,10,14-tetramethylpentadecane, Sigma-Aldrich, St. Louis, MO).[15] Organs were harvested between 1 week and 9 months after injection as indicated. Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (approval codes G37/11 and G45/12).

Morphological studies

Glomerular abnormalities were determined in 50 glomeruli per mouse in a blinded manner as published.[15,39] These included glomerular hypercellularity, crescent formation, fibrinoid necrosis, segmental proliferation, hyalinosis, and capillary wall thickening. Lung tissue was perfused with 500 μl formalin and fixed overnight, washed with ethanol, paraffin-embedded, and periodic acid–Schiff-stained.[40] Leukocyte infiltration, granuloma formation, alveolar-wall

thickening, and alveolar hemorrhage were assessed in a semi-quantitative score according to the percentage of tissue affected (0–25% = 1, 25–50% = 2, 50–75% = 3, >75% = 4). Mean scores from 15 high power fields were calculated. For immunohistochemistry, tissue was stained with antibodies against CD3 (A0452; Dako, Hamburg, Germany), F4//80 (BM8, BMA Biomedicals, Hiddenhausen, Germany), MAC2 (M3/38; Cedarlane-Laboratories, Burlington, Ontario, Canada), GR-1 (NIMP-R14, Hycult biotech, Uden, the Netherlands), Foxp3 (FJK-16 s, eBioscience, San Diego, CA) or KI67 (D3B5, Cell Signaling, Danvers, MA) and developed with a polymer-based secondary antibody–alkaline phosphatase kit (POLAP; Zytomed, Berlin, Germany), as published previously. Fifty glomerular cross-sections per kidney and 30 high power fields (magnification ×400) per kidney and lung section were counted in a blinded fashion.

Isolation of leukocytes from various tissues

Spleens were harvested in Hanks' Balanced Salt Solution and passed through 70-μm nylon meshes. After lysis of erythrocytes, cells were washed and passed over 40-μm meshes, counted, and resuspended in phosphate-buffered saline for either culture or fluorescence-activated cell sorting analysis. Kidneys were minced and incubated in the digestion medium (RPMI 1640 medium containing 10% fetal calf serum, 1% HEPES, 1% Penicillin/Streptomycin, 8 μg/ml Collagenase D, and 0.4 μg/ml DNase) at 37 °C for 40 min. After homogenization and lysis of erythrocytes, cells were filtered over 40-μm meshes and sedimented for 15 min at 4 °C. The upper leukocyte fraction was aspirated and filtered through another 40-μm nylon mesh. The peritoneal cavity was washed with 5 ml ice-cold phosphate-buffered saline to harvest cells at day 7 after pristane administration. For analysis of leukocytes from lungs, organs were removed, minced, and incubated in the digestion medium at 37 °C for 45 min. Tissues were then homogenized using a Gentle MACS dissociator (Miltenyi, Bergisch Gladbach, Germany), filtered over a 70-μm mesh and purified using a Percoll-gradient centrifugation.[40] Peripheral blood was drawn into EDTA-coated tubes and red blood cell lysis was performed after staining for surface markers.

Systemic cellular and humoral immune responses

Splenocytes (4×10^6 cells/ml) were cultured under standard conditions in the presence of 1 μg/ml anti-CD3 (eBioscience) and supernatants were harvested after 72 h. Commercially available ELISAs were used for detection of IFNγ, IL-4, IL-17A (Biolegend, San Diego, CA), IL-13 (eBioscience), and TGFßl (R&D Systems, Minneapolis, MN).[41] Circulating anti–Ul-RNP (Arotec, Wellington, New Zealand) and anti–ds-DNA antibodies (precoated plates from Alpha Diagnostic International, San Antonio, TX) were analyzed by ELISA[15,39] (the following antibodies were used: total IgG [Biozol, Eching, Germany], IgG1, IgG2b, IgG2c, and IgG3 [Invitrogen, Frederick, MD]).

Flow cytometry

Cells were stained with fluorochrome-labeled antibodies against CD45, CD3, CD4, CD8, CD19, CD44, CD69, CD62L, CCR6, γδTCR, Ly6G, CCR6, CD11c (eBioscience), and CD11b (BD Biosciences, Heidelberg, Germany) as previously described.[27,42] For intracellular and intranuclear staining, samples were processed using a commercial intranuclear staining kit (Foxp3-kit, eBioscience). Fluorochrome-labeled antibodies against IL-4, IL-5, IL-13, IL-17, IFNγ, Foxp3, T-bet (eBioscience), RORγt, and Gata-3 (BD Biosciences) were used as recently published.[27,43] For cytokine staining, cells were activated with Phorbol-12-myristat-13-acetate (PMA) (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem-Merck, Billerica, MA) for 5 h. After 30 min of incubation, brefeldin A (10 μg/ml; Sigma-Aldrich) was added. LIVE/DEAD staining (Invitrogen Molecular Probes, Eugene, OR) was used to exclude dead cells during flow cytometry. Experiments were performed on a BD LSRII Cytometer (Becton Dickinson, Heidelberg, Germany).

Quantitative real-time PCR analysis

Total RNA of renal cortex was isolated according to a standard Trizol protocol and purified by utilizing a Nucleospin kit (Macherey-Nagel, Düren, Germany). Real-time PCR was performed as described previously (all primer sequences available upon request) and results were normalized to expression of 18 S rRNA.[27]

Treg suppression assay

$CD4^+$ splenocytes were enriched by using magnetic-activated cell sorting according to the manufacturer's protocol (MACS $CD4^+$ T cell Kit II, Miltenyi Biotec). Tregs and effector T cells from $Foxp3^{Cre}$ and $Foxp3^{Cre} \times Stat3^{fl/fl}$ mice were then isolated, utilizing the Foxp3-driven YFP expression, by fluorescence-activated cell sorting in the institutional HEXT FACS Sorting Core facility (performed on a BD ARIAIII Cytometer, Becton Dickinson). A total of 1×10^5 $CD45^+CD4^+YFP^-$ effector T cells from $Foxp3^{Cre}$ mice were then cultured for 72 h in anti-CD3 monoclonal antibody (5 μg/ml, BD Biosciences) pre-coated 96-well plates either alone or in co-culture with 1×10^5 $CD45^+CD4^+YFP^+$ Tregs from either $Foxp3^{Cre}$ or $Foxp3^{Cre} \times Stat3^{fl/fl}$ mice.[27] IFNγ production was determined by cytokine ELISA performed from the supernatants.

Statistical analyses

Results are expressed as mean ± s.e.m. Groups were compared by Student's *t*-test and survival analyses were performed by log-rank test. A *P*-value < 0.05 was considered statistically significant.

Ethics

Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (approval codes G37/11 and G45/12).

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Peritoneal immune responses.
Figure S2. Immune responses at 4 and 9 months after pristane injection.
Figure S3. Serum auto-antibody subclasses.
Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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RORγt$^+$Foxp3$^+$ Cells are an Independent Bifunctional Regulatory T Cell Lineage and Mediate Crescentic GN

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ABSTRACT

Cells expressing both the regulatory T cell (Treg)-inducing transcription factor Foxp3 and the Th17 transcription factor RORγt have been identified (biTregs). It is unclear whether RORγt$^+$Foxp3$^+$ biTregs belong to the Th17-specific Treg17 cells, represent intermediates during Treg/Th17 transdifferentiation, or constitute a distinct cell lineage. Because the role of biTregs in inflammatory renal disease is also unknown, we studied these cells in the nephrotoxic nephritis (NTN) model of acute crescentic GN. Induction of NTN resulted in rapid renal and systemic expansion of biTregs. Notably, analyses of the biTreg expression profile revealed production of both anti-inflammatory (IL-10, IL-35) and proinflammatory (IL-17) cytokines. Additionally, biTregs expressed a signature of surface molecules and transcription factors distinct from those of Th17 cells and conventional Tregs (cTregs), and biTregs were identified in Treg17-deficient mice. Finally, fate reporter and cell transfer studies confirmed that biTregs are not Treg/Th17 transdifferentiating cells. Therapeutic transfer of biTregs suppressed the development of nephritis to an extent similar to that observed with transferred cTregs, but *in vitro* studies indicated different mechanisms of immunosuppression for biTregs and cTregs. Intriguingly, as predicted from their cytokine profile, endogenous biTregs displayed additional proinflammatory functions in NTN that were abrogated by cell-specific deletion of RORγt. In summary, we provide evidence that RORγt$^+$Foxp3$^+$ biTregs are a novel and independent bifunctional regulatory T cell lineage distinct from cTregs, Treg17 cells, and Th17 cells. Furthermore, biTregs appear to contribute to crescentic GN and hence may be novel therapeutic targets.

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Even after decades of research, the immune system keeps surprising us with its complexity and ever-expanding number of cellular mediators. Especially in the field of regulatory T cells (Treg) observations of recent years have suggested a previously unrecognized high degree of diversity.[1] Importantly, it was suggested that lineage-specific Tregs might exist which correspond to their proinflammatory Th counterpart.[2–4] Treg1 cells preferentially downregulate Th1 responses, while Treg17 cells dampen Th17 responses. Interestingly, programming of these lineage-specific Tregs seems to rely on some of the same transcription factors needed for induction of the opposing Th cell population. Recently, using the nephrotoxic nephritis (NTN) model of crescentic GN, we could show that activation of the transcription factor Stat3 is not only crucial for generation of nephritogenic Th17 cells but also programs their protective Treg17 cell counterpart.[4] This prompted the question whether other Th17-related transcription factors are active in regulatory T cells as well. Interestingly, pioneering work by the group of Eberl could show that not only Stat3 but also the second Th17 master transcription factor

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RORγt is expressed by a subpopulation of Foxp3$^+$ Tregs (hereafter referred to as biTregs). These cells were shown to possess potent suppressive capacity and to secrete large amounts of IL-10[5], identifying them as Tregs. However in a follow-up study the same authors reported proinflammatory IL-17 expression in RORγt$^+$Foxp3$^+$ biTregs under inflammatory conditions mimicking fungal infection.[6] This finding indicates both pro- and anti-inflammatory functions of biTregs and suggests a physiologic role during infection.

Importantly, RORγt$^+$Foxp3$^+$ biTregs were described to be regularly present in healthy humans as well, demonstrating trans-species conservation.[7,8] Multiple studies by several independent groups could also show the presence of IL-17 secreting biTregs in patients with different inflammatory pathologies including ulcerative colitis,[9] colonic cancer,[10] psoriasis,[11] peridontitis,[12] and juvenile arthritis.[13] While all these studies reported an association of biTregs with inflammation and tumors, their functional role remains widely unknown. Also, controversy exists over whether biTregs resemble a stable and unique T cell lineage or represent intermediates of Th17/Treg transdifferentiation.[14–16] Given these uncertainties, it is not surprising that nothing is known about biTregs with respect to renal disease. Their contribution to renal injury, however, is quite likely because multiple studies by us and others could show crucial dependency of acute glomerulonephritis on Foxp3$^+$ Tregs.[17–21] Administration of Foxp3$^+$ Tregs resulted in protection from GN while depletion of Foxp3$^+$ Tregs was shown to greatly aggravate renal injury. Conversely, RORγt is the most potent master transcription factor for induction of pathogenic Th17 responses.[22] In line, both RORγt and its target cytokine IL-17 were found to be central proinflammatory mediators of GN.[4,20,23,24] It is thus tempting to speculate that cells expressing not only one but both of these potent transcription factors, RORγt and Foxp3, play a significant role in inflammatory renal disease. Importantly, given the dominant role of RORγt in development of pathogenic Th17 responses, multiple research groups and companies are in the process of establishing blocking agents.[25–28] Therefore it is of great interest to better characterize all cell populations expressing RORγt, especially in the light of potential clinical applications. We thus decided to study the NTN model of acute GN to address the following aspects: (*1*) characterize RORγt$^+$Foxp3$^+$ biTreg dynamics during the course of GN, (*2*) determine whether biTregs represent Treg17 cells, Treg/Th17 intermediates or a unique cell population, (*3*) investigate the functional role of biTregs in GN.

RESULTS

biTregs Rapidly Expand During the Course of Crescentic GN

RORγt$^+$Foxp3$^+$ biTregs were found at low frequencies both systemically and locally in the kidneys of healthy mice. Induction of NTN, however, resulted in their rapid expansion in spleens and kidneys as compared with non-immunized control animals (Figure 1A and B). Over time, percentages decreased and were comparable to control levels at day 28 after NTN induction (Figure 1B). Interestingly, the dynamics of biTregs paralleled RORγt$^+$ Th17 responses (Figure 1C). Percentages of conventional Foxp3$^+$ Tregs (cTreg) in contrast, followed a different, more delayed time course and remained increased in the nephritic kidneys (Figure 1D). Expansion of biTregs probably occurred via proliferation, as indicated by very high Ki67 expression, which even exceeded levels observed in Th17 cells and cTregs (Figure 1E).

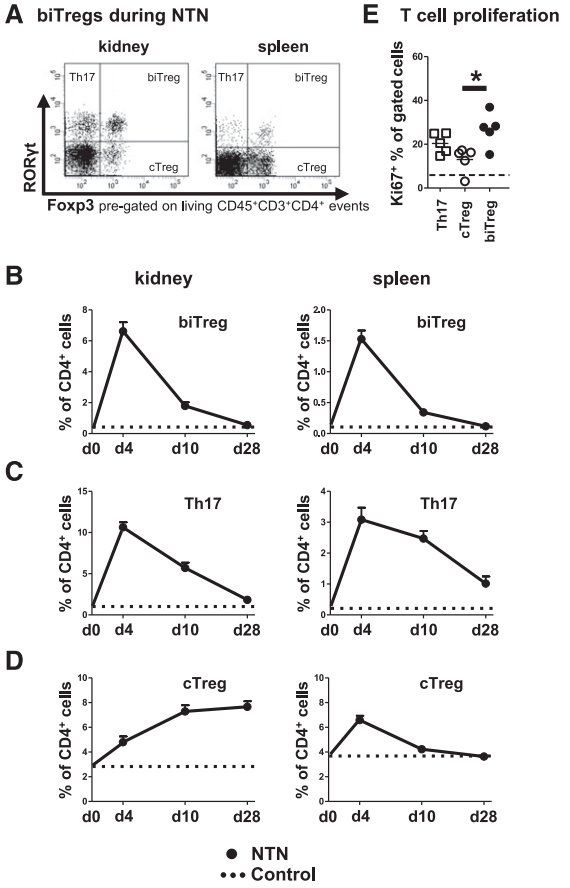

Figure 1. biTregs rapidly expand during the course of crescentic glomerulonephritis. (A) Representative FACS analysis of renal (left) and systemic (right) RORγt$^+$Foxp3$^-$ Th17, RORγt$^-$Foxp3$^+$ conventional Tregs (cTreg) and RORγt$^+$Foxp3$^+$ biTregs 10 days after NTN induction in wild-type (WT) mice. (B–D) For a time course study, frequencies of renal (left panels) and splenic (right panels) biTregs (B), Th17 cells (C) and cTregs (D) were determined at days 4 (n=7), 10 (n=10), and 28 (n=6). Dotted lines in (B–D) indicate untreated WT control mice (n=5). (E) Frequencies of Ki67$^+$ proliferating cells among the indicated T cell subsets. Dashed line represents total CD4$^+$ T cells (n=5). Circles and squares represent individual animals, horizontal lines indicate mean values. Error bars represent standard deviation. *P<0.05.

biTregs Show Co-Expression of Proinflammatory and Immune-Regulatory Molecules

Analysis of splenic and renal biTregs revealed robust expression of IL-17, together with very high levels of the chemokine receptor CCR6 at days 4 and 10 after NTN induction (Figure 2A). In contrast, no IL-17 expression was detected in cTregs. Expression of the Th1-associated molecules IFNγ and chemokine receptor CXCR3 was only found at low levels in biTregs (not shown). Interestingly, analysis of mRNA from FACS sorted spleen cells of RORγt Foxp3 double reporter mice revealed strong expression of anti-inflammatory IL-10 and the IL-35 subunit EBI-3 (Figure 2B) in biTregs at days 4 and 8 after immunization with sheep IgG. In line with their observed rapid expansion, biTregs also showed very high levels of activation (Figure 2C). In addition, biTregs co-expressed a distinctive pattern of regulatory molecules and transcription factors, clearly differentiating them from Th17 cells and cTregs. Surface expression of ICOS, CTLA-4, and CD103 was strikingly enhanced. CD25 and GITR expression were similar to cTregs, while PD-1 and Helios expression in contrast were significantly reduced (Figure 2D). Furthermore, analyses of transcription factor mRNAs revealed lack of PZLF expression in biTregs, distinguishing them from Th17 cells (Supplemental Figure 1A). Finally, as opposed to cTregs, Blimp-1 expression was low in biTregs and similar to levels in Th17 cells (Supplemental Figure 1B).

biTregs are Different from Treg17 Cells and do not Derive from Th17 Cells or cTregs

Next we wanted to establish whether biTregs are different from recently described Stat3-dependent Treg17 cells. Indeed, FACS

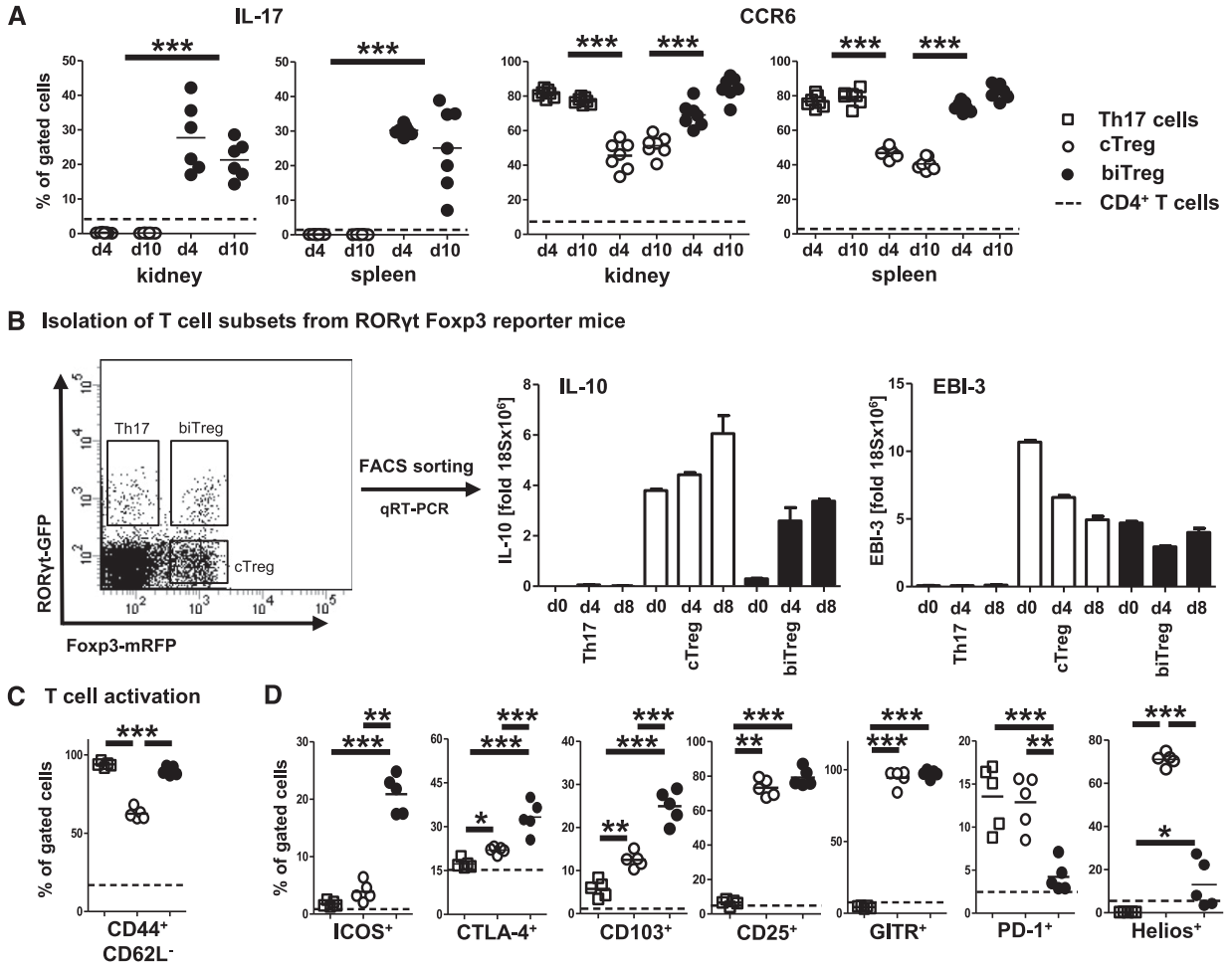

Figure 2. biTregs co-express proinflammatory and immune-regulatory molecules. (A) Renal and spleen cell IL-17 secretion (left) and CCR6 expression (right) was determined by flow cytometry for the indicated T cell subsets at 4 and 10 days after NTN induction in wild-type (WT) mice. (B) Th17 cells, biTregs, and cTregs were FACS sorted from MACS-enriched splenic CD4$^+$ T cells from RORγt^{GFP}Foxp3^{mRFP} double reporter mice (FACS plot pre-gated on CD45$^+$CD3$^+$CD4$^+$ events). Quantitation of IL-10 and EBI-3 by qRT-PCR before and at 4 and 8 days after sIgG immunization is shown. (C) T cell activation and (D) regulatory molecules for the indicated T cell subsets from WT spleen cells were FACS analyzed 6 days after sIgG immunization. Circles and squares represent individual animals, horizontal lines indicate mean values. Error bars represent standard deviation. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

analyses of kidneys and spleens from Treg17-deficient Foxp3^{Cre} × STAT3^{flox} mice revealed similar percentages of biTregs at day 4 after NTN induction, indicating their independent nature (Figure 3A). In order to investigate whether biTregs are a thymic or a peripherally induced Treg population, we analyzed thymi of 4- and 8-week-old naïve wild-type mice. Our analyses revealed complete absence of RORγt expression in CD4$^+$Foxp3$^+$ thymic Tregs in all animals studied (Figure 3B). Because this finding indicated a peripheral origin of biTregs, we next evaluated whether biTregs derive from RORγt$^+$Foxp3$^-$ Th17 cells or Foxp3$^+$RORγt$^-$ cTregs. Both populations were highly purified by FACS sorting from spleens of RORγt Foxp3 double reporter mice and independently transferred into CD45.1 recipient mice. Analysis of spleens and kidneys at 6 days after NTN induction showed that none of the transferred RORγt$^+$ Th17 cells had upregulated Foxp3 and likewise, none of the Foxp3$^+$ cTregs had upregulated RORγt (Figure 3C). Because transient upregulation of Foxp3 during T helper cell activation has been postulated, we next performed fate reporter studies. Analyses of Foxp3$^{Cre\text{-}yfp}$ activity reporter × Ai9 fate reporter mice showed that almost all cells in spleens of naïve mice that had expressed Foxp3 at some stage in life remained Foxp3 positive. NTN induction, however, resulted in growing percentages of fate positive Foxp3 cells that had lost Foxp3 expression (ex-Foxp3 cells). Interestingly, loss of Foxp3 occurred late during NTN between days 7 and 12 (Figure 3D), which coincides with retraction of the pool of biTregs (Figure 1B).

biTregs do not Trans-Differentiate into cTregs or Th17 Cells

Next, we wanted to clarify the underlying mechanism of the observed contraction of biTregs in the later course of NTN. For this purpose, RORγt$^+$Foxp3$^+$ biTregs were highly purified by FACS sorting from CD45.2 double activity reporter mice and transferred into CD45.1 recipients (Figure 4A). Analyses at day 10 after NTN induction revealed significantly greater enlargement of spleens in transferred versus control animals. Calculated numbers of recovered CD45.2 cells in spleens alone were about 8 times higher than the transferred 2×10^5 cells (Figure 4B). In line, the transferred CD45.2 population in spleens and kidneys showed high proliferative activity much exceeding recipient CD45.1 cells (Figure 4C). Tracking the fate of the transferred biTregs interestingly revealed that the vast majority had downregulated both RORγt and Foxp3 in spleens and kidneys. Minor populations of similar percentages had downregulated either RORγt or Foxp3 while a fourth small population had maintained the RORγt$^+$Foxp3$^+$ biTreg phenotype (Figure 4D).

Exogenous biTregs Suppress Crescentic GN by Mechanisms Different from cTregs

In a next step, we wanted to study the functional role of biTregs in GN. We thus adoptively transferred 2×10^5 biTregs or treated mice with PBS and analyzed kidneys at 10 days after NTN induction. Strikingly, glomerular injury, as measured by crescent formation and fibrinoid necrosis, was almost completely prevented by transfer of biTregs, while controls developed significant disease (Figure 5A). Similarly, tubulointerstitial injury was greatly ameliorated in the group receiving biTregs (Figure 5A). In line, the renal proinflammatory cell infiltrate was significantly reduced in the biTreg group (Supplemental Figure 2A, C–E), while renal Foxp3$^+$ Tregs were similar between the groups (Supplemental Figure 2B). In order to evaluate whether transfer of biTregs would also result in improved long-term outcome of nephritis, we repeated the experiment and evaluated renal damage at 30 days after NTN induction. Again we found significant protection from disease by transfer of biTregs (Figure 5B). Next, we wanted to compare the effects of exogenous biTregs with cTregs and Foxp3$^-$CD4$^+$ T effector cells (Teff). Analysis of kidneys from mice receiving either biTregs or cTregs showed significant and similar amelioration of renal injury at day 7 after NTN induction, when compared with Teff transferred mice (Figure 5C). Because both Treg populations effectively suppressed NTN, we next wanted to compare their mechanisms of immunosuppression by *in vitro* suppression assays. Surprisingly, analyses showed that only cTregs but not biTregs sufficiently reduced IL-2 levels in the supernatant of co-cultures with Teff. In contrast, however, biTregs much increased levels of the immunosuppressive cytokine IL-10 (Figure 5D), indicating different modes of action.

IL-17 Production by biTregs is Dependent on RORγt

Next, we wanted to study the functional role of RORγt expression in biTregs. For this purpose, we generated Foxp3^{Cre} × RORC$^{flox/flox}$ mice, because our experiments had shown that biTregs are the only Foxp3$^+$ cell population expressing RORγt. In contrast to RORγt pan-knockout mice, Foxp3^{Cre} × RORC$^{flox/flox}$ mice did not lack lymph nodes, indicating preserved RORγt activation in lymphoid tissue inducer cells. In line, spleen cell numbers and subset composition including total Foxp3$^+$ Tregs and CD4$^+$ T helper cells were similar in naïve wild-type and knockout mice (not shown). Importantly, however, analysis of systemic immunity at day 6 after immunization with sIgG showed complete absence of RORγt selectively in Foxp3$^+$ cells of knockout animals (Figure 6A), proving defective RORγt activation in biTregs. Spleen cell numbers (Figure 6B) and IFNγ$^+$ Th1, IL-17$^+$ Th17 as well as total Foxp3$^+$ Treg percentages were not different between the groups (Figure 6C). Most notably, however, IL-17 production by Foxp3$^+$ T cells and thus by biTregs was abrogated in Foxp3^{Cre} × RORC$^{flox/flox}$ mice (Figure 6D). T cell activation and proliferation were comparable (Figure 6, E and F). Similarly, production of various Th1, Th17, and Treg hallmark cytokines by spleen cells was not different (Figure 6G). Finally, we found that *in vitro* suppressive activity of RORγt-deficient Tregs was unchanged in comparison to wild-type Tregs (Figure 6H).

GN is Ameliorated by Deletion of RORγt in biTregs

Next, we wanted to know whether RORγt signaling in biTregs confers them with pathogenic properties. Indeed, analyses

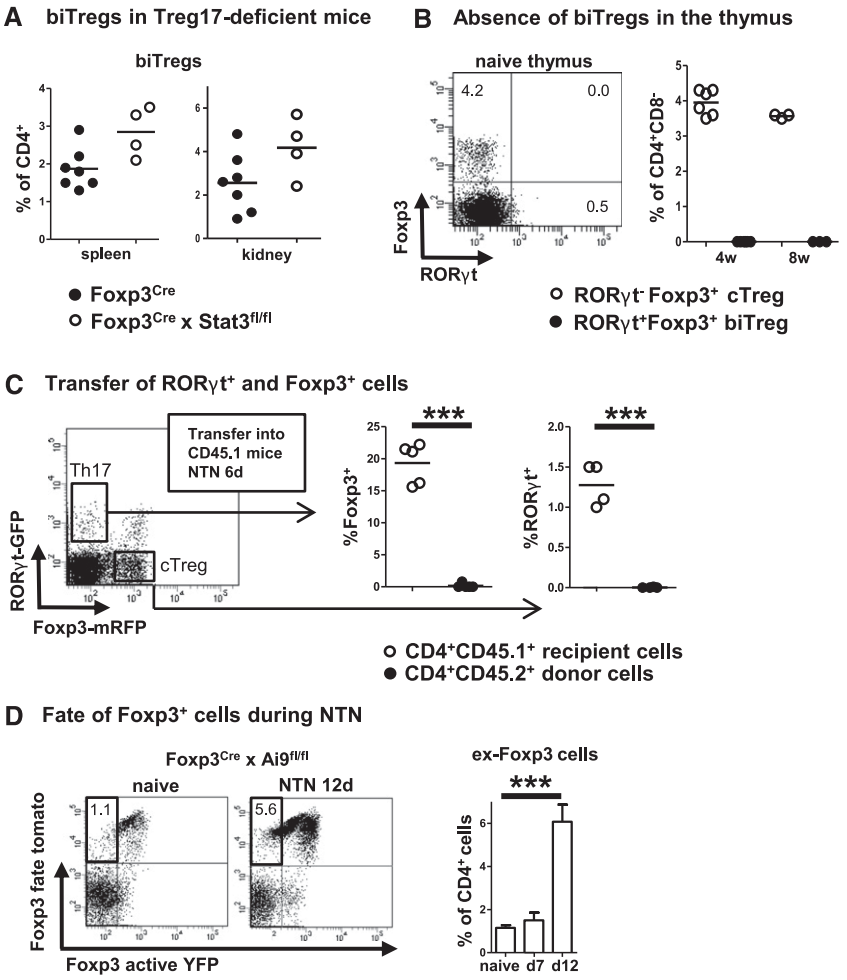

Figure 3. biTregs are different from Treg17 cells and do not derive from Th17 or cTregs. (A) Frequencies of splenic and renal biTregs 4 days after NTN induction in Treg17-deficient Foxp3^{Cre}xStat3$^{fl/fl}$ mice and Foxp3^{Cre} controls. (B) FACS analysis of thymocytes from naïve 4- and 8-week-old wild-type (WT) mice. A representative FACS plot from a 4-week-old mouse is shown on the left (pre-gated on CD45$^+$CD3$^+$CD4$^+$CD8$^-$ cells). Quantitation of the indicated cell populations is shown on the right. (C) RORγt$^+$Foxp3$^-$ Th17 cells and Foxp3$^+$RORγt$^-$ cTregs were FACS sorted (plot pre-gated on CD45$^+$CD3$^+$CD4$^+$ events) and transferred into CD45.1 recipients. Percentages of spleen cell Foxp3 and RORγt expression among live CD4$^+$CD45.1$^+$ recipient and CD4$^+$CD45.2$^+$ donor cells are shown (data from one representative of two independent experiments for each transfer study are shown). (D) Representative FACS plots of spleen cells from Foxp3$^{Cre\text{-}YFP}$ activity × Ai9^{tomato} fate reporter mice from naïve and NTN treated mice at 12 days after induction (pre-gated on CD45$^+$CD3$^+$CD4$^+$ events). ex-Foxp3 cells are found in the upper left quadrant. Quantification of ex-Foxp3 cells is shown as percentage of CD4$^+$ cells (n=4 per group). Numbers in FACS plots indicate percentages of gated cells. Circles represent individual animals, horizontal lines indicate mean values. Error bars represent standard deviation. ***P<0.001.

at day 10 after NTN induction revealed amelioration of nephritis in Foxp3^{Cre} × RORC$^{flox/flox}$ animals in terms of histologic damage and albuminuria (Figure 7A). Furthermore, proinflammatory renal leukocyte infiltration was significantly reduced while renal Foxp3$^+$ total Treg frequencies remained similar (Figure 7B). Likewise, percentages of renal Th1 and Th17 cells were comparable (Figure 7C). Importantly, however, RORγt expression was specifically absent in renal Foxp3$^+$ cells of Foxp3^{Cre} × RORC$^{flox/flox}$ animals, confirming lack of RORγt activation in biTregs (Figure 7D). In line, we found robust expression of IL-17 in renal Tregs of wild-type mice which was completely abrogated in knockouts (Figure 7E). Analysis of systemic immune responses showed slightly reduced spleen cell numbers in knockout mice but otherwise comparable subset composition. In particular, similar percentages of total Tregs and Th17 cells were observed (Supplemental Figure 3A). Also, levels of IL-17 production by spleen cells were not different while IL-6 production was even enhanced in knockouts. Similarly, Treg characteristic cytokines were unaltered (TGF-β) or even elevated (IL-10, Supplemental Figure 3B). Humoral immune responses as measured by antigen-specific antibody production were not altered by RORγt deficiency in biTregs (Supplemental Figure 3C). In order to validate these findings in a second model, we studied accelerated NTN (aNTN).[20] Mice were preimmunized with sheep IgG and nephritis was induced 5 days later, at a time point when biTregs have massively expanded. Analysis at day 7 after aNTN induction again showed ameliorated renal damage in Foxp3^{Cre} × RORC$^{flox/flox}$ mice (Supplemental Figure 4A). Renal proinflammatory leukocyte infiltration was reduced while frequencies of renal Tregs were similar (Supplemental Figure 4B). The renal Th1 and Th17 equilibrium again remained unchanged (Supplemental Figure 4C).

DISCUSSION

Our study aimed to better characterize the biology of RORγt$^+$Foxp3$^+$ biTregs and define their role in acute crescentic GN. This is of special importance because the Th17 defining transcription factor RORγt has proven to be a potent proinflammatory mediator during glomerulonephritis[20] and multiple blocking agents have been developed and await clinical testing.[25–28] Our analyses showed regular presence of biTregs in both spleens and kidneys of healthy mice. Interestingly, we observed a rapid and

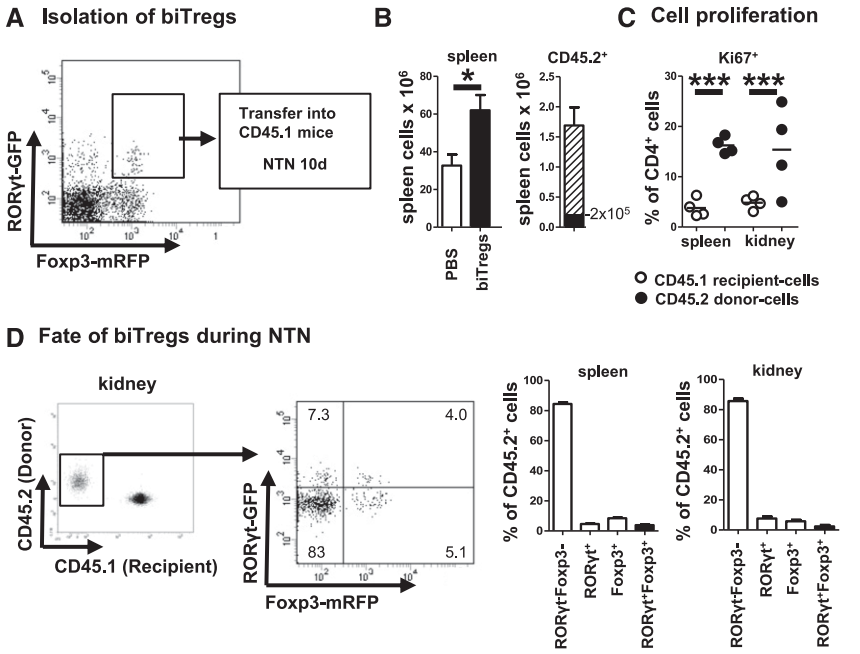

Figure 4. biTregs do not trans-differentiate into Th17 or conventional Tregs. (A) biTregs were FACS sorted from spleens of CD45.2 Foxp3^{mRFP} × RORγt^{GFP} double reporter mice (representative FACS plot, pre-gated on CD45$^+$CD3$^+$CD4$^+$ events) and transferred into CD45.1 WT mice 1 day prior to the induction of NTN. (B) Total spleen cells (left panel) were quantified at day 10 of NTN following the transfer of 2 × 10^5 biTregs (n=4 animals, black bar) or PBS (n=8 controls, white bar). Splenic CD45.2 donor cells had massively expanded (right panel, shaded bar). (C) Proliferation of renal and splenic donor and recipient CD4$^+$ T cells as determined by Ki67 expression. (D) Splenic and renal RORγt and Foxp3 expression of CD45.2 donor cells at day 10 after NTN (FACS plots show a representative kidney, pre-gated on CD3$^+$CD4$^+$ events. Data from one representative of two independent transfer studies are shown). Quantification of the indicated subpopulations in spleens and kidneys is shown. Circles represent individual animals, horizontal lines indicate mean values. Error bars represent standard deviation. *P<0.05; ***P<0.001.

massive biTreg expansion early during the course of NTN which paralleled Th17 cells. Conventional Tregs (cTregs), in contrast, showed different and more delayed dynamics. Proliferation rates of biTregs were very high, indicating that they increase numbers by cell division rather than recruitment or transdifferentiation from other cell types. Further characterization of biTregs showed a bifunctional pro- and anti-inflammatory profile. They express high levels of IL-17 and the Th17-characteristic chemokine receptor CCR6. At the same time, however, biTregs secrete high amounts of anti-inflammatory IL-10 and IL-35. biTregs also showed quite high levels of activation, indicating their functional importance. Detailed analyses of surface expression of immune-modulatory molecules revealed a unique signature differing from both Th17 cells and cTregs. As a main hallmark, we found enhanced levels of ICOS on biTregs, which is in line with two previous reports.[5,29] Interestingly, ICOS expression on Tregs has also been reported to be associated with IL-17 production.[30] Furthermore, biTregs showed a uniquely strong expression of CTLA-4 and also the Integrin CD103 which was previously described to be a marker for regulatory T cells with the highest suppressive capacity.[31] CD25 and GITR expression was high and similar to cTregs, PD-1, and Helios expression in contrast was significantly reduced. Further analyses also revealed a unique transcription factor signature, different from Th17 cells and cTregs. biTregs lacked mRNA expression of the transcription factor PZLF, which was recently shown to be characteristic for naturally occurring nTh17 cells.[32] Similarly, we noted that Blimp-1, a transcription factor implicated in Treg effector functions,[33] was only weakly expressed in biTregs. Collectively, these findings suggested that biTregs represent a unique and independent cell lineage with both suppressive and proinflammatory properties at the same time.

Next, we wanted to study the developmental origin of biTregs. Recently, a Treg subset specialized at downregulating Th17 responses has been described. These Treg17 cells depend on activation of the transcription factor Stat3.[4,34] Because Stat3 is a known inducer of RORγt, we aimed to investigate whether biTregs might belong to this newly identified Treg subset. However, biTregs were present at normal percentages in mice lacking Stat3 activation in Tregs, indicating that they are a population different from Treg17. Next, we addressed the question of whether biTregs are a thymic or a peripherally induced Treg subset. To this end, analyses of thymi showed complete absence of biTregs, which indicates peripheral induction. We thus aimed to investigate whether biTregs might arise in the periphery from RORγt single positive Th17 cells or Foxp3 single positive cTregs and performed fate reporter and cell transfer studies. Highly purified RORγt$^+$ Foxp3$^-$ Th17 cells and Foxp3$^+$RORγt$^-$ cTregs were transferred and their fate was analyzed in spleens and kidneys at day 6 after NTN induction, a time point when biTregs are fully expanded. Results showed that none of the Th17 cells had upregulated Foxp3 and likewise none of the cTregs had started to express RORγt. We could thus conclude that biTregs do not derive from Th17 cells or cTregs. Our findings therefore support the Foxp3 lineage heterogeneity model recently proposed by Hori[35] rather than Foxp3/Th17 lineage plasticity. However, it has also been postulated that Foxp3 might be transiently induced in non-Treg CD4$^+$ T cells during their activation.[36–39] We therefore tracked the fate of Foxp3$^+$ cells continuously during NTN using fate reporter

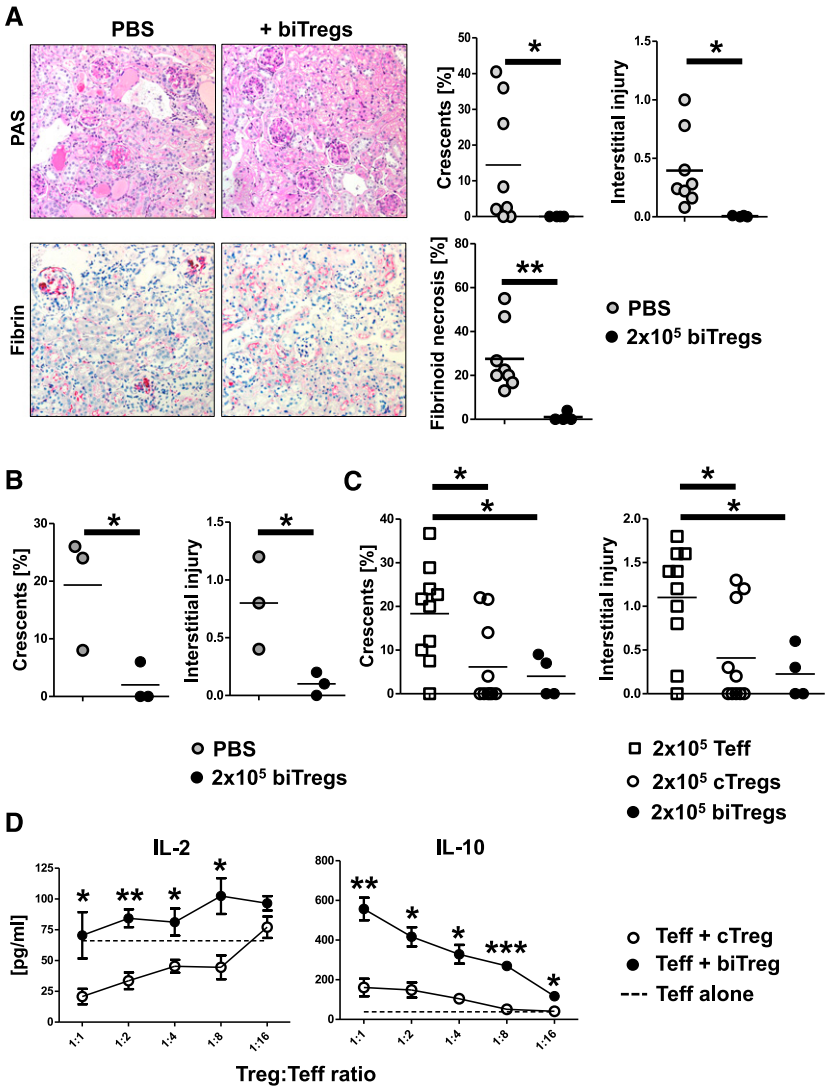

Figure 5. Exogenous biTregs suppress GN by mechanisms different from cTregs. (A) Analyses of renal injury at day 10 of NTN in mice injected with PBS or 2×10^5 biTregs. Representative photographs of PAS or fibrin-stained (red) kidney sections (original magnification, ×200) and quantification of renal histologic damage (crescents, interstitial injury, and fibrinoid necrosis as indicated). (B) Quantification of renal histologic damage at day 30 of NTN in PBS or 2×10^5 biTreg injected mice. (C) Renal histologic damage was quantified at day 7 of NTN after transfer of either 2×10^5 Teff, cTregs, or biTregs. (D) *In vitro* suppression assays were performed by co-culturing Teff with either cTregs or biTregs at the indicated ratios (n=3 per group). Cytokine levels of IL-2 and IL-10 were analyzed in co-culture supernatants as indicated. Circles and squares represent individual animals, horizontal lines indicate mean values. Error bars represent standard deviation. *P<0.05; **P<0.01; ***P<0.001.

mice. Analyses showed that almost all Foxp3$^+$ cells in naïve mice had maintained Foxp3 activation during their life. Likewise, during the first 7 days after NTN induction we did not detect loss of Foxp3 expression in fate positive cells, excluding unspecific and temporary Foxp3 upregulation during T effector cell activation. During later stages of NTN, however, a growing fraction of Foxp3 fate positive cells showed loss of Foxp3. These ex-Foxp3 cells were generated at a time point (12 days after NTN) at which numbers of cTregs are stable while numbers of biTregs rapidly decline. We thus wanted to answer the question of whether the observed retraction of biTregs might be due to loss of Foxp3 and/or transdifferentiation into Th17 cells. To answer this question, we adoptively transferred highly purified CD45.2 biTregs cells into CD45.1 animals. At day 10 of NTN we found massive enlargement of spleens in the recipient animals containing numbers of CD45.2 cells that exceeded the transferred numbers by 10-fold. In line, CD45.2 donor-derived cells had a high proliferative activity much exceeding that of CD4$^+$CD45.1 recipient cells. Interestingly, analysis of donor cell fate revealed that the vast majority had downregulated both Foxp3 and RORγt activation. Small and similarly sized fractions had downregulated either Foxp3 or RORγt with no tendency for preferential transdifferentiation into RORγt$^+$Foxp3$^-$ Th17 or conventional Foxp3$^+$RORγt$^-$ cTregs. These findings support a concept in which RORγt$^+$Foxp3$^+$ biTregs represent a distinct cell lineage which rapidly expands from the pre-existing pool in naïve mice by inflammation-induced proliferation. Subsequently the cells retract and downregulate both transcription factors. After clarifying the dynamics and fate of biTregs, we aimed to study their function. We thus adoptively transferred biTregs into wild-type recipient mice and subsequently induced nephritis. In line with their Foxp3 expression and similar to the observations from Lochner *et al.*,[5] biTregs showed regulatory capacity and potently protected from renal injury. Importantly, protective effects by exogenous biTregs were maintained long term and also ameliorated renal injury at 30 days after disease induction. Furthermore, additional transfer studies comparing biTregs with cTregs showed a similar degree of protection from glomerulonephritis by both populations. However, given the striking differences between cTregs and biTregs on multiple levels as cytokine profile, surface molecule signature and transcription factor expression, we wanted to explore whether they also differ functionally. Indeed, *in vitro* suppression assays indicated different mechanisms of immunosuppression. While only cTregs sufficiently suppressed IL-2 levels in co-culture with effector T cells, biTregs much enhanced secretion of the anti-inflammatory cytokine IL-10. These

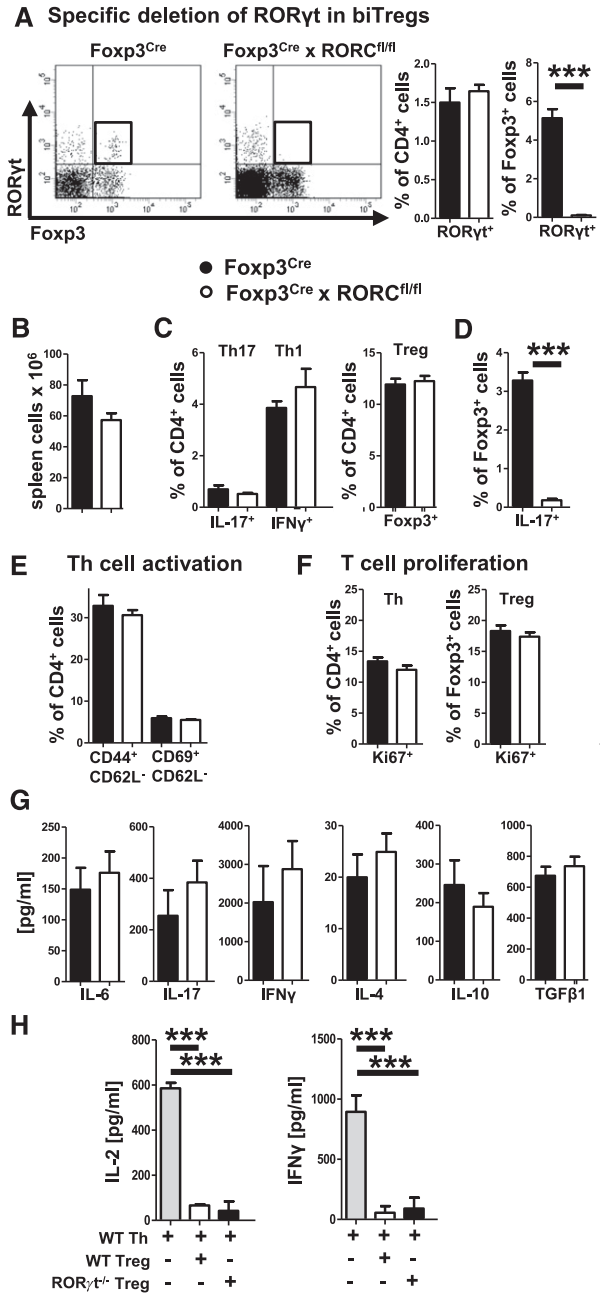

Figure 6. IL-17 production by biTregs is dependent on RORγt. (A) Representative FACS plots of spleen cells 6 days after sheep IgG immunization of indicated mice (pre-gated on $CD45^+CD3^+CD4^+$ events). Quantification of RORγt in $CD4^+$ and $Foxp3^+$ T cells in $Foxp3^{Cre}$ (n=11) and $RORC^{flox/flox} \times Foxp3^{Cre}$ mice (n=15). (B) Quantification of spleen cell numbers. (C) Splenic $CD4^+$ subsets determined by FACS (gated as indicated). (D) IL-17 production by splenic $Foxp3^+$ T cells. (E) T cell activation and (F) proliferation of the indicated subsets as determined by flow cytometry from spleen cells. (G) Spleen cell secretion of the indicated cytokines determined by ELISA. (H) *In vitro* suppression of T helper cell (Th) produced IL-2 and IFNγ by WT and $ROR\gamma t^{-/-}$ Tregs as determined by ELISA. Error bars represent standard deviation. ***$P<0.001$.

data identified biTregs as unique regulatory T cells with a sum effect that is anti-inflammatory in nephritis. However, we suspected that biTregs might have some pathogenic potential as well. In particular, because RORγt expression is strongly associated with proinflammatory properties[20] and we observed robust secretion of IL-17 by biTregs. We thus generated mice with selective RORγt deficiency in $Foxp3^+$ Tregs. Because our previous experiments showed that no other $Foxp3^+$ cell population apart from biTregs activates RORγt, this is effectively a knockout of RORγt in biTregs. Development of mice with RORγt-deficient biTregs was normal and they did not show any signs of spontaneous autoimmunity, indicating intact Treg suppressive function. Analysis of immune responses, however, showed complete lack of IL-17 secretion by biTregs in the knockouts. It is of note that Treg and Th17 responses appeared otherwise unaltered, which further underlines the independent character of biTregs. Importantly, NTN severity was much ameliorated in the knockout mice, proving a proinflammatory role of RORγt expression in biTregs. In order to validate these findings in a second model, we also studied the course of accelerated NTN[20] and again found protection from renal injury in the absence of RORγt activation in biTregs.

Our data thus lead to the conclusion that biTregs possess both potent anti-inflammatory functions but also some proinflammatory, RORγt-mediated properties. This bifunctional nature might seem illogical at first sight. However, biTregs could have evolutionarily developed to fill an important gap between mediators of host defense and tissue protection. Their unique properties equip them to fight pathogens while they can protect us from collateral tissue injury and development of autoimmunity at the same time. Importantly, their proinflammatory functions can be blocked by abrogation of RORγt signaling, which makes biTregs a promising target for RORγt-directed therapies of inflammatory diseases.

In summary our studies identify biTregs as novel mediators of glomerulonephritis. biTregs represent a previously unrecognized independent and bifunctional regulatory T cell lineage with great potential for future therapeutic strategies.

CONCISE METHODS

Animals

LoxP-site flanked $RORC^{fl/fl}$ and $RORC^{-/-}$ mice were obtained from The Jackson Laboratory. $Stat3^{fl/fl}$ mice were a generous gift from Shizuo Akira, Osaka University, Japan. $Foxp3^{YFP\text{-}Cre}$ mice were a kind gift from Alexander Y. Rudensky, Memorial Sloan-Kettering Cancer Center, New York. BAC-transgenic *Rorc(γt)-Gfp*^{TG} *x* Fir (Foxp3-IRES-mRFP) mice[5] were kindly provided by Gerard Eberl, Paris, France and Matthias Lochner, Hannover, Germany. Ai9 mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J) were kindly provided by Edgar Kramer, Hamburg, Germany. CD45.1 mice initially derived from The Jackson Laboratory. All animals used in this study were on a C57BL/6 background and were raised under specific pathogen-free conditions at our animal facility.

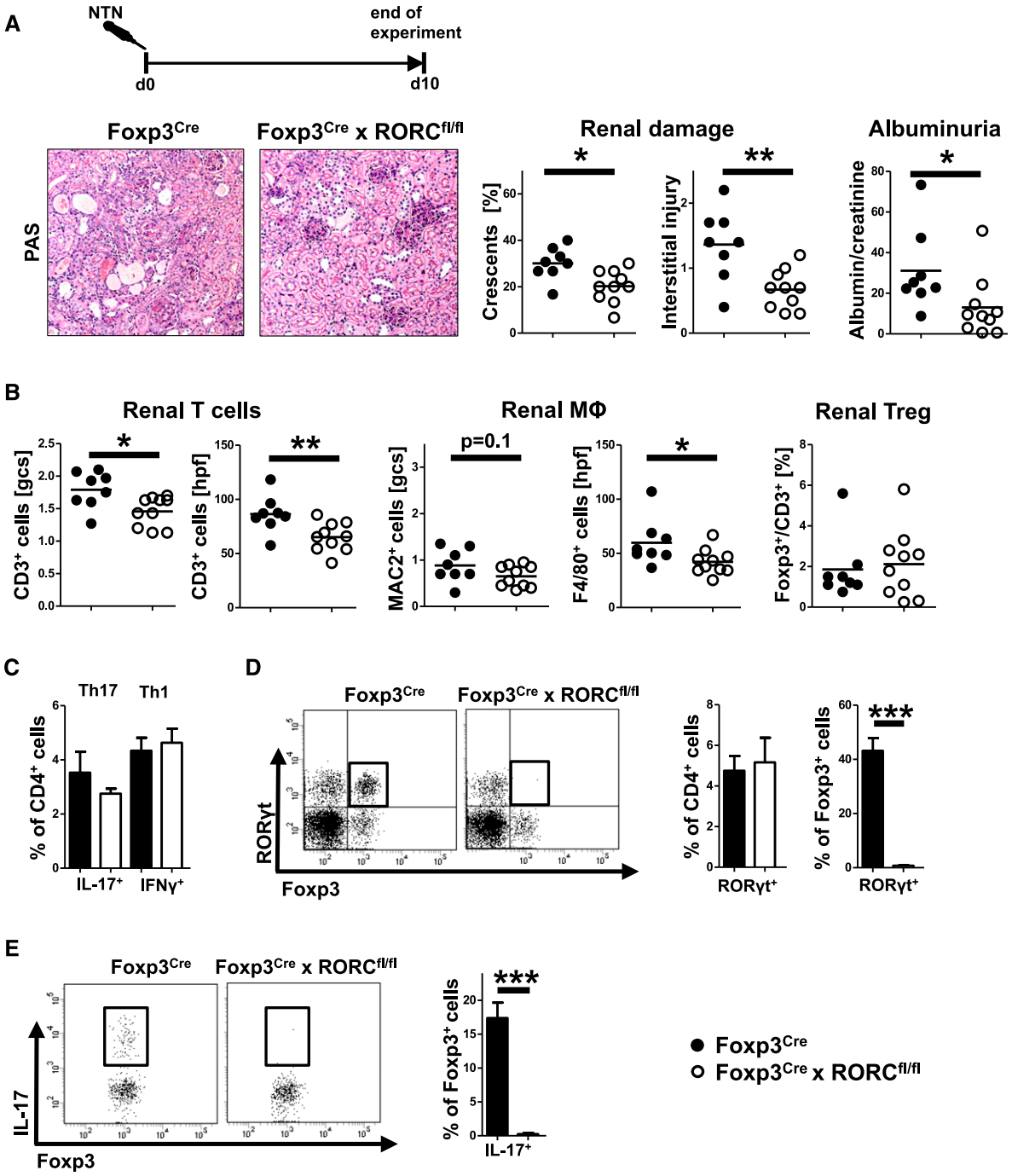

Figure 7. Crescentic GN is ameliorated by deletion of RORγt in biTregs. (A) Experimental set-up and representative photographs of PAS-stained kidney sections (original magnification, ×200) with quantification of renal histologic damage (crescents and interstitial damage) and albuminuria (albumin/creatinine). (B) Quantification of glomerular and interstitial T cells (CD3), macrophages (MAC2, F4/80), and Treg percentages (Foxp3/CD3). (C) FACS analyses of renal Th1 and Th17 cells. (D) Analysis of RORγt expression in the indicated renal leukocyte subsets (a representative FACS plot is shown, pre-gated on $CD45^+CD3^+CD4^+$ events.). (E) Analysis of IL-17 production by renal $Foxp3^+$ cells (a representative FACS plot is shown, pre-gated on $CD45^+CD3^+CD4^+Foxp3^+$ events). Circles represent individual animals, horizontal lines indicate mean values. Error bars represent standard deviation. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Animal Experiments and Functional Studies

Nephrotoxic nephritis was induced in 8- to 10-week-old male mice of the indicated genotypes by i.p. injection of 2.5 mg of nephrotoxic sheep serum per gram body weight.[4] For accelerated NTN (aNTN), mice were i.p. pre-immunized with 0.5 mg sheep IgG in complete Freund's adjuvant at day −5. NTN was induced at day 0.[20] For immunization studies, mice were i.p. immunized with 0.5 mg sheep IgG in complete Freund's adjuvant and organs were harvested at day 6. For transdifferentiation studies, FACS sorted CD45.2 splenic Foxp3$^+$ (5×10^5 cells per animal) or RORγt$^+$ single positive CD4$^+$ T cells (1.5×10^5 cells per animal) from naïve *Rorc(γt)-Gfp^{TG} x* Fir (Foxp3-IRES-mRFP) double reporter donor mice were i.v. injected into the CD45.1 recipient tail vein. For nephritis studies, FACS sorted RORγt$^+$Foxp3$^+$ biTregs, RORγt$^-$Foxp3$^+$ cTregs or Foxp3$^-$CD4$^+$ T effector cells (2×10^5 cells per animal) from spleens of double reporter donor mice were i.v. injected into the wild-type recipient tail vein. Organs were harvested between 4 and 30 days after immunization or NTN induction as indicated. Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (approval codes G37/11, G45/12, 73/14, and 07/15). Urine samples were collected after housing the mice in metabolic cages. Albuminuria was determined by standard ELISA (Bethyl Laboratories). Blood urea nitrogen (BUN) and urinary creatinine were quantified using standard laboratory methods.

Morphologic Studies

Crescent formation and glomerular necrosis were determined in a minimum of 50 glomeruli per mouse in 2 μm thick PAS-stained kidney sections in a blinded manner. Semiquantitative analysis of tubulointerstitial damage was performed using ten randomly selected cortical areas (×200) as described previously.[20] Paraffin-embedded sections were stained with antibodies directed against murine fibrin alpha chain (UC45; Abcam, Inc., Cambridge, UK), CD3 (A0452; Dako, Hamburg, Germany), F4/80 (BM8; BMA Biomedicals, Hiddenhausen, Germany), MAC2 (M3/38; Cedarlane-Laboratories, Burlington, ON, Canada), GR-1 or Foxp3 and developed with a polymer-based secondary antibody–alkaline phosphatase kit (POLAP; Zytomed, Berlin, Germany), as published previously.[40] Fifty glomerular cross-sections (gcs) and 30 tubulointerstitial high power fields (hpf, magnification, ×400) per kidney section were counted in a blinded fashion.

Isolation of Leukocytes from Various Tissues

Spleens or thymi were harvested in HBSS and passed through 70 μm nylon meshes. After lysis of erythrocytes with ammonium chloride, cells were washed and passed over 40 μm meshes. Cells were then washed again, counted and resuspended in PBS for either culture or FACS analysis. Kidneys were minced and incubated in digestion medium (RPMI 1640 medium containing 10% FCS, 1% HEPES, 1% penicillin/streptomycin, 8 μg/ml collagenase D and 0.4 μg/ml DNase) at 37°C for 40 minutes. Tissues were then homogenized, passed over 70 μm nylon meshes and centrifuged at 300 *g* at 4°C for 8 minutes. After lysis of erythrocytes with ammonium chloride, cells were filtered over 40 μm meshes, washed again and sedimented for 15 minutes at 4°C. The upper leukocyte fraction was aspirated and filtered through another 40 μm nylon mesh. Cells were washed, counted and resuspended in PBS for staining and FACS analysis.

Antigen-Specific Systemic Cellular and Humoral Immune Responses

Splenocytes (4×10^6 cells/ml) were cultured under standard conditions in the presence of normal sheep IgG (10 μg/ml, Sigma-Aldrich, Taufkirchen, Germany) and supernatants were harvested after 72 hours. Commercially available ELISAs were used for detection of IFNγ, IL-4, IL-6, TNFα, IL-10, IL-17A (Biolegend, San Diego, CA), and TGFβ1, IL-2 (R&D Systems, Minneapolis, MN). Circulating sheep-globulin-specific serum IgG titers were analyzed by ELISA (for total IgG; Biozol, Eching, Germany, for IgG1, IgG2c and IgG3; Invitrogen, Frederick, MD).

Flow Cytometry

Cells were surface-stained for 30 minutes at 4°C with fluorochrome-labeled antibodies against CD45, CD45.1, CD45.2, CD3, CD4, CD8, CD19, CD25, CD44, CD69, CD62L, CCR6, CXCR3, ICOS, CTLA-4, PD-1, GITR, and CD103 (Ebioscience, San Diego, CA) as previously described.[4]

For intracellular and intranuclear staining, samples were processed using a commercial intranuclear staining kit (Foxp3-Kit; Ebioscience). Fluorochrome-labeled antibodies against IL-17, IFNγ, Foxp3, Ki67, T-Bet (all Ebioscience), RORγt (BD Biosciences, Heidelberg, Germany), and Helios (Biolegend) were employed as recently published.[4] For intracellular cytokine staining, cells were activated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem-Merck) for 4 hours. After 30 minutes of incubation, Brefeldin A (10 μg/ml; Sigma-Aldrich) was added. LIVE/DEAD staining (Invitrogen Molecular Probes, Eugene, OR) was used to exclude dead cells during flow cytometry and to ensure viability of the cells after the stimulation procedure. Experiments were performed on a BD LSRII cytometer (Becton Dickinson, Germany). FACS sorting was performed from single-cell suspensions enriched for CD4$^+$ T cells by MACS sorting (T-cell isolation kit II; Miltenyi Biotec, Germany) from the indicated tissues and animal strains by the institutional HEXT FACS Sorting Core facility using a BD ARIAIII Cytometer (Becton Dickinson, Germany) as previously described.[4]

Treg Suppression Assay

Total Tregs and effector T cells from wild-type and RORC$^{-/-}$ mice were isolated from splenic single-cell suspensions by MACS according to the manufacturer's instructions (MACS CD4$^+$ T-Cell-Isolation Kit; Miltenyi Biotec). Briefly, CD4$^+$ T cells were enriched using a biotinylated antibody cocktail, depleting all other blood cell types with anti-biotin microbeads. CD4$^+$CD25$^+$ regulatory T cells were isolated by positive selection using PE-labeled anti-CD25 mAb and anti-PE microbeads. For comparison of cTregs with biTregs, Foxp3$^+$ RORγt$^-$ cTregs, RORγt$^+$Foxp3$^+$ biTregs and CD4$^+$Foxp3$^-$Teff were isolated by FACS sorting from spleens of naïve *Rorc(γt)-Gfp^{TG} x* Fir (Foxp3-IRES-mRFP) double reporter mice. 1×10^5 effector T cells were cultured for 72 hours in anti-CD3 mAb (5 μg/ml; BD Biosciences) pre-coated 96-well plates either alone or in co-culture with total Tregs, cTregs or biTregs at different ratios as indicated. Suppressive

capacity was determined by cytokine ELISAs performed from the supernatants.[18]

Quantitative Real-Time PCR Analysis

Quantitative RT-PCR from RNA derived from FACS sorted cells was performed in a Stepone Plus detector (Applied Biosystems) as described before.[41] For detection of IL-10 and 18S, the sybr green method was used (primer sequences available upon request) while detection of EBI-3, PZLF and Blimp-1 was performed using Taqman probes (Applied Biosystems assay id: Mm00469294_m1, Mm01176868_m1, Mm00476128_m1). Samples were run in duplicates and normalized to 18S rRNA.

Statistical Analyses

Results are expressed as mean±SD. In the case of two groups, comparison was performed by *t* test and a P value <0.05 was considered statistically significant. For studies with more than two experimental groups, one-way ANOVA was applied using Tukey *post hoc* testing.

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DISCLOSURES

None.

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RORγt expression in T_{regs} promotes systemic lupus erythematosus via IL-17 secretion, alteration of T_{reg} phenotype and suppression of Th2 responses

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Summary

Systemic lupus erythematosus (SLE) is a common autoimmune disorder with a complex and poorly understood immunopathogenesis. However, a pathogenic role for the T helper type 17 (Th17) axis was demonstrated by many studies, while regulatory T cells (T_{regs}) were shown to mediate protection. Recently, we and others characterized a novel and independent T cell population expressing both the T_{reg} characteristic transcription factor forkhead box protein 3 (FoxP3) and the Th17-defining retinoic acid receptor-related orphan nuclear receptor γt (RORγt). Studies in a model of acute glomerulonephritis unveiled potent regulatory, but also proinflammatory, functions of RORγt$^+$FoxP3$^+$ T_{regs}. This bi-functional nature prompted us to suggest the name 'biT_{regs}'. Importantly, the pathogenic biT_{reg} effects were dependent upon expression of RORγt. We thus aimed to evaluate the contribution of RORγt$^+$FoxP3$^+$ biT_{regs} to pristane-induced SLE and explored the therapeutic potential of interference with RORγt activation. Our analyses revealed expansion of IL-17 producing biT_{regs} in a distinctive time–course and organ-specific pattern, coincident with the development of autoimmunity and tissue injury. Importantly, specific ablation of RORγt activation in endogenous biT_{regs} resulted in significant amelioration of pristane-induced pulmonary vasculitis and lupus nephritis. As potential mechanisms underlying the observed protection, we found that secretion of IL-17 by biT_{regs} was abrogated completely in FoxP3^{Cre} × RORC$^{fl/fl}$ mice. Furthermore, T_{regs} showed a more activated phenotype after cell-specific inactivation of RORγt signalling. Finally, and remarkably, biT_{regs} were found to potently suppress anti-inflammatory Th2 immunity in a RORγt-dependent manner. Our study thus identifies biT_{regs} as novel players in SLE and advocates RORγt-directed interventions as promising therapeutic strategies.

Keywords: immunology, lupus nephritis, lymphocytes, systemic lupus, transcription factors

Introduction

Systemic lupus erythematosus (SLE) is a complex and relatively common autoimmune disorder, which can affect multiple organs including skin, joints, lungs, kidneys and the central nervous system [1,2]. As SLE causes high morbidity and mortality in a rather young collective of patients, the search for new therapeutic strategies is a priority for scientists worldwide [3,4]. Despite intensive research, the events which lead to development of SLE and eventually cause the associated organ pathologies still remain widely elusive [5,6]. However, a central role for CD4$^+$ T helper cells in disease pathogenesis has been demonstrated by multiple studies in mice and humans, including our own [7–11]. Recently, we and others particularly highlighted the importance of the T helper type 17/interleukin-17 (Th17/IL-17) axis for systemic autoimmunity and lupus nephritis [10,12,13]. Importantly, mice deficient in IL-17A or F were shown to be protected from disease in

three independent studies, using the murine model of pristane-induced SLE [14–16]. Furthermore, recent studies could identify a specialized regulatory T cell subset, which is tailor-made for down-regulation of pathogenic Th17 responses [17,18]. Lack of these T regulatory 17 ($T_{reg}17$) cells resulted in much increased SLE associated mortality and organ pathologies [19]. A crucial role for both, Th17 cells and T_{regs} in SLE is therefore evident. Interestingly, we and others could recently describe an intriguing novel T cell subset, expressing the unusual combination of the T_{reg} master transcription factor forkhead box protein 3 (FoxP3), together with the Th17 master regulator retinoic acid receptor-related orphan nuclear receptor γt (RORγt) [20–22]. Functional characterization revealed that these RORγt FoxP3 double-positive T cells displayed both regulatory as well as proinflammatory functions in a model of acute crescentic glomerulonephritis [22]. $ROR\gamma t^{+}FoxP3^{+}$ T cells were shown to produce the proinflammatory cytokine IL-17, but at the same time they secreted high amounts of immunoregulatory IL-10, transforming growth factor (TGF)-β and IL-35. Furthermore, they potently suppressed T effector cell (T_{eff}) responses *in vitro*, identifying them as T_{regs} [20,22]. Given their bi-functional properties, we proposed to name these $ROR\gamma t^{+}FoxP3^{+}$ T cells 'biT_{regs}'. In accordance with their unique cytokine profile and transcription factor expression, biT_{regs} showed both pro- and anti-inflammatory *in-vivo* functions in crescentic glomerulonephritis [22]. Exogenous biT_{reg} transfer ameliorated renal inflammation to a similar extent as conventional RORγt-negative T_{regs}, although the suppressive mechanisms seem to be different. Conversely, endogenous biT_{regs} displayed additional proinflammatory functions. Interestingly, these proinflammatory biT_{reg} functions depended upon activation of RORγt [22]. This is clinically highly relevant, as multiple pharmaceutical companies are currently developing RORγt blocking agents and some Phases I and II studies are already ongoing [23–25]. Furthermore, a landmark study which was published during preparation of this paper confirmed the biological relevance of biT_{regs} and surprisingly identified them as potent down-regulators of anti-inflammatory Th2 immunity [26]. This effect, which might explain some of the proinflammatory biT_{reg} functions, also depended upon activation of the transcription factor RORγt. Given their multiple immune modulatory functions, biT_{regs} are highly likely to contribute to development of SLE. Because, however, nothing is known to date about the clinical relevance of biT_{regs}, we decided to study their role in SLE. A major hallmark of human SLE is activation of the Type I interferon pathway, as has just recently been highlighted again [27]. We thus chose to use the pristane model of SLE, which is currently the only available murine model characterized by a strong interferon signature [28]. In particular, our study addressed the following aspects: (1) characterization of biT_{reg} dynamics in the different organ systems affected during the course of SLE, (2) analysis of the role of RORγt in biT_{regs} with special focus on IL-17 secretion and regulation of Th2 immunity and (3) assessment of the contribution of biT_{regs} to organ pathologies in pristane-induced SLE.

Materials and methods

Animals

LoxP-site flanked $RORC^{fl/fl}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). $FoxP3^{YFP\text{-}Cre}$ mice were a kind gift from Alexander Y. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). All animals used in this study were on a C57BL/6 background and were raised under specific pathogen-free conditions at our animal facility.

Animal experiments and functional studies

Pristane disease was induced in 8–10-week-old male or female $FoxP3^{Cre} \times RORC^{fl/fl}$ mice and matched $FoxP3^{Cre} \times RORC^{wt/wt}$ (referred to as $FoxP3^{Cre}$) littermate controls by single intraperitoneal (i.p.) injection of 500 μl of pristane oil (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, St Louis, MO, USA) [14,19]. Organs were harvested between 1 week and 9 months after injection, as indicated. Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (approval codes 37/11, 45/12, 73/14 and 07/15).

Morphological studies

Glomerular abnormalities were determined in 50 glomeruli per mouse in a blinded manner, as published previously [14,29]. These included glomerular hypercellularity, crescent formation, fibrinoid necrosis, segmental proliferation, hyalinosis and capillary wall thickening. Lung tissue was perfused with 500 μl formalin and fixed overnight, washed with ethanol, paraffin-embedded and periodic acid-Schiff (PAS)-stained [30]. Leucocyte infiltration, granuloma formation, alveolar-wall thickening and alveolar haemorrhage were assessed in a semi-quantitative score according to the percentage of tissue affected (0–25% = 1, 25–50% = 2, 50–75% = 3, >75% = 4). Mean scores from 15 high-power fields were calculated. Numbers of pulmonary granulomas were determined in 10 low-power fields (lpf, magnification × 100) per tissue section; their size was quantified using the Zeiss Axio Vision software (Carl Zeiss, Jena, Germany). For immunohistochemistry, tissue was stained with antibodies against CD3 (A0452; Dako, Hamburg, Germany), F4/80 (BM8; BMA Biomedicals, Hiddenhausen, Germany), MAC2 (M3/38; Cedarlane-Laboratories, Burlington, ON, Canada), granulocyte-differentiation antigen-1 (GR-1) (NIMP-R14; Hycult Biotech, Uden, the Netherlands), FoxP3 (FJK-16s; eBioscience, San Diego, CA, USA) or KI67 (D3B5; Cell

Signaling, Danvers, MA, USA) and developed with a polymer-based secondary antibody–alkaline phosphatase kit (POLAP; Zytomed, Berlin, Germany), as published previously. Fifty glomerular cross-sections (gcs) per kidney and 30 high-power fields (hpf, magnification × 400) per kidney and lung section were counted in a blinded fashion.

Isolation of leucocytes from various tissues

Spleens were harvested in Hanks' balanced salt solution (HBSS) and passed through 70-μm nylon meshes. After lysis of erythrocytes, cells were washed and passed over 40-μm meshes, counted and resuspended in phosphate-buffered saline (PBS) for either culture or fluorescence activated cell sorter (FACS) analysis. Kidneys were minced and incubated in digestion medium [RPMI-1640 medium containing 10% fetal calf serum (FCS), 1% HEPES, 1% penicillin/streptomycin, 8 μg/ml collagenase D and 0·4 μg/ml DNase] at 37°C for 40 min. Kidney tissue was then homogenized using a gentle magnetic affinity cell sorter (MACS) dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany) and purified using Percoll-gradient centrifugation [31]. The peritoneal cavity was washed with 5 ml ice-cold PBS to harvest cells at the indicated time-points after pristane administration [19]. For analysis of leucocytes from lungs, organs were removed, minced and incubated in digestion medium at 37°C for 45 min. Tissues were then homogenized using a gentle MACS dissociator (Miltenyi Biotech), filtered over a 70-μm mesh and purified using Percoll-gradient centrifugation [30]. Peripheral blood was drawn into ethylenediamine tetraacetic acid (EDTA)-coated tubes and red blood cell lysis was performed.

Systemic humoral immune responses

Circulating anti-ds-DNA and anti-U1-ribonucleoprotein (RNP) antibodies from serum were analysed by ELISA at the indicated dilutions after coating microtitre plates with either poly-L-lysine (Sigma-Aldrich) and calf thymus DNA (Worthington) or U1-RNP (Arotec). For analysis of total non-antigen-specific immunoglobulins, ELISA plates were precoated with anti-mouse immunoglobulin (Ig)G antibodies (Jackson Immuno Research). For all analyses, serum samples were applied at the dilutions indicated. The following secondary antibodies were used for detection: total IgG, IgG1 (both Southern Biotech), IgG2b (Invitrogen), IgG2c (Bethyl) and IgG3 (Jackson Immuno Research). For detection of IgE, sera were diluted 1 : 200 and a commercially available ELISA was utilized (Biolegend, San Diego, CA, USA), following the manufacturer's instructions.

Analyses of cytokines from serum and spleen cell cultures

Splenocytes (4×10^6 cells/ml) were cultured under standard conditions in the presence of 1 μg/ml anti-CD3 (eBioscience, San Diego, CA, USA) and supernatants were harvested after 72 h. Commercially available ELISAs were used for detection of IFN-γ and IL-17A (Biolegend). Serum was obtained from mice at 5 and 9 months after pristane induction and was analysed for levels of IL-17 and IFN-γ by ELISA (Biolegend) [19].

Flow cytometry

Cells were stained with fluorochrome-labelled antibodies against CD45, CD3, CD4, CD8, CD19, CD44, CD69, CD62L, CCR6, γδTCR, inducible T cell co-stimulator (ICOS), glucocorticoid-induced TNFR family-related gene (GITR), CD103, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), CXCR3 (eBioscience), C-X-C chemokine receptor type 5 (CXCR5), programmed death 1 (PD-1), IgD, IgM, CD138 and PD-L1 (Biolegend). For intracellular and intranuclear staining, samples were processed using a commercial intranuclear staining kit (FoxP3 kit; eBioscience). Fluorochrome-labelled antibodies against IL-4, IL-5, IL-13, IL-17, IFN-γ, FoxP3 (eBioscience), RORγt, KI67 (both BD Biosciences, Heidelberg, Germany) and Gata-3 (Biolegend) were employed, as published recently [19,22]. For cytokine staining, cells were activated with phorbol myristate acetate (PMA) (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem-Merck, Temecula, CA, USA) for 3.5 h. After 30 min of incubation, brefeldin A (10 μg/ml; Sigma-Aldrich) was added. LIVE/DEAD staining (Invitrogen Molecular Probes, Eugene, OR, USA) was used to exclude dead cells during flow cytometry. Experiments were performed on a BD LSRII Cytometer (Becton Dickinson, Heidelberg, Germany).

T$_{reg}$ suppression assay

CD4$^+$ spleen cells were enriched using magnetic-activated cell sorting according to the manufacturer's protocol (MACS CD4$^+$ T cell Kit II; Miltenyi Biotec). T$_{regs}$ and T$_{eff}$ cells were isolated by FACS sorting (performed on a BD ARIAIII Cytometer; Becton Dickinson). A total of 1×10^5 CD45$^+$CD4$^+$yellow fluorescent protein (YFP)$^-$ effector T cells from FoxP3^{Cre} mice were then cultured for 72 h in anti-CD3 monoclonal antibody (mAb) (5 μg/ml; BD Biosciences) precoated 96-well plates either alone or in co-culture with CD45$^+$CD4$^+$YFP$^+$ T$_{regs}$ from FoxP3^{Cre} or FoxP3^{Cre} × RORC$^{fl/fl}$ mice at the ratios indicated. Suppressive capacity was determined by IL-2 ELISA performed from the supernatants, as published recently [17,22,32]. For analyses of IFN-γ, IL-10, IL-4, IL-13 and TNF-α in the supernatants, cytometric bead array assays were performed using a commercial kit (LEGENDplex^{TM} mouse Th cytokine mix and match subpanel; BioLegend). To assess T$_{eff}$ cell proliferation, cells from the above culture experiments were harvested and analysed for their KI67 expression by FACS.

Quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA of renal cortex and spleen tissue was isolated according to a standard Trizol protocol and purified utilizing a Nucleospin kit (Macherey & Nagel, Düren, Germany).

Real-time PCR was performed after cDNA transcription as described previously (all primer sequences are available upon request) and results were normalized to expression of 18S rRNA [17].

Statistical analyses

Results are expressed as mean ± standard error of the mean (s.e.m.). Groups were compared by Student's *t*-test. A *P*-value $< 0{\cdot}05$ was considered statistically significant.

Results

IL-17$^+$ biT$_{regs}$ expand during systemic pristane-induced lupus

In order to characterize more clearly the pristane model of SLE, we initially performed time–course analyses of the multiple organ manifestations. After pristane injection, mice develop acute sterile peritonitis and non-immune complex pulmonary capillaritis. Approximately 3 weeks later, immunologically active peritoneal and pulmonary granulomas become apparent. Subsequently, autoimmunity is established with increasing serum levels of various autoantibodies, which we found to be present already in low amounts at week 8. Deposition of immune complexes then leads to progressive development of lupus nephritis which, according to our analyses, became evident by light microscopy at approximately 5 months (data not shown). In Fig. 1a a schematic overview is depicted. In order to assess the potential role of biT$_{regs}$, we analysed their frequencies in multiple organs before and at different time–points after injection of pristane. biT$_{regs}$ were found to be present in both peritoneal lavage cells and lungs of healthy animals. In acute antigen-independent sterile peritonitis and pulmonary vasculitis, their population expanded only slightly (Fig. 1b,c). However, at later time-points, coincident with development of peritoneal and pulmonary granulomas, biT$_{reg}$ percentages increased significantly ($2{\cdot}5 \pm 0{\cdot}27\%$ of peritoneal FoxP3$^+$ T$_{regs}$ at baseline *versus* $5{\cdot}56 \pm 0{\cdot}98\%$ at 5 weeks and $7{\cdot}57 \pm 0{\cdot}99\%$ at 8 weeks; $5{\cdot}5 \pm 0{\cdot}46\%$ of pulmonary FoxP3$^+$ T$_{regs}$ at baseline, $18{\cdot}96 \pm 3{\cdot}17\%$ at 5 weeks and $21{\cdot}4 \pm 0{\cdot}86\%$ of FoxP3$^+$ T$_{regs}$ at week 8) (Fig. 1b,c). In contrast, spleens which are only mildly affected during pristane-induced SLE showed merely a slight expansion of biT$_{regs}$ (Fig. 1d). Finally, we studied biT$_{regs}$ during the course of immune complex lupus nephritis. Interestingly, our analyses revealed a distinctive pattern of renal infiltration. While only few biT$_{regs}$ were detected at 2 months after pristane injection ($1{\cdot}3 \pm 0{\cdot}38\%$ of FoxP3$^+$ T$_{regs}$), their frequencies increased to reach a maximum at 5 months ($10{\cdot}62 \pm 1{\cdot}7\%$ of FoxP3$^+$ T$_{regs}$). Afterwards, biT$_{reg}$ proportions declined steadily and were almost back to baseline levels ($0{\cdot}3 \pm 0{\cdot}04\%$ of FoxP3$^+$ T$_{regs}$) at 9 months ($1{\cdot}3 \pm 0{\cdot}38\%$ of FoxP3$^+$ T$_{regs}$) (Fig. 1e). A significant proportion of biT$_{regs}$ produced IL-17 in all examined organs at all time-points (Fig. 1b–e). A representative FACS plot of pulmonary biT$_{regs}$, including their IL-17 secretion, is shown in Fig. 1f.

RORγt activation in biT$_{regs}$ aggravates pulmonary capillaritis

In order to study the role of RORγt in biT$_{regs}$, we generated FoxP3^{Cre} × RORC$^{fl/fl}$ mice, which specifically lack RORγt activation in T$_{regs}$. As pristane-injected mice develop severe, often lethal, haemorrhagic pulmonary capillaritis, we first studied lung pathology in FoxP3^{Cre} × RORC$^{fl/fl}$ and FoxP3-^{Cre} control mice. At 3 weeks after pristane injection, no macroscopic or histological differences were noted (Supporting information, Fig. S1a,b). However, at later stages, when biT$_{regs}$ expand and pulmonary inflammation becomes granulomatous, we found striking protection of mice lacking RORγt in T$_{regs}$. Macroscopic pulmonary haemorrhage was reduced significantly in FoxP3^{Cre} × RORC$^{fl/fl}$ mice (haemorrhage score $2{\cdot}9 \pm 0{\cdot}3$ *versus* $1{\cdot}67 \pm 0{\cdot}3$) (Fig. 2a). In line with this, histological signs of vasculitis were ameliorated (vasculitis score $1{\cdot}63 \pm 0{\cdot}3$ *versus* $0{\cdot}77 \pm 0{\cdot}2$) (Fig. 2a). Interestingly, granulomas were also reduced in numbers ($6 \pm 1{\cdot}0$ *versus* $2{\cdot}5 \pm 0{\cdot}6$) and size ($41{\cdot}23 \pm 4{\cdot}3$ *versus* $26{\cdot}88 \pm 5{\cdot}4 \times 10^3$ μm^2) in mice with T$_{reg}$-specific deficiency of RORγt (Fig. 2b). Furthermore, our analyses revealed a significant reduction of infiltrating T cells, both by immunohistochemistry ($17{\cdot}16 \pm 1{\cdot}6$ *versus* $11{\cdot}52 \pm 1{\cdot}4$ CD3$^+$ cells/hpf) as well as FACS analysis ($55{\cdot}66 \pm 1{\cdot}5$ *versus* $49{\cdot}48 \pm 2{\cdot}4\%$ CD4$^+$ of total CD3$^+$ cells) of lungs (Fig. 2c). Similarly, numbers of GR-1-positive neutrophils were also reduced in FoxP3^{Cre} × RORC$^{fl/fl}$ mice ($9{\cdot}72 \pm 0{\cdot}7$ *versus* $6{\cdot}8 \pm 0{\cdot}4$ GR-1$^+$ cells/hpf) (Fig. 2d).

Lupus nephritis is ameliorated in FoxP3^{Cre} × RORC$^{fl/fl}$ mice

At later stages, pristane-injected mice develop proliferative immune complex glomerulonephritis. We thus studied renal disease and found significant amelioration of lupus nephritis at 9 months in FoxP3^{Cre} × RORC$^{fl/fl}$ mice, as evidenced by protection from glomerular injury ($58{\cdot}9 \pm 6{\cdot}3$ *versus* $26{\cdot}18 \pm 4{\cdot}4\%$ abnormal glomeruli) (Fig. 3a). In line with this, glomerular cell proliferation, a hallmark of lupus nephritis, was also reduced ($1{\cdot}0 \pm 0{\cdot}07$ *versus* $0{\cdot}67 \pm 0{\cdot}07$ KI67$^+$ cells/gcs) (Fig. 3b). Importantly, protection from histological damage also resulted in less functional injury, as indicated by lower blood urea nitrogen (BUN) levels ($24{\cdot}4 \pm 1{\cdot}2$ *versus* $19{\cdot}6 \pm 1{\cdot}6$ mg/dl) (Fig. 3c). Finally, we found that loss of RORγt activation in T$_{regs}$ also protected from development of albuminuria ($239{\cdot}5 \pm 20{\cdot}4$ *versus* $151{\cdot}7 \pm 18{\cdot}7$ mg/g albumin per creatinine) (Fig. 3d).

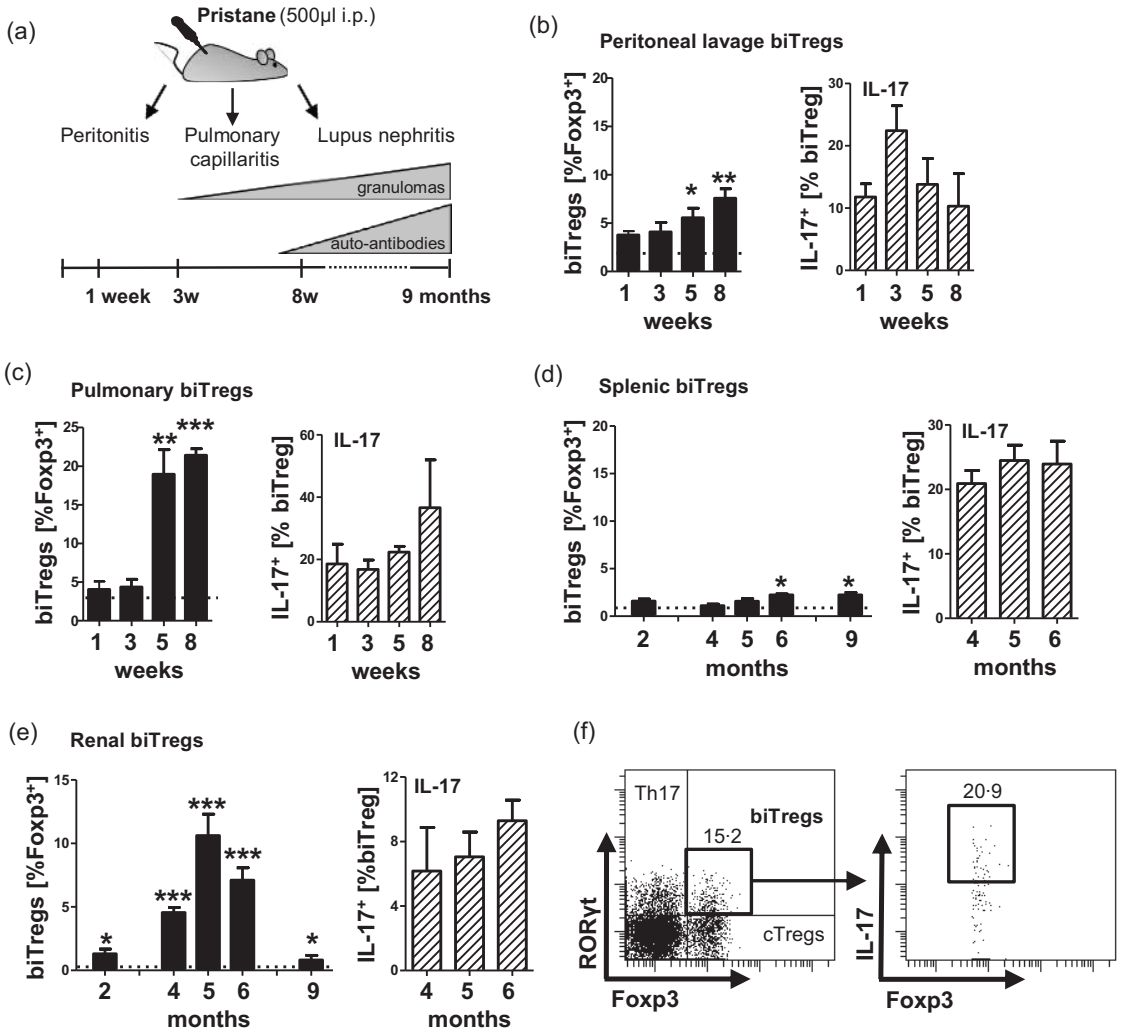

Fig. 1. (a) The temporal course of pristane-induced inflammation and organ-specific pathologies is shown. Intraperitoneal injection leads to development of acute innate mediated peritonitis and non-immune complex pulmonary capillaritis. Subsequently peritoneal and pulmonary granulomas develop and autoantibody formation is initiated. During the following months, immune complex lupus nephritis develops. (b) Retinoic acid receptor-related orphan nuclear receptor γt (RORγt)$^+$forkhead box protein 3 (FoxP3)$^+$ bi-functional regulatory T cell (biT$_{reg}$) percentages among FoxP3$^+$ peritoneal lavage cells at the indicated time-points after pristane injection (left) and interleukin (IL)-17 production by biT$_{regs}$ (right). (c) Pulmonary (d) splenic and (e) renal biT$_{reg}$ percentages among FoxP3$^+$ T$_{regs}$ and percentages of IL-17$^+$ biT$_{regs}$ at the indicated time-points after pristane injection. (f) A representative fluorescence activated cell sorter (FACS) plot from inflamed lungs at 5 weeks after pristane injection shows RORγt$^+$FoxP3$^+$ biT$_{regs}$, RORγt$^+$FoxP3$^-$ T helper type 17 (Th17) cells and FoxP3$^+$RORγt$^-$ conventional T$_{regs}$ (cT$_{regs}$). IL-17 production by biT$_{regs}$ is shown on the right. Numbers indicate percentages of FoxP3$^+$ cells. Dotted lines indicate basal biT$_{reg}$ percentages in healthy mice. Bars show mean ± standard error of the mean (s.e.m.). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ *versus* basal percentages.

Renal inflammatory cell infiltration is reduced in mice with RORγt-deficient T$_{regs}$

Because infiltrating renal leucocytes are key players of renal tissue injury, we next analysed renal inflammatory cell infiltration. In line with amelioration of renal damage, immunohistochemistry revealed a significant reduction of both glomerular ($2{\cdot}9 \pm 0{\cdot}3$ *versus* $1{\cdot}84 \pm 0{\cdot}2$ CD3$^+$ cells/gcs) and interstitial ($29{\cdot}58 \pm 1{\cdot}1$ *versus* $24{\cdot}08 \pm 1{\cdot}5$ CD3$^+$ cells/hpf) T cells in FoxP3^{Cre} × RORC$^{fl/fl}$ mice (Fig. 4a). Percentages of regulatory T cells, however, were not significantly different ($0{\cdot}022 \pm 0{\cdot}002$ *versus* $0{\cdot}017 \pm 0{\cdot}002$ FoxP3$^+$/CD3$^+$ cells per hpf) (Fig. 4b). Similarly, we found unchanged proportions of renal Th1 and Th17 cells (Supporting information, Fig. S2a,b). Interestingly, however, percentages of total IL-17$^+$ renal leucocytes were significantly lower in kidneys of FoxP3^{Cre} × RORC$^{fl/fl}$ mice ($2{\cdot}11 \pm 0{\cdot}16$ *versus* $1{\cdot}6 \pm 0{\cdot}07\%$ IL-17$^+$ of CD45$^+$ cells), indicating reduced influx of IL-17$^+$ populations different from Th17 cells (Fig. 4c). Furthermore, we also detected

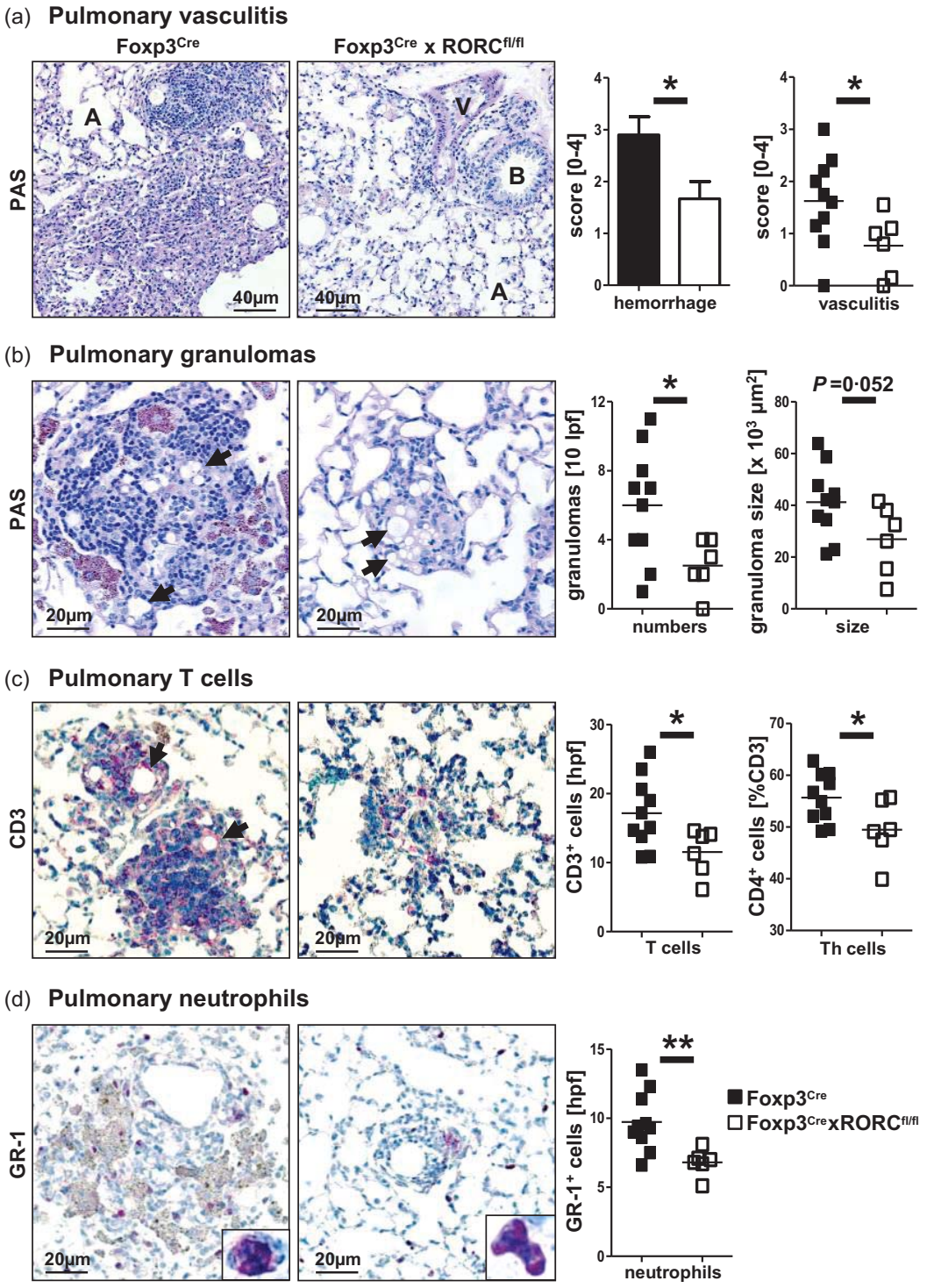

Fig. 2. (a) Representative photographs of periodic acid-Schiff (PAS)-stained lungs from indicated mouse strains at 8 weeks after pristane injection (left). Quantification of macroscopic haemorrhage as well as vasculitis score is shown (right) (original magnification ×200). (b) Representative photographs of pulmonary granulomas and quantification of granuloma numbers and size (original magnification ×400). (c) Immunohistochemical staining for pan T cell marker CD3 (left). Quantification of pulmonary $CD3^+$ T cell numbers by immunohistochemistry and $CD4^+$ T helper cell percentages by fluorescence activated cell sorter (FACS) as indicated (original magnification ×200). (d) Immunohistochemical staining and quantification of infiltrating granulocyte-differentiation antigen-1 $(GR\text{-}1)^+$ neutrophils (original magnification ×200). A = alveolus; B = bronchus; V = vessel; arrows indicate pristane droplets. Squares represent individual animals, horizontal lines indicate means. Bars show mean ± standard error of the mean (s.e.m.). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

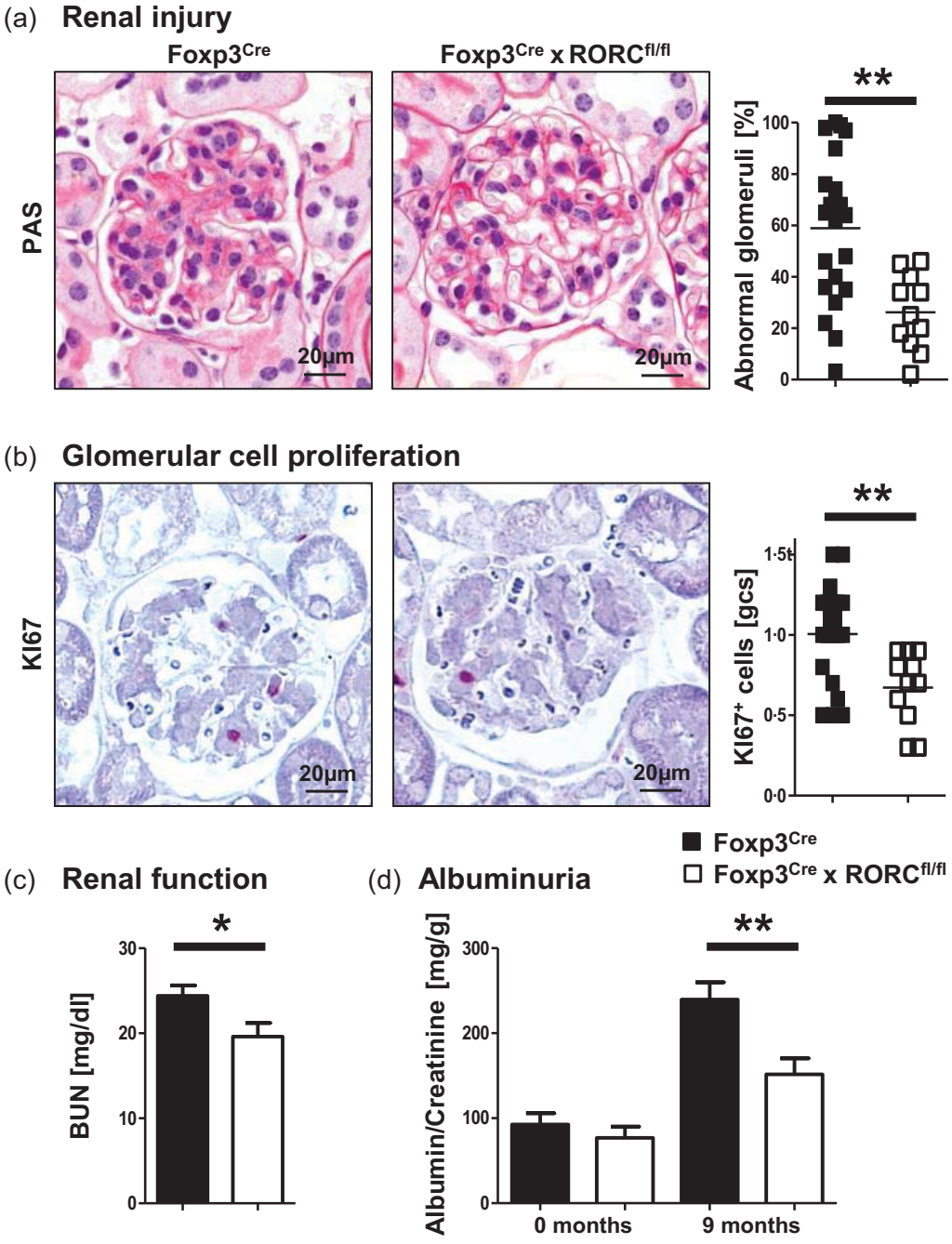

Fig. 3. (a) Representative photographs of periodic acid-Schiff (PAS)-stained kidney sections at 9 months after pristane injection, as well as quantification of glomerular injury (original magnification ×400). (b) Representative photographs of immunohistochemical staining of KI67 and quantification of glomerular KI67$^+$ cells (original magnification ×400). (c) Quantification of serum blood urea nitrogen (BUN) levels. (d) Quantification of urinary albumin/creatinine ratios at the indicated time-points before and after injection of pristane. Bars show mean ± standard error of the mean (s.e.m.). Squares represent individual animals, horizontal lines indicate means. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

lower numbers of glomerular ($3{\cdot}74 \pm 0{\cdot}55$ *versus* $2{\cdot}2 \pm 0{\cdot}27$ MAC-2$^+$ cells/gcs) (Fig. 4d) and interstitial ($21{\cdot}53 \pm 2{\cdot}1$ *versus* $13{\cdot}86 \pm 1{\cdot}2$ F4/80$^+$ cells/hpf) (Fig. 4e) macrophages in mice with T$_{reg}$-specific deletion of RORγt.

Humoral autoimmunity remains unaltered in FoxP3^{Cre} × RORC$^{fl/fl}$ mice

Next, we wanted to explore the mechanisms leading to tissue protection from pristane-induced SLE in FoxP3^{Cre} × RORC$^{fl/fl}$ mice. We therefore assessed the effects of RORγt deactivation

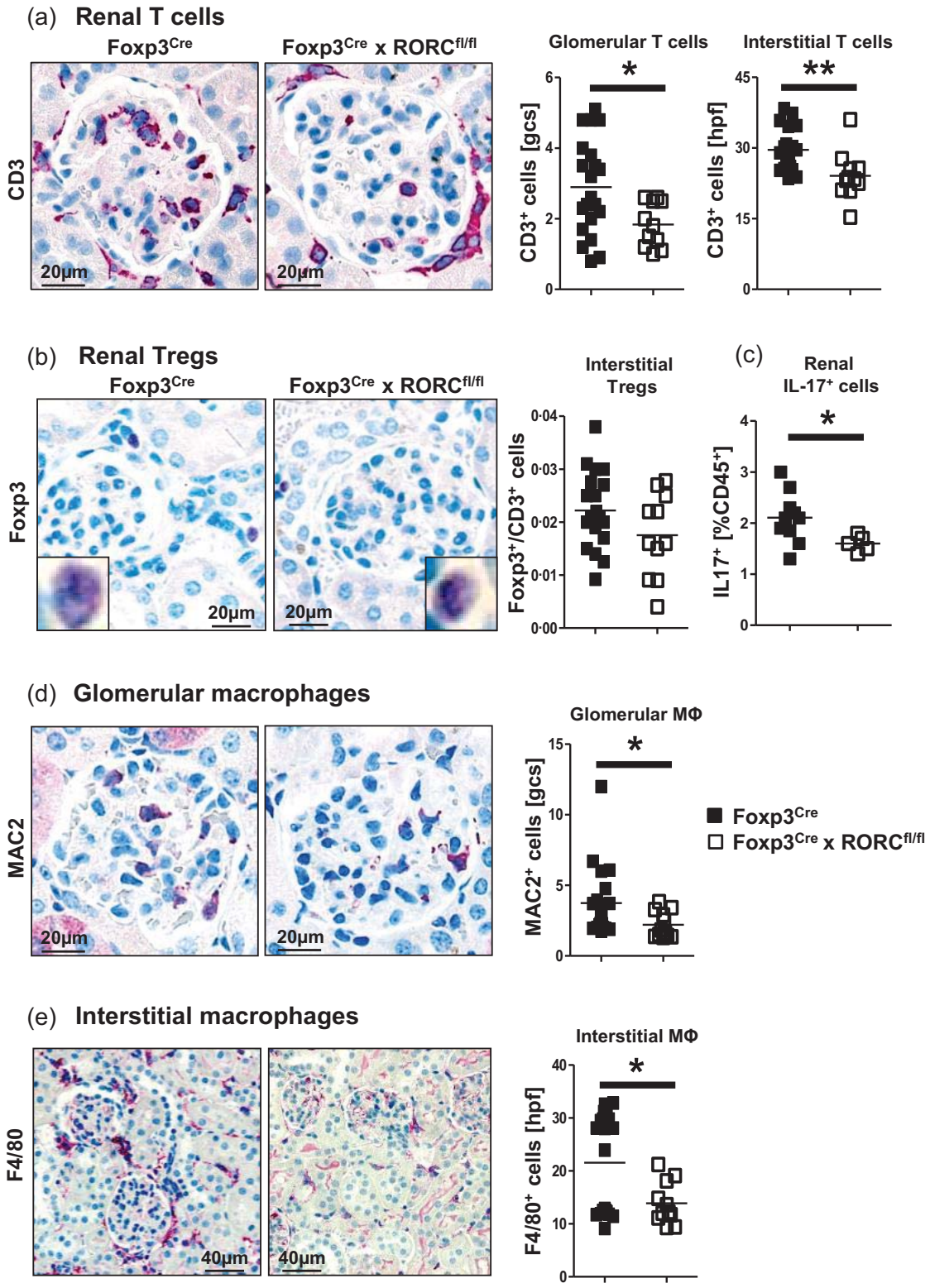

Fig. 4. Representative photographs and quantification of glomerular and interstitial renal (a) $CD3^+$ T cell and (b) forkhead box protein 3 $(FoxP3)^+$ regulatory T cell (T_{reg}) infiltration at 9 months after pristane injection (original magnification ×400). (c) Fluorescence activated cell sorter (FACS) analysis of total renal infiltrating interleukin (IL)-17^+ leucocytes (one of two sets shown). (d) Representative photographs and quantification of glomerular $MAC2^+$ monocyte/macrophage infiltration (original magnification ×400). (e) Representative photographs and quantification of renal interstitial $F4/80^+$ monocyte/macrophage infiltration (original magnification ×200). Squares represent individual animals, horizontal lines indicate means. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

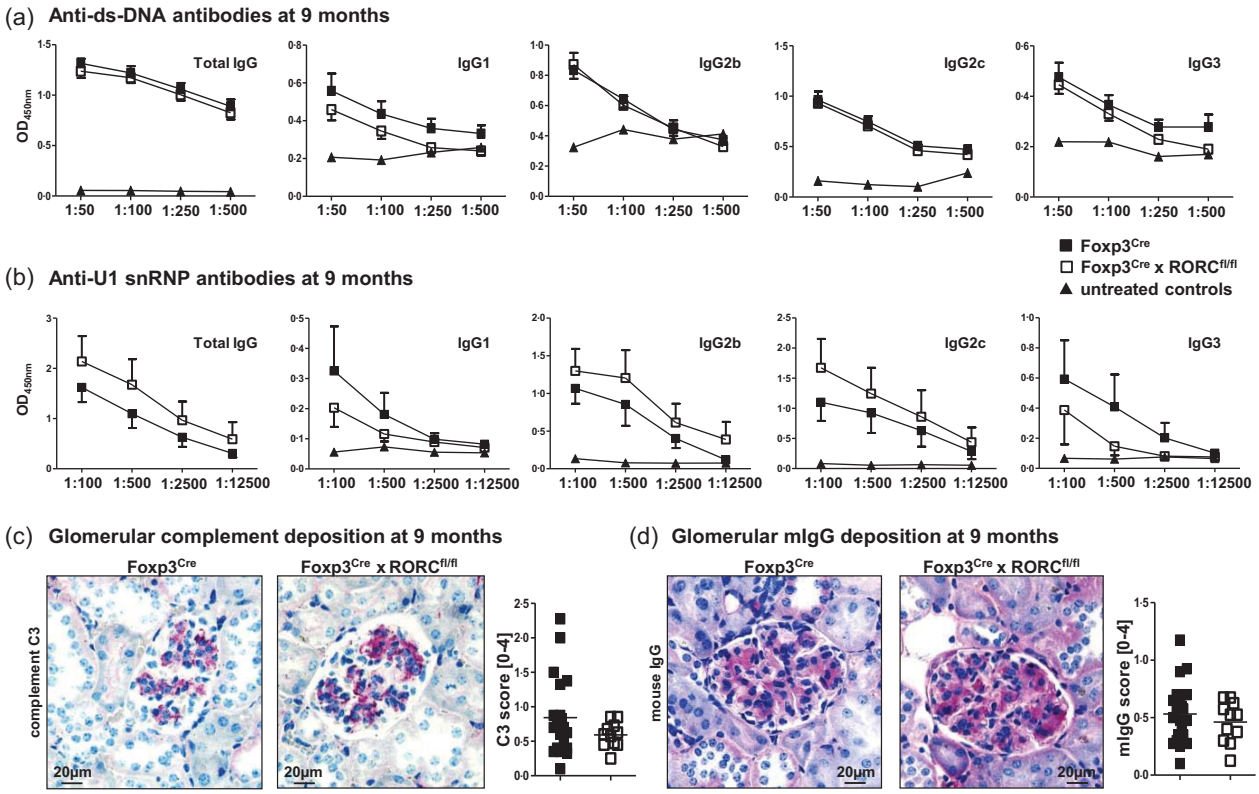

Fig. 5. (a) Quantification of immunoglobulin (Ig)G anti-ds-DNA antibodies, as well as the indicated IgG subclasses from serum of forkhead box protein 3 (FoxP3)^{Cre} wild-type and FoxP3^{Cre} × retinoic acid receptor-related orphan nuclear receptor (ROR)C$^{fl/fl}$ mice in serial dilutions by enzyme-linked immunosorbent assay (ELISA). (b) Quantification of serum anti-U1-ribonucleoprotein (RNP) autoantibodies of the indicated IgG subclasses in serial dilutions by ELISA. (c) Immunohistochemical staining and quantification of glomerular complement C3 deposition (original magnification ×400). (d) Immunohistochemical staining and quantification of glomerular mouse IgG (mIgG) deposition (original magnification ×400). Squares represent individual animals, horizontal lines indicate means.

in biT$_{regs}$ on humoral immune responses. In this respect, we found unaltered levels of serum total IgG as well as identical amounts of all analysed subclasses at 9 months after pristane injection (Supporting information, Fig. S3a). Similarly, formation of IgG autoantibodies of all subclasses against dsDNA (Fig. 5a) as well as U1-RNP was unchanged in FoxP3^{Cre} × RORC$^{fl/fl}$ mice (Fig. 5b). In line with unchanged serum antibody levels, we documented a similar immune complex deposition in kidneys of pristane-injected mice, as evidenced by complement C3 (0·84 ± 0·1 *versus* 0·59 ± 0·05 C3 deposition score) (Fig 5c) and mouse IgG (mIgG) staining (0·53 ± 0·06 *versus* 0·46 ± 0·05 mIgG deposition score) (Fig. 5d). Similarly, analyses of splenic B cell populations showed similar percentages of total B cells (16·7 ± 1·0 *versus* 16·38 ± 2·2% CD19$^+$CD138$^-$ of CD45$^+$ cells), switched memory B cells (9·24 ± 3·6 *versus* 14·55 ± 1·3% IgM$^-$IgD$^-$ of CD19$^+$CD138$^-$ cells) as well as plasma cells (1·34 ± 0·39 *versus* 0·52 ± 0·07% CD138^{high} of CD45$^+$ cells) in both strains of mice (Supporting information, Fig. S3b–d). Finally, we found that percentages of splenic T follicular helper cells (TfH) were also unaffected by abrogation of RORγt activation in T$_{regs}$ (1·3 ± 0·29 *versus* 2·48 ± 0·85% CXCR5$^+$PD-1$^+$ of CD4$^+$FoxP3$^-$ cells) (Supporting information, Fig. S3e).

IL-17 secretion by biT$_{regs}$ is dependent upon RORγt

In a next step, we aimed to explore alterations of cellular immune responses. As IL-17 is a known downstream target of RORγt, we analysed IL-17 expression in FoxP3^{Cre} × RORC$^{fl/fl}$ mice. Analysis of peritoneal lavage cells at 3 weeks after pristane injection confirmed specific and complete absence of RORγt in FoxP3$^+$ T$_{regs}$ (4·58 ± 1·5 *versus* 0·28 ± 0·3% biT$_{regs}$ of FoxP3$^+$ cells) (Fig. 6a, left). In line with our hypothesis, IL-17 production by T$_{regs}$ was abrogated totally in FoxP3^{Cre} × RORC$^{fl/fl}$ mice, indicating absolute dependency on RORγt (2·33 ± 0·48 *versus* 0·15 ± 0·06% IL-17$^+$ of FoxP3$^+$ cells) (Fig. 6a, right). Similarly, analysis of vasculitic lungs showed absence of RORγt$^+$ biT$_{regs}$ (4·06 ± 1·0 *versus* 0·55 ± 0·33% biT$_{regs}$ of FoxP3$^+$ cells) as well as abolished IL-17 production by T$_{regs}$ (2·9 ± 0·74 *versus* 0·35 ± 0·19% IL-17$^+$ of FoxP3$^+$ cells) (Fig. 6b). Finally, we analysed nephritic kidneys at 5 months after pristane injection and again found absence of biT$_{regs}$ in FoxP3^{Cre} × RORC$^{fl/fl}$ mice (10·62 ± 1·7 *versus*

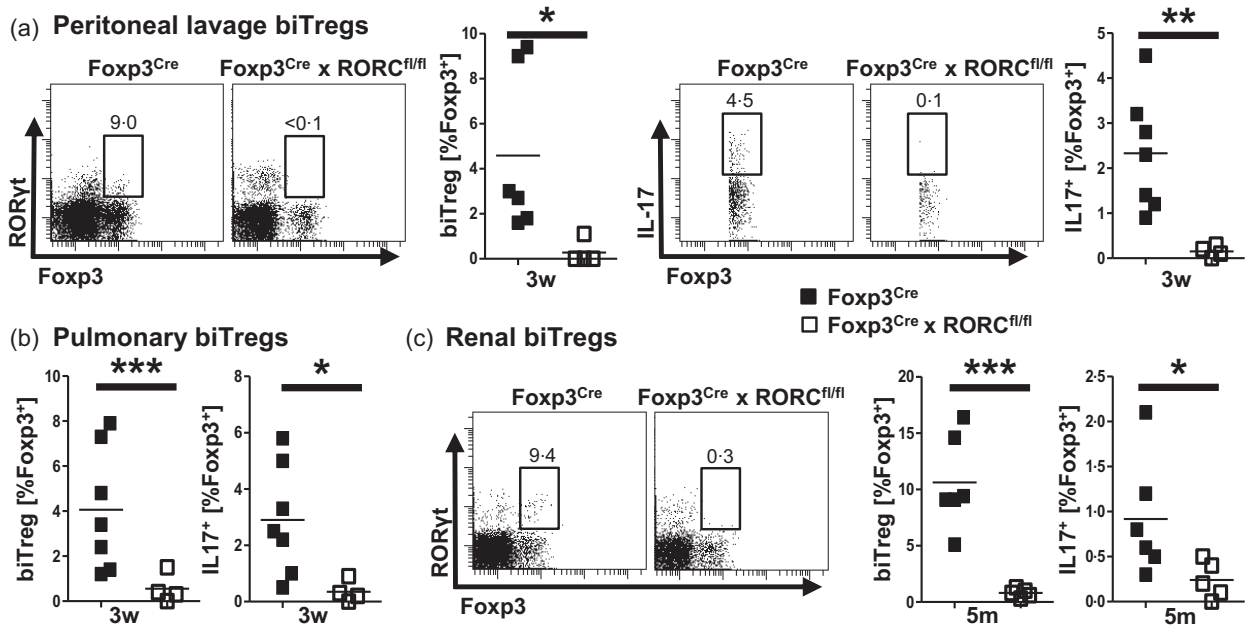

Fig. 6. (a) A representative fluorescence activated cell sorter (FACS) plot and quantification of peritoneal lavage bi-functional regulatory T cells (biT_{regs}) (left) as well as interleukin (IL)-17 production by biT_{regs} (right) at 3 weeks after pristane injection in forkhead box protein 3 $(FoxP3)^{Cre}$ wild-type and $FoxP3^{Cre}$ × retinoic acid receptor-related orphan nuclear receptor $(ROR)C^{fl/fl}$ mice (right). (b) Quantification of pulmonary biT_{regs} (left) and IL-17 production (right) at 3 weeks after pristane injection in the indicated mouse strains. (c) A representative FACS plot and quantification of renal biT_{regs} (left) and their IL-17 production (right) at 5 months after pristane injection. Numbers in FACS plots indicate percentages of $FoxP3^+$ cells. Squares represent individual animals, horizontal lines indicate means. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

$0{\cdot}8 \pm 0{\cdot}17\%$ biT_{regs} of $FoxP3^+$ cells) as well as abrogation of IL-17 secretion by renal $FoxP3^+$ T_{reg} cells ($0{\cdot}92 \pm 0{\cdot}27$ *versus* $0{\cdot}24 \pm 0{\cdot}09\%$ IL-17^+ of $FoxP3^+$ cells) (Fig. 6c).

RORγt deficiency alters the phenotype of T_{regs}

In order to evaluate whether RORγt also affects intrinsic T_{reg} functions, we studied FoxP3 protein expression on the single-cell level. Interestingly, we found significantly higher FoxP3 mean fluorescence intensities in T_{regs} from the blood of naive $FoxP3^{Cre}$ × $RORC^{fl/fl}$ mice [1376 ± 24 *versus* 1567 ± 36 FoxP3 mean fluorescence intensity (MFI)]. The same was true for T_{regs} from both peritoneal lavage cells ($702{\cdot}6 \pm 79{\cdot}8$ *versus* $1212 \pm 200{\cdot}3$ FoxP3 MFI) and lungs ($500{\cdot}7 \pm 55{\cdot}7$ *versus* $778{\cdot}5 \pm 120$ FoxP3 MFI) 3 weeks after pristane injection (Fig. 7a). Detailed analysis of T_{reg} activation marker molecules revealed enhanced surface expression of CD103 ($7{\cdot}9 \pm 1{\cdot}0$ *versus* $15{\cdot}33 \pm 1{\cdot}7\%$ of $FoxP3^+$ cells), GITR ($7{\cdot}67 \pm 0{\cdot}68$ *versus* $22{\cdot}9 \pm 1{\cdot}1\%$ of $FoxP3^+$ cells) and ICOS ($9{\cdot}06 \pm 0{\cdot}91$ *versus* $21{\cdot}95 \pm 2{\cdot}14\%$ of $FoxP3^+$ cells) on RORγt-deficient T_{regs}. Levels of CTLA-4 and PD-L1 remained unaffected (Fig. 7b). In congruence with elevated expression of activation markers, we found higher T_{reg} activation ($37{\cdot}07 \pm 2{\cdot}6$ *versus* $45{\cdot}53 \pm 0{\cdot}86\%$ $CD69^+CD62L^-$ and $21{\cdot}68 \pm 1{\cdot}2$ *versus* $36{\cdot}57 \pm 2{\cdot}5\%$ $CD44^+CD62L^-$ of $FoxP3^+$ cells) (Fig. 7c) as well as proliferative activity ($77{\cdot}09 \pm 2{\cdot}1$ *versus* $83{\cdot}43 \pm 1{\cdot}5\%$ $KI67^+$ of $FoxP3^+$ cells) (Fig. 7d). *In-vitro* suppression of cytokine production by T_{regs}, including IL-2, Th1 characteristic IFN-γ, Th2 prototype IL-4 and IL-13, as well as Th17-associated TNF-α, were unchanged in co-culture experiments with T_{eff} cells (Supporting information, Fig. S4a–d). Induction of IL-10 production was also similar between the groups (Supporting information, Fig. S4e). Finally, suppression of T_{eff} cell proliferation was unaffected in $FoxP3^{Cre}$ × $RORC^{fl/fl}$ mice (Supporting information, Fig. S4f).

biT_{regs} control Th2 immunity in a RORγt-dependent manner

Next, we wanted to assess whether the observed absence of IL-17 secretion and enhanced T_{reg} activation in $FoxP3^{Cre}$ × $RORC^{fl/fl}$ mice might affect systemic immunity. We therefore analysed immune responses in the blood of naive mice. Remarkably, we found a spontaneous hyper-Th2 phenotype with an increase in Gata3 ($0{\cdot}47 \pm 0{\cdot}04$ *versus* $0{\cdot}69 \pm 0{\cdot}05\%$ $Gata3^+$ among $CD4^+FoxP3^-$ cells), IL-5 ($0{\cdot}27 \pm 0{\cdot}09$ *versus* $0{\cdot}64 \pm 0{\cdot}08\%$ IL-5^+ among $CD4^+FoxP3^-$ cells) and IL-13 ($1{\cdot}9 \pm 0{\cdot}41$ *versus* $4{\cdot}3 \pm 0{\cdot}29\%$ IL-13^+ among $CD4^+FoxP3^-$ cells)-positive T helper cells in $FoxP3^{Cre}$ × $RORC^{fl/fl}$ animals (Fig. 8a). In line with this, we also found enhanced Th2 responses in peritoneal lavage cells (Fig. 8b, Supporting information, Fig. S5a) and lungs (Fig. 8c) during pristane-induced inflammation. Analyses of the renal cellular infiltrate supported these

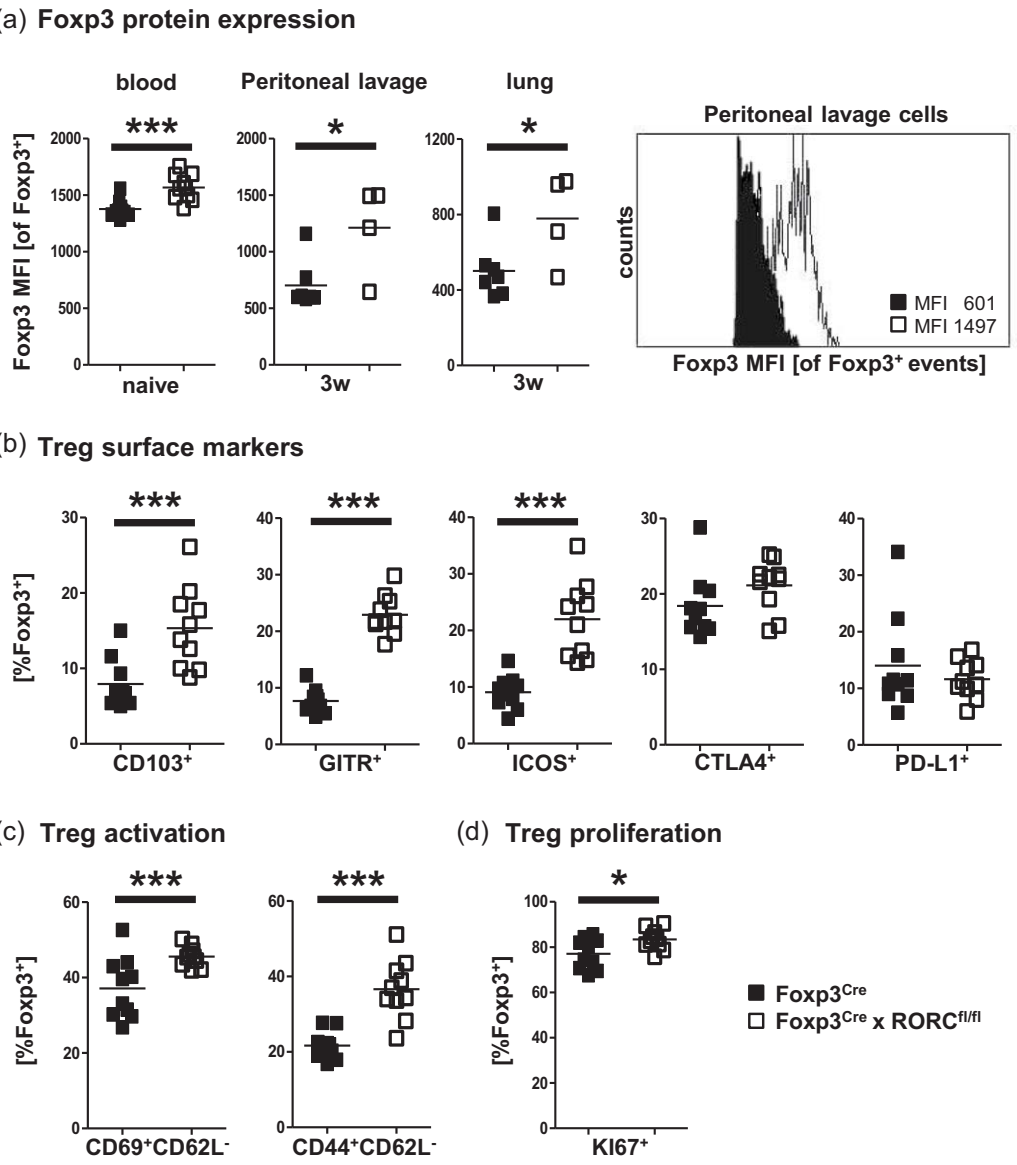

Fig. 7. (a) Quantification of forkhead box protein 3 (FoxP3) mean fluorescence intensity (MFI) in regulatory T cells (T_{regs}) from blood of naive mice as well as peritoneal lavage and pulmonary T_{regs} at 3 weeks after pristane injection from indicated mouse strains (left) and a representative fluorescence activated cell sorter (FACS) plot of FoxP3 MFI in peritoneal lavage T_{regs} (right). (b) Analysis of the indicated surface markers on T_{regs} from blood of naive mice. (c) Quantification of activation markers on T_{regs} from blood of naive mice of the indicated genotypes. (d) Assessment of T_{reg} proliferative activity by FACS analysis of KI67 expression in naive blood T_{regs}. Squares represent individual animals, horizontal lines indicate means. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

findings and also showed broad skewing towards Th2 immunity ($0{\cdot}41 \pm 0{\cdot}08$ *versus* $0{\cdot}73 \pm 0{\cdot}1\%$ IL-4^+, $0{\cdot}95 \pm 0{\cdot}14$ *versus* $2{\cdot}0 \pm 0{\cdot}5\%$ IL-5^+ and $4{\cdot}14 \pm 0{\cdot}2$ *versus* $6{\cdot}62 \pm 0{\cdot}7\%$ IL-13^+ among $CD4^+FoxP3^-$ cells) (Fig. 8d). Conversely, Th1 and Th17 responses remained unaffected by knock-out of RORγt in T_{regs} in all examined organs (Supporting information, Fig. S5b–f). Similarly, serum levels of IFN-γ and IL-17 were identical (Supporting information, Fig. S5g), as was splenic and renal IFN-γ mRNA expression (Supporting information, Fig. S5h). Finally, we analysed humoral Th2 immunity and found strikingly elevated levels of IgE antibodies in serum of naive $FoxP3^{Cre} \times RORC^{fl/fl}$ mice ($358 \pm 26{\cdot}8$ *versus* $801{\cdot}2 \pm 110$ ng/ml) as well as after disease induction ($670{\cdot}9 \pm 116$ *versus* 1633 ± 194 ng/ml) (Fig. 8e).

Discussion

Our study aimed to evaluate the role of the newly identified $ROR\gamma t^+FoxP3^+$ biT$_{regs}$ [20,22,26,33] in systemic lupus erythematosus. As nothing is known about the occurrence and biology of these multi-potent cells in SLE, we started with a

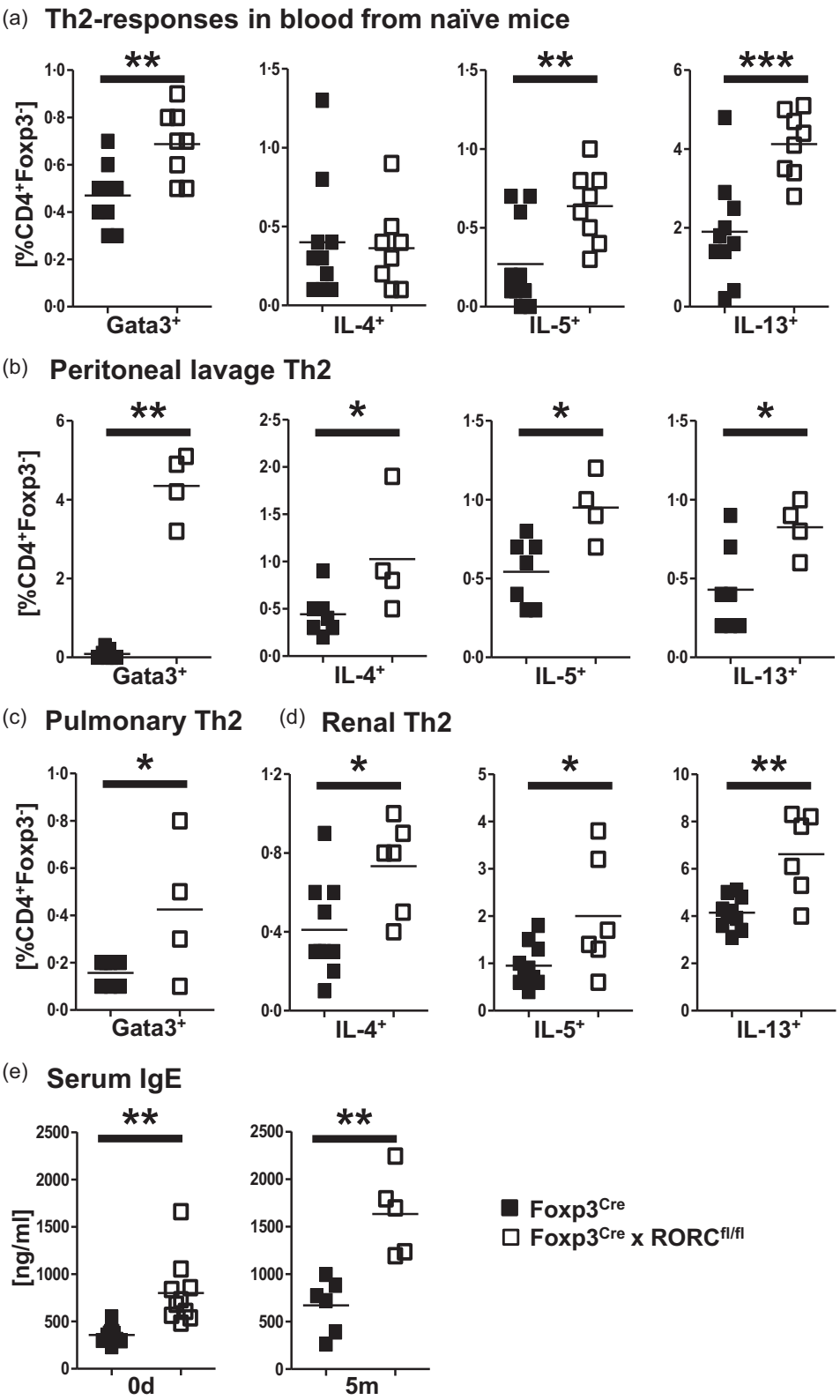

Fig. 8. (a) Quantification of T helper (Th) cells expressing the Th2 characteristic transcription factor Gata3 or the indicated Th2 cytokines in peripheral blood from naive mice by flow cytometry. (b) Flow cytometric analysis of peritoneal Th cells expressing Gata3 or the indicated Th2 prototype cytokines from peritoneal lavage cells 3 weeks after pristane injection. (c) Gata3 expression by T helper cells in lungs at 3 weeks after pristane injection. (d) Quantification of renal T helper cells expressing the indicated Th2 cytokines at 2 months after pristane injection. (e) Quantification of immunoglobulin (Ig)E levels by enzyme-linked immunosorbent assay (ELISA) from serum of naive mice and at 5 months after pristane injection. Squares represent individual animals, horizontal lines indicate means. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

thorough analysis of the temporal and organ-specific pattern of biT_{reg} expansion. In the first 2 weeks after pristane injection mice develop sterile peritonitis, which is mediated by innate immunity [34]. During this acute, non-autoantigen-dependent inflammation, biT_{reg} levels remained at baseline. Another early and antigen-independent manifestation of

pristane is acute pulmonary vasculitis with diffuse alveolar haemorrhage [35]. Similarly to peritonitis, we did not find relevant expansion of biT$_{regs}$ during this innate phase of pulmonary inflammation. However, the persistence of inflammatory responses after pristane injection leads to chronically progressive development of autoimmunity and formation of peritoneal and pulmonary lymphogranulomas, which became apparent from approximately 3 weeks post-injection. Interestingly, during this stage of disease, biT$_{reg}$ percentages increased significantly in both organs, indicating a role for establishment of granulomas and autoimmunity. In addition, these findings suggest that biT$_{reg}$ expansion occurs in an antigen-dependent manner, which is in full agreement with a recent report [26]. With increasing time after pristane injection, various types of autoantibodies develop [36,37]. These are deposited in the kidneys and result in progressive immune complex-dependent lupus nephritis. During this process, renal biT$_{regs}$ showed a very distinct and concerted time–course. Their population started to expand early, in parallel with increasing renal inflammation and reached a maximum at approximately 5 months. Subsequently, biT$_{reg}$ percentages decreased slowly and were almost back to baseline levels at 9 months after pristane injection. Interestingly, this distinguished time–course of renal biT$_{regs}$ in chronically developing lupus nephritis parallels their dynamics in the nephrotoxic nephritis model of acute glomerulonephritis [22]. In contrast to the massive peritoneal, pulmonary and renal biT$_{reg}$ expansion, they increased only slightly in spleens. This concurs with the fact that pristane injection results only in a mild splenic inflammatory response. In summary, biT$_{regs}$ thus seem to be early mediators of adaptive inflammation and tissue injury during pristane-induced SLE. Importantly, and in accordance with previous findings by us and others [21,22,38,39], a robust fraction of biT$_{regs}$ produces IL-17 in all organs and at all investigated time-points. Given the observed expansion of biT$_{regs}$ in all affected organs, we hypothesized that biT$_{regs}$ might play key functional roles during SLE development. As our earlier data suggest that activation of RORγt mediates the pathogenic functions of biT$_{regs}$ [22], we generated mice with selective deficiency of RORγt in FoxP3^{+} T$_{regs}$. In a first step, we wanted to explore whether abrogation of RORγt activation would result in protection from pulmonary vasculitis, which is a rare but life-threatening complication of SLE in humans [40]. Analysis of lungs at an early time-point, before development of autoimmunity and expansion of biT$_{regs}$, showed similar degrees of injury. However, at later stages, FoxP3^{Cre} × RORC$^{fl/fl}$ mice were protected significantly from pulmonary vasculitis in terms of haemorrhage, leucocyte infiltration and histological damage. Furthermore, pulmonary granulomas were reduced both in size and numbers. Next, we aimed to study the development of lupus nephritis, which is one of the most severe organ manifestations of SLE in humans and associates with a bad prognosis. In this respect, we found that FoxP3^{Cre} × RORC$^{fl/fl}$ mice were protected significantly, as evidenced by less histological injury and glomerular cell proliferation. In line with this, functional parameters of renal injury as serum BUN levels and albuminuria were also reduced significantly. Furthermore, we found much decreased renal proinflammatory leucocyte infiltration in mice lacking RORγt activation in T$_{regs}$. Interestingly, frequencies of IL-17-producing total renal leucocytes were also reduced, despite unaltered renal Th17 responses. This indicates effects of T$_{reg}$-specific RORγt deletion on IL-17^{+} leucocyte populations different from Th17 cells as, for example, γδTcells and T$_{regs}$ themselves. In order to explore the mechanisms by which abrogation of RORγt activation in biT$_{regs}$ protects from SLE organ manifestations, we next assessed development of humoral autoimmunity. However, our analyses revealed unchanged levels of total serum IgG as well as all measured subclasses in FoxP3^{Cre} × RORC$^{fl/fl}$ mice. Similarly, serum levels of SLE characteristic anti-ds-DNA, and anti-U1-RNP autoantibodies of all subclasses remained unaltered. Consistent with unaffected serum antibodies, we found the same extent of renal complement C3 and IgG deposition in both groups of mice. In congruence, analyses of splenic B cell subpopulations, plasma cells and T follicular helper cells showed no difference.

Given the unchanged humoral autoimmunity, we next explored T cell immune responses. Strikingly, and in line with the importance of RORγt for Th17 cells [41], proinflammatory IL-17 expression by biT$_{regs}$ was abrogated completely in FoxP3^{Cre} × RORC$^{fl/fl}$ mice. This observation might, at least in part, account for the observed amelioration of SLE-induced tissue injury. In order to identify further potential mechanisms of protection, we explored whether RORγt would also affect T$_{reg}$ suppression and activity. Similar to our previous data, analysing T$_{regs}$ from RORC pan-knock-out mice [22], we found unchanged *in-vitro* suppressive capacity. *In vivo*, however, T$_{reg}$-selective deletion of RORγt resulted in enhanced levels of FoxP3 protein as well as increased expression of T$_{reg}$ activation markers. These findings indicate that RORγt expression in T$_{regs}$ impairs their *in-vivo* regulatory function. Therefore, the high activation status of RORγt^{+} biT$_{regs}$, which we and others have described previously [22,33,42,43], does not seem to be induced by RORγt. Rather, additional transcription factors, which are co-activated in RORγt^{+} biT$_{regs}$ as, for example, interferon regulatory factor (IRF)4 [26], runt-related transcription (Runx)1, Runx3 [42] or unknown others, might be responsible. Next, we wanted to explore whether these observed T$_{reg}$ alterations would result in any effects on systemic immunity. Interestingly, we found significantly elevated type 2 immune responses in naive and pristane-challenged FoxP3^{Cre} × RORC$^{fl/fl}$ mice, both systemically and in all inflamed target organs. Th1 and Th17 responses, in contrast, remained unaltered. Importantly, these observations are in full agreement with a very recent report, which was published during preparation of this paper [26]. This emergence of biT$_{regs}$ as potent regulators

of type 2 immunity is particularly remarkable, as the mechanisms regulating Th2 responses remain widely unknown to date. In this regard, our data do not indicate a direct effect of biT_{regs} on suppression of Th2 cells, as generation of Th2 immunity was not altered in our *in-vitro* co-culture assays of T_{eff} with RORγt-deficient T_{regs}. Rather, as suggested by Ohnmacht *et al.*, indirect mechanisms, involving interactions between T_{regs} and dendritic cells, seem to be important [26].

Taken together, we found that biT_{reg} selective deletion of RORγt resulted in abrogation of their IL-17 secretion, enhancement of Th2 immunity and increased T_{reg} activation. Importantly, these changes were associated with protection from pulmonary vasculitis and lupus nephritis. It remains unclear, however, to what extent each of these three RORγt-dependent immune alterations contribute to disease amelioration. While it is likely that protection results from a mixed phenotype, involving all three mechanisms, future studies will need to address this aspect in more detail.

In summary, our study provides the first evidence, to our knowledge, for a crucial role of the newly defined biT_{regs} in SLE. Our findings thus further support research into biT_{reg} biology and favour RORγt-directed interventions as novel therapeutic options for SLE.

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Disclosure

None.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. (a) Quantification of macroscopic haemorrhage as well as vasculitis score is shown as indicated. (b) Quantification of granuloma numbers and size. Squares represent individual animals, horizontal lines indicate means. All comparisons between the groups $P > 0{\cdot}05$ not significant (n.s.).

Fig. S2. (a) Representative fluorescence activated cell sorter (FACS) plots and (b) quantification of $CD45^+CD3^+CD4^+$ forkhead box protein 3 $(FoxP3)^-$ T effector cells (T_{eff}) secreting the indicated cytokines from kidneys of mice at 9 months after pristane injection. Numbers in fluorescence activated cell sorter (FACS) plots indicate percentages. Squares represent individual animals, horizontal lines indicate means. All comparisons between the groups $P > 0{\cdot}05$ not significant (n.s.).

Fig. S3. (a) Levels of total immunoglobulin (Ig)G and the indicated IgG subclasses were determined by enzyme-linked immunosorbent assay (ELISA) from serum of mice at 9 months after pristane injection at serial dilutions. (b) Percentages of splenic total B cells, (c) switched-memory B cells (sMB) and (d) plasma cells were not different between the groups at 9 months after pristane injection. (e) Frequencies of splenic T follicular helper cells (TfH) were similar at 9 months after pristane injection. All comparisons between the groups $P > 0{\cdot}05$ not significant (n.s.).

Fig. S4. (a–f) *In-vitro* suppression assays were performed by co-culturing wild-type $CD4^+$ T effector cells (T_{eff}) with regulatory T cells (T_{regs}) from forkhead box protein 3 $(FoxP3)^{Cre}$ × retinoic acid receptor-related orphan nuclear receptor (ROR)Cfl/fl mice or $FoxP3^{Cre}$ controls at the indicated ratios ($n = 4$ per group). (a) Cytokine levels of interleukin (IL)−2 were analysed in co-culture supernatants by enzyme-linked immunosorbent assay (ELISA). Cytokine levels of (b) Th1 cytokine interferon (IFN)-γ, (c) T helper type 2 (Th2) cytokines IL-4 and IL-13, (d) Th17-associated cytokine tumour necrosis factor (TNF)-α and (e) IL-10 were analysed in co-culture supernatants by cytometric bead array. (f) Suppression of T_{eff} proliferation was analysed by quantification of KI67 expression. Dotted lines represent T_{eff} alone without T_{regs} ($n = 4$). All comparisons between the groups $P > 0{\cdot}05$ not significant (n.s.).

Fig. S5. (a) Representative fluorescence activated cell sorter (FACS) plots of expression of Gata3 and the indicated T helper type 2 (Th2) cytokines in T effector cells (T_{eff}) of peritoneal lavage cells at 3 weeks after pristane injection. (b–e) Fluorescence activated cell sorter (FACS) analyses of indicated Th1 and Th17 cytokine and transcription factor expression in T_{eff} from (b) blood of naive mice, (c) peritoneal lavage cells at 3 weeks, (d)

pulmonary leucocytes at 3 weeks and (e) renal infiltrating leucocytes at 8 weeks after pristane injection. (f) Enzyme-linked immunosorbent assay (ELISA) analysis of spleen cell cytokine production at 9 months. (g) ELISA analyses of serum cytokine levels at the indicated time-points after pristane injection. (h) Interferon (IFN)-γ mRNA expression levels in spleens and kidneys at 9 months after pristane injection. Numbers in FACS plots indicate percentages. Squares represent individual animals, horizontal lines indicate means. Bars represent mean ± standard error of the mean (s.e.m.). All comparisons between the groups $P > 0{\cdot}05$ not significant (n.s.).