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Régulation de la fonction des lymphocytes T par les facteurs de transcription NF- κ B: implications dans le cancer et l'auto-immunité.

Yenkel Grinberg-Bleyer

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Université Claude Bernard



MEMOIRE

Pour l'obtention du diplôme

D'HABILITATION A DIRIGER DES RECHERCHES

Présenté par

Yenkel GRINBERG-BLEYER

Régulation de la fonction des lymphocytes T par les facteurs de transcription NF- κ B: implications dans le cancer et l'auto-immunité.

Soutenance prévue le 4 Novembre 2021 devant un jury composé de :

Pr. Charles DUMONTET Président

Dr. Nicolas BIDERÉ Rapporteur

Dr. Uzma HASAN Rapportrice

Dr. Vanja SISIRAK Rapporteur

Dr. Jacqueline MARVEL Examinatrice

Dr. Eliane PIAGGIO Examinatrice

Dr. Benoit SALOMON Examineur

Remerciements

Je tiens tout d'abord à remercier Uzma Hasan, Nicolas Bidère et Vanja Sisirak pour avoir accepté de faire partie de mon jury en tant que rapporteurs : merci pour le temps que vous me consacrez, je suis certain que vos commentaires et opinions feront grandement progresser mes recherches.

Merci également à Jacqueline Marvel et Charles Dumontet, dont l'expertise en immunologie ne pourra être que bénéfique à la progression de mes projets.

Merci infiniment à mes directeurs de thèse, Eliane Piaggio et Benoit Salomon. Vous m'avez tout appris, des bases de l'immunologie aux techniques de pointe ; vous avez patiemment supporté mon inexpérience et mon caractère pendant des années. Je vous dois toute ma (courte) carrière de chercheur et c'est un honneur de vous compter parmi les membres de mon jury.

Merci à Alain Puisieux et Patrick Mehlen pour avoir misé sur moi et permis de créer mon équipe indépendante, alors même que mes papiers de post-doc n'étaient pas encore publiés.

Merci à Christophe Caux et à son équipe pour m'avoir accueilli au CRCL, m'avoir ouvert les portes de leur labo et partagé leurs connaissances et leur matériel. J'espère que je ne vous ai pas trop embêté avec mes questions et remarques incessantes !

Merci à Julien Marie et son équipe, nos colocataires, pour leur compréhension et leur volonté d'évoluer en bonne intelligence avec une équipe de petits jeunes.

Merci à tous nos collaborateurs, en particulier Ulf Klein, ainsi que Julie Caramel, Stéphane Dalle et leur équipe, toujours à l'écoute et disponibles pour parler de science.

Merci aux membres de mon équipe, qui mettent en œuvre les projets et sont donc les vrais responsables du labo ; merci notamment à Philippe Cassier, dont le franc-parler, l'honnêteté et les immenses connaissances en médecine et en science sont un apport inestimable à l'équipe.

Enfin, merci à ma mère et à Gaëlle, qui supportent mon caractère et mes projets depuis toujours. Et bien sûr à Noam, qui me rappelle tous les jours qu'il y a des choses bien plus importantes que les manip et le labo, et dont les rires me permettent toujours de bien commencer et terminer mes journées.

Résumé

Les lymphocytes T (LT), cellules du système immunitaire adaptatif, jouent des rôles multiples dans le maintien de l'homéostasie et en situation pathologique : réponse aux agents pathogènes, auto-immunité, cancer... Cette diversité fonctionnelle repose sur l'existence de multiples variétés de lymphocytes T, des lymphocytes T cytotoxiques CD8⁺, aux LT régulateurs Foxp3⁺, en passant par les différents sous-types de LT 'helper'.

L'étude de ces fonctions a permis le développement des immunothérapies qui ont révolutionné la prise en charge des patients atteints de certaines maladies auto-immunes et cancers. Si la composition en sous-types de LT dans les tissus enflammés et le micro-environnement tumoral est généralement bien décrite, les mécanismes moléculaires, c'est-à-dire les voies de signalisation et facteurs de transcription, qui orchestrent leur développement et leur fonction, sont eux encore mal compris. Nos travaux récents et projets en cours visent à étudier le rôle de NF-kappaB (NF-κB) dans ces processus.

NF-κB est une famille de facteurs de transcription composée de 5 membres : NF-κB1, NF-κB2, RelA, RelB et c-Rel. Si NF-κB a été largement impliquée dans différents aspects de la biologie des LT, le rôle de chacune des sous-unités (SU) est encore peu défini. Nous posons l'hypothèse que ces protéines exercent des rôles critiques et sélectifs dans la différenciation, la prolifération et la fonction des sous-populations de LT, à l'état basal et pendant la réponse immunitaire.

Dans les LT régulateurs, nous avons pu démontrer que les sous-unités RelA et NF-κB2 préviennent le développement de l'auto-immunité, alors que c-Rel est spécifiquement impliqué dans l'inhibition de la réponse anti-tumorale. Dans la continuité de ces résultats, nos projets actuels visent à explorer la régulation de la biologie des LT helper et cytotoxiques par NF-κB. Pour ce faire, nous avons développé des modèles de souris porteuses d'inactivation conditionnelles de chaque SU dans les différentes populations de LT; nous avons également mis au point un système d'ablation des SU dans des LT primaires humains, via la stratégie CRISPR/Cas9. Ces outils vont nous permettre d'aborder le rôle des SU de NF-κB à l'état basal, dans le cadre de l'auto-immunité, et dans le contexte de la croissance tumorale et des immunothérapies du cancer. De plus nous cherchons à savoir si l'activité spécifique de ces protéines dans les cellules tumorales elles-mêmes, influent sur la composition et la fonction du microenvironnement tumoral. Enfin, dans le cadre d'un projet de recherche translationnelle, nous étudions la distribution et la régulation des différents types de LT dans les lymphomes T périphériques nodaux, avec pour objectif la mise en évidence de cibles d'immunothérapie.

Nous pensons que ces travaux auront une portée à la fois fondamentale sur la compréhension des réponses T, et thérapeutique à travers le ciblage des SU de NF-κB ou des gènes qu'elles contrôlent.

Table des matières

CURRICULUM VITAE	4
LISTE DES FIGURES	12
1^E PARTIE : TRAVAUX EFFECTUES	13
1- Travaux de thèse : Stimulation des lymphocytes T régulateurs Foxp3⁺ par l'IL-2 et le TNF : implication dans les maladies auto-immunes et potentiel thérapeutique.	14
1.1 <i>Introduction</i>	14
1.2 <i>L'IL-2, une nouvelle immunothérapie du diabète de type 1</i>	14
1.3 <i>La fonction paradoxale du TNF-α dans la stimulation des Treg</i>	15
1.4 <i>L'axe TNF/TNFR2 comme cible thérapeutique dans la sclérose en plaques</i>	16
2. Travaux de post-doctorat : Le rôle sélectif des facteurs de transcription de la famille NF-kappaB dans la biologie des Treg.	18
2.1 <i>Introduction : NF-κB, un régulateur crucial de l'immunité</i>	18
2.2 <i>Les SU de NF-κB contrôlent l'identité moléculaire des Treg</i>	19
2.3 <i>Le rôle spécifique de la SU c-Rel dans l'immunité anti-tumorale.</i>	20
2E PARTIE : PROJETS	23
1. Introduction générale	24
2. Vers une vision holistique de l'orchestration de la fonction des LT par les SU de NF-κB .. 25	
2.1 <i>NF-κB comme régulateur central de l'identité des LT CD4 et CD8 chez la souris et l'homme.</i> ..	25
2.2 <i>NF-κB dans la différenciation des LT CD4⁺ helper : implication dans la physiopathologie des maladies auto-immunes</i>	28
2.3 <i>La voie canonique de NF-κB dans la fonction des LT effecteurs CD4⁺ et CD8⁺ : immunité anti-tumorale et réponse aux thérapies par inhibiteurs de checkpoint.</i>	30
3. Fonction intrinsèque des SU de NF-κB dans les cellules de mélanome : rôle dans l'établissement du microenvironnement immunitaire	33
4. Analyse du microenvironnement immunitaire dans les lymphomes T périphériques nodaux : impact pronostic et ciblage thérapeutique	35
5. Discussion générale	38
REFERENCES	42
ANNEXES	47

CURRICULUM VITAE

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Poste actuel

Chercheur CRCN INSERM

Chef de l'équipe ATIP-Avenir « Régulation Moléculaire de l'Immunité dans le Cancer ».

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INSERM U1052, CNRS UMR 5286, Centre Léon Bérard, Université Claude Bernard Lyon 1, LabEx DEV2CAN

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Formation

2007-2010 Doctorat en Immunologie, Université Pierre et Marie Curie (Paris VI)

2005-2007 Master de Biologie Moléculaire et Cellulaire, spécialité Immunologie, Université Pierre et Marie Curie (Paris VI)

2001-2005 DEUG et Licence de Biologie, parcours Physiologie-physiopathologie, Université Pierre et Marie Curie (Paris VI)

2001 Baccalauréat série S, Paris.

Expérience

2018- Chercheur CRCN INSERM au CRCL, chef d'équipe.

2012-2018 Chercheur post-doctorant, Sankar Ghosh lab, Columbia University Medical Center, New York, USA: Etude des facteurs de transcription de la famille NF- κ B dans les lymphocytes T régulateurs dans l'autoimmunité et le cancer.

2011 Chercheur post-doctorant, laboratoire I3 (INSERM U959, Paris), sous la supervision de Benoit Salomon : Rôle de l'axe TNFR/TNFR2 dans la physiologie des lymphocytes T régulateurs, dans un modèle murin de scléroses en plaques.

2007-2010 Doctorant, laboratoire I3 (INSERM U959, Paris), sous la supervision de Benoit Salomon et Eliane Piaggio : Stimulation des lymphocytes T régulateurs chez la souris : implications dans la physiopathologie et la thérapie du diabète auto-immun.

2008 Stage d'apprentissage de la microscopie confocale, laboratoire de Jeffrey Bluestone, sous la supervision de Qizhi Tang, UCSF, San Francisco, USA.

Activités de direction et encadrement scientifique

J'anime depuis 2018, l'équipe Régulation Moléculaire de l'Immunité dans le Cancer (labellisée ATIP-Avenir en 2019), composée de :

Philippe Cassier, médecin de Centre
Allison Voisin, chercheuse post-doctorante (financement Fondation ARC)
Maud Ligier, chercheuse post-doctorante (financement Labex DEVweCAN)
Guilhem Lalle, doctorant 3^e année (financement Labex DEVweCAN)
Pierre Stéphan, doctorant 2^e année (financement FRM)
Raphaëlle Lutraite, doctorante 2^e année (financement ARS Auvergne)
Julie Twardowski, ingénieure d'études CDD (financement Labex DEV2CAN)
Marlène Perrin-Niquet, technicienne CDI (Centre Léon Bérard)

Encadrement de travaux de recherche

En cours

- 2019-2022 Post-doctorat d'Allison Voisin : «Evaluation de la fonction de la voie canonique de NF- κ B dans les lymphocytes T CD8+ chez l'homme et la souris »
- 2021 Post-doctorat de Maud Ligier : « Régulation du transcriptome des lymphocytes T par NF- κ B : implication dans l'immunité anti-tumorale »
- 2019-2022 Doctorat de Guilhem Lalle : « Contribution sélective des sous-unités de NF- κ B dans les lymphocytes T effecteurs CD4+ au cours du cancer »
- 2020-2022 Doctorat de Pierre Stéphan (en co-supervision avec Olivier Trédan): « Analyse du micro-environnement immunitaire dans les lymphomes T nodaux : impact pronostic et ciblage thérapeutique »
- 2020-2023 Doctorat de Raphaëlle Lutraite (en co-supervision avec Christophe Caux) : « Régulation du développement et de la pathogénicité des lymphocytes Thelper par NF- κ B dans l'autoimmunité »

Passés

Nom – Prénom	Etablissement d'inscription	Formation	% encadrement	Date de l'encadrement	Publications
CARON Rachel	Columbia University	Doctorat	50%	01/09/2016-20/02/2018	Grinberg-Bleyer et al., Cell 2017, JI 2018
PLANCHET Constance	UCBL	Master 2	100%	04/02/2019-07/06/2019	
SYLVANDER Elise	UCBL	Master 2	100%	01/09/2020-30/10/2020	
TWARDOWSKI Julie	UCBL	Master 2	100%	02/01/2020-30/06/2020	Lalle et al. Cells 2021
STEPHAN Pierre	UCBL	Master 2	100%	04/02/2020-30/06/2020	Stéphan et al., Cancers 2020
REBUSTELLA Céline	UCBL	Master 1	50%	02/05/2021-31/07/2021	

Membre de jury de thèse

2019 Martina Lubrano di Ricco, UPMC, Paris : "The role of Tumor Necrosis Factor Receptor Family (TNFRF) members in regulatory T cell biology" (examineur)

Membre de comités de suivi de thèse

2020- Valentin Benbouker (CRCL, Lyon) : « Expression de ZEB1 dans le mélanome : impact sur l'immunité anti-tumorale »

2019- Olivia Le Saux (CRCL, Lyon) : « fonction et la valeur prédictive des différentes sous-populations de lymphocytes B dans le cancer de l'ovaire »

Enseignement

Depuis 2019 : Master 2 Biologie de la peau, UCBL, Cours sur l'Immunothérapie du mélanome (2h/an)

Depuis 2018 : Master 1 Cancer, UCBL, Cours et TD sur les lymphocytes T régulateurs dans le cancer (2x1h30/an+ évaluations écrites et orales)

Création d'une UE du Master Cancer de l'UCBL "Critical thinking and efficient communication in science", pour la rentrée 2022, 8h/an)

Support de la recherche

Financement des projets de recherche

- 2021-2024 LabEx DEV2CAN (220.000 €) : « Régulation Moléculaire de l'Immunité dans le Cancer »
- 2019-2022 ATIP-Avenir (180.000 €+ salaire post-doctorant) : "Investigating the roles of NF- κ B subunits as master regulators of effector T cell function and response to cancer immunotherapies."
- 2018-2021 LabEx DEVweCAN (250.000 €) : « Rôle des facteurs de transcription NF- κ B dans l'immunité anti-tumorale »
- 2020-2021 Fondation ARSEP (50.000 €) : « Etude des rôles des sous-unités de NF- κ B dans la fonction des lymphocytes Thelper 17 au cours de l'EAE. »
- 2020-2021 Fondation ARC (50.000 €) : « Etude du rôle de NF- κ B dans les réponses lymphocytaires T dans le mélanome »
- 2020-2021 Fondation BMS (50.000 €) : « Etude du rôle de NF- κ B RelA dans la réponse à la thérapie anti-PD-1 dans le mélanome »

Financements doctoraux et post-doctoraux

- 2013-2016 Financement de post-doctorat, Cancer Research Institute (CRI), USA
- 2011-2012 Financement de post-doctorat, Fondation ARSEP
- 2007-2010 Bourse doctorale, Ministère de l'enseignement supérieur de la recherche

Récompenses et honneurs

- 2019 Prix Institut Necker-Fondation TOURRE
- 2018 Bright Sparks award, European Federation of Immunological Societies.
- 2013 Award for postdoctoral research, Phillippe Foundation, New York, USA.
- 2010 Prix de la meilleure thèse; Société française d'Immunologie et BD Biosciences

Collaborations principales

- Véronique Baud, Paris: Analyses biochimiques de l'activité NF- κ B.
- Christine Caux, Lyon: Régulation des cellules TH1.17 dans le cancer chez l'homme.
- Françoise Durand-Dubief, Lyon: Analyse de l'inflammation dans la SEP.
- Ulf Klein, Leeds, UK : Modèles murins.
- Benoit Salomon, Paris: Rôle des voies TNFR/NF- κ B dans les Treg.
- Joost Van Meerwijk, Toulouse: Rôle de NF- κ B dans la circulation des Treg.
- Caroline Wilson, Newcastle, UK : Modèles murins.

Animation scientifique

Activité d'expertise pour journaux à comité de relecture

Je fais partie du Review Board de Frontiers in Immunology, et j'exerce une activité de rapporteur pour JITC, European Journal of Immunology, Cells, Cancers, Scientific Reports...

Participation à la vie collective du laboratoire et du centre

-Membre du comité de pilotage des plateformes animalerie, cytométrie et imagerie.

-Membre du comité d'animation du CRCL.

-Création et animation d'un groupe de travail inter-équipes sur l'onco-immunologie et les immunothérapies, au CRCL.

Organisation de congrès

-Membre du comité d'organisation du 2^e congrès IRCI (Immune Responses in Cancer and Infection), Lyon, Juin 2022

Membre de sociétés savantes

Membre de la Société Française d'Immunologie depuis 2009

Publications et communications scientifiques

Articles publiés dans des revues à comité de lecture international, par ordre chronologique inverse ; les noms des étudiants que j'ai encadrés sous soulignés.

Ronin E., Pouchy C, Khosravi M., Grégoire S., Kassem S., Sleurs D., Martin GH., Chanson N., Lombardi Y., Lalle G., Wajant H., Auffray C., Lucas B., Marodon G., **Grinberg-Bleyer Y.*** and Salomon B.L.*(co-senior, co-corresponding authors). "Tissue-restricted control of established central nervous system autoimmunity by TNF receptor 2 expressing Treg cells" **PNAS**, 2021, Vol. 118 No. 13

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Voisin A., **Grinberg-Bleyer Y.** "The many-sided contributions of NF- κ B to T-cell biology in health and disease"(review). **International Review of Cell and Molecular Biology**. 2020, doi.org/10.1016/bs.ircmb.2020.10.006

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Differentiation that Activates and Organizes Asymmetric Cell Division.” **Cell reports**, 2016 May 24;15(8):1615.

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Grinberg-Bleyer Y., Baeyens, A., You, S, Fourcade, G., Gregoire, S., Cagnard, N., Carpentier, W., Boissonnas, A., Tang, Q., Bluestone, J., Chatenoud, L., Klatzmann, D., Salomon, B.L., and Piaggio, E. “IL-2 reverses established type one diabetes by a local effect on pancreatic regulatory T cells”. **J Exp Med**, 2010, 207(9):1871-8.

Brevet international

Ghosh S., **Grinberg-Bleyer Y.** Use of pentoxifylline with immune checkpoint-blockade therapies for the treatment of melanoma. **Patent # WO2017058881 A1**

Présentations orales en congrès internationaux.

4th International Cancer Symposium / Cancer Research Center of Lyon, Lyon, France. October 2-4, 2019. Regulation of T-cell immunity and tolerance to cancer by the NF- κ B family of transcription factors.

European Congress of Immunology, Bright Sparks lectures, Amsterdam, Netherlands, September 2-5, 2018. NF- κ B c-Rel is crucial for the regulatory T cell immune checkpoint in cancer.

Annual Meeting of the French Society for Immunology, Montpellier, France, November 8-10, 2011 TNF α /TNFR2 pathway is essential for Treg homeostasis during EAE.

Meeting of the French Club for Neuro-immunology, Paris, France, May 19, 2011. Regulatory role of TNF α in EAE and in Treg homeostasis in the CNS.

Annual Meeting of the French Society for Immunology, Marseille, France, November 24-26, 2010 Paradoxical protective effect of pathogenic T cells in murine autoimmune diabetes through a regulatory T cell boost.

Liste des figures

Figure 1 : Modèle de résolution de l'inflammation par un boost Teff->Treg

Figure 2 : Voies de signalisation associées à NF- κ B dans les lymphocytes T

Figure 3 : Les sous-unités de NF- κ B dans le développement, l'activation et la fonction des Treg.

Figure 4 : Cadre général du travail.

Figure 5 : Stratégie de recherche générale.

Figure 6 : Analyse de l'identité des Tconv CD4⁺ murins déficients pour Rel ou RelA.

Figure 7 : Cartographie de la régulation de la réponse T par les SU de NF- κ B.

Figure 8 : Analyse de la fonction de RelA et c-Rel dans les LT humains.

Figure 9 : RelA, mais pas c-Rel, est requis pour la différenciation des TH17 *in vitro*.

Figure 10 : Fonction intrinsèque de RelA dans la pathogénicité des Teff CD4 au cours de l'EAE.

Figure 11 : RelA participe à la fonction des Teff CD4⁺ dans la tumeur.

Figure 12 : Contribution sélective des SU de NF- κ B dans la croissance du mélanome.

Figure 13 : Stratégie d'analyse du microenvironnement des PTCL.

1^e PARTIE
TRAVAUX EFFECTUES

1- Travaux de thèse : Stimulation des lymphocytes T régulateurs Foxp3⁺ par l'IL-2 et le TNF : implication dans les maladies auto-immunes et potentiel thérapeutique.

1.1 Introduction

J'ai initialement rejoint l'équipe de Benoit Salomon au sein du laboratoire dirigé par David Klatzmann en 2006 pour un stage de Master ; notre relation de travail a été si forte que j'y suis en fait resté près de 6 ans en cumulant Master, thèse et postdoc ! Le travail de l'équipe de Benoit se concentrait (et se concentre toujours) sur l'étude de la physiologie et le potentiel thérapeutique des lymphocytes T (LT) régulateurs CD4⁺Foxp3⁺ (Treg), acteurs majeurs de la suppression des réponses immunitaires. Via une multitude de mécanismes moléculaires, les Treg sont capables d'inhiber l'activation, l'expansion et/ou la fonction des autres cellules immunitaires, en particulier leurs contreparties LT « conventionnels » (Tconv). Ceci leur confère une fonction protectrice dans l'auto-immunité ou la transplantation, illustrée par le syndrome auto-immun systémique et léthal observé chez les souris déficientes pour *Foxp3* (syndrome *Scurfy*) ; un phénotype comparable se développe chez les patients porteurs de mutations dans *FOXP3* (syndrome IPEX pour Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome). Dans le même ordre d'idée, de nombreuses maladies auto-immunes, comme le diabète de Type 1 ou la sclérose en plaques, sont caractérisées par un défaut quantitatif et/ou fonctionnel des Treg. A l'inverse, ce pouvoir inhibiteur confère aux Treg un rôle le plus souvent délétère dans le cancer (nous y reviendrons plus tard).

Au vu du fort potentiel thérapeutique des Treg dans les maladies auto-immunes, différents types de traitements sont envisageables : les thérapies cellulaires via un transfert de Treg, et l'administration de molécules capables d'améliorer le compartiment Treg *in vivo*. Au cours de ma thèse, je me suis concentré sur cette seconde approche. Les travaux que j'ai conduit sous la direction des Dr. Benoit Salomon et Eliane Piaggio, ont conduit à la découverte de deux facteurs solubles produits par les Tconv et capables d'augmenter la prolifération et la fonction des Treg dans le diabète de type 1 : l'Interleukine-2 (IL-2), et le Tumor Necrosis Factor- α (TNF- α).

1.2 L'IL-2, une nouvelle immunothérapie du diabète de type 1 (Grinberg-Bleyer et al., 2010a)

L'IL-2 a tout d'abord été identifiée comme une molécule importante pour la prolifération des LT, d'où son appellation initiale « T-cell growth factor » (Smith, 1980). Toutefois le syndrome lymphoprolifératif détecté chez les souris invalidées pour l'IL-2 a largement remis ce concept en cause (Schorle et al., 1991). Il est désormais établi que l'IL-2 permet avant tout la survie et la prolifération des Treg ; nous parlerons donc plutôt d'un « Treg growth factor » (Nelson, 2004). Il a été démontré que le déficit génétique impactant la production d'IL-2 chez la souris Non Obese Diabetic (NOD, qui développe spontanément un diabète auto-immun) (Denny et al., 1997), provoque un défaut de survie des Treg dans le pancréas enflammé et est l'un des responsables de la rupture de tolérance conduisant au diabète. De ce fait, l'injection répétée de faibles doses d'IL-2 permet une prévention quasi-complète de l'apparition du diabète de type 1 (Tang et al., 2008).

Nous nous sommes alors demandé si l'IL-2 pouvait également être efficace en thérapie curative du diabète auto-immun. De façon remarquable, 5 injections de faibles doses d'IL-2 humaine (25.000 unités/injection) suffisent à induire une rémission chez près de 60% des souris diabétiques. De plus, l'administration de 5 doses supplémentaires permet de restaurer une glycémie normale chez 40% des souris nos répondeuses au traitement initial.

Cet effet curatif est associé à une augmentation spécifique de la proportion de Treg dans le pancréas, et à une amélioration de leur expression de CD25 et Foxp3, sans que les tissus lymphoïdes soient impactés. Ainsi, plutôt que d'induire une immunosuppression généralisée, le traitement par IL-2 à faible dose permet spécifiquement de restaurer le déficit en Treg au sein du tissu ciblé par l'attaque auto-immune. Enfin, nous avons également montré que l'effet bénéfique de la thérapie IL-2 passait probablement par son effet sur les Treg, car elle est inefficace chez les souris *Cd28^{-/-}* dépourvues de Treg.

Ces données ont mis en évidence l'IL-2 comme candidat médicament dans le diabète de type 1. A l'époque, il ne s'agissait que de la deuxième molécule décrite comme ayant un effet curatif dans cette maladie, après les anticorps anti-CD3. Ceci a conduit à la mise en place d'un essai clinique à l'hôpital de la Pitié-Salpêtrière (identifiant NCT01353833). Nos travaux novateurs ont par la suite permis à différentes équipes de tester le potentiel thérapeutique de l'IL-2 dans de très nombreux modèles précliniques d'auto-immunité, d'allergie et de transplantation, avec des résultats souvent probants (Klatzmann and Abbas, 2015).

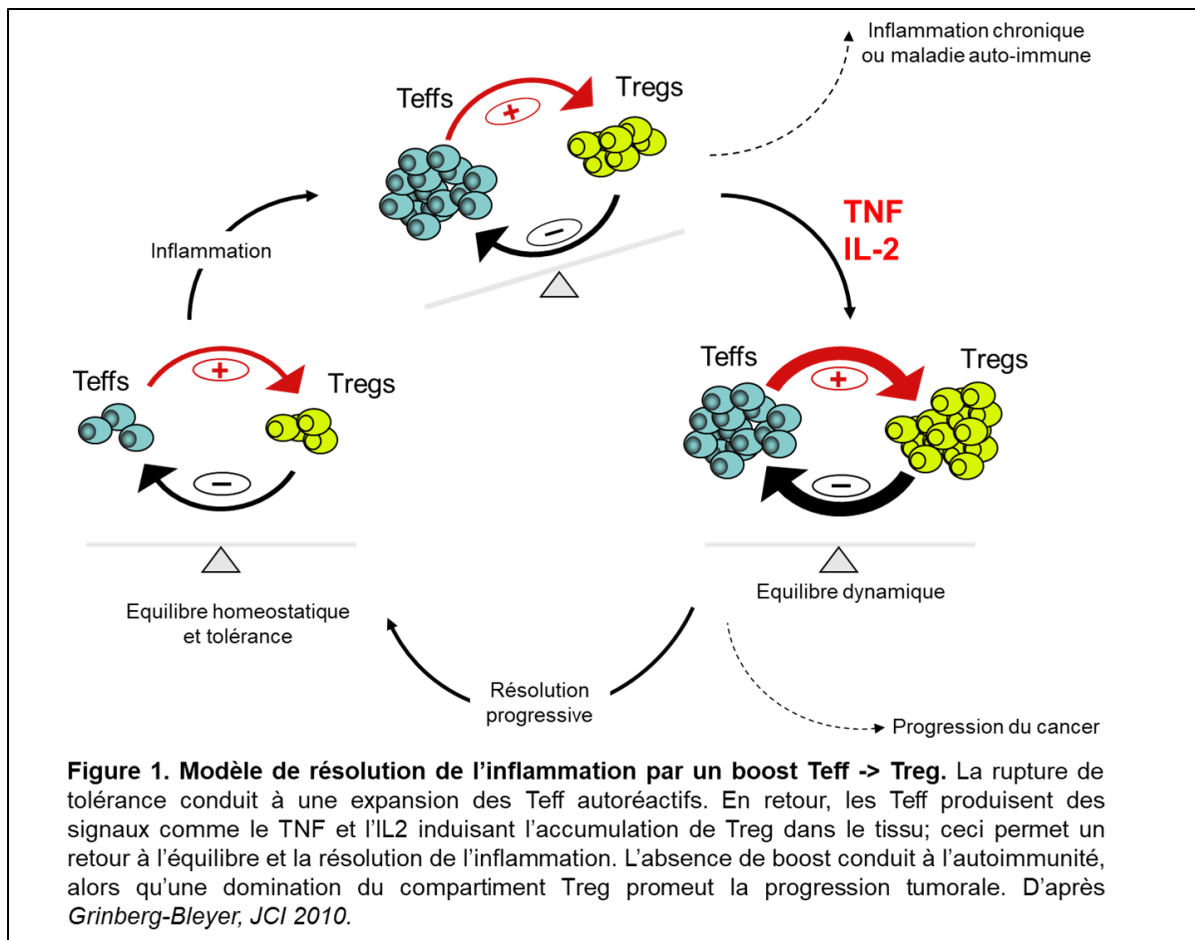
1.3 La fonction paradoxale du TNF- α dans la stimulation des Treg (Grinberg-Bleyer et al., 2010b)

Mon second projet de thèse avait un aspect plus fondamental, et s'intéressait aux mécanismes de régulation de l'activité des Treg. Si les mécanismes de suppression des Tconv par les Treg sont bien décrits, la question inverse n'était à l'époque pas ou peu explorée. J'étais donc en charge de répondre à une question simple : les Tconv sont-ils capables d'impacter l'activation ou la fonction des Treg ?

Nous avons tenté de répondre à ces questions dans un modèle murin de diabète auto-immun. Nous avons fait une observation tout à fait paradoxale : le transfert de Tconv spécifiques d'un antigène pancréatique augmente fortement l'expansion et la fonction suppressive des Treg dans le pancréas et les ganglions drainant, induisant une prévention de la maladie à long terme. Ainsi, la même cellule qui induit le diabète, peut aussi exercer un rétrocontrôle via un « effet boost » sur les Treg (Figure 1).

J'ai ensuite tenté de déterminer le(s) mécanisme(s) moléculaire(s) de cette expansion des Treg. L'analyse de la signature transcriptionnelle des Treg a mis en évidence que les voies de signalisation déclenchées par le TNF, molécule clé de l'inflammation, étaient fortement utilisées par les Treg *boostés* ; notamment l'axe TNFR2/NF- κ B dont nous parlerons plus longuement par la suite. Des expériences de blocage *in vivo* nous ont confirmé l'implication cruciale du TNF dans le boost des Treg, mettant ainsi en lumière une nouvelle facette de cette cytokine dans l'inhibition des réponses auto-immunes.

Ces travaux ont depuis été confirmés dans de nombreux autres modèles, et nous-même avons ensuite disséqué les mécanismes moléculaires et cellulaires permettant cet effet paradoxal du TNF.



1.4 L'axe TNF/TNFR2 comme cible thérapeutique dans la sclérose en plaques (Ronin et al., 2021)

Dans ce troisième projet, nous avons choisi comme modèle d'étude l'EAE (Experimental Autoimmune Encephalomyelitis), modèle murin de sclérose en plaques (SEP). Dans cette maladie, les molécules thérapeutiques ciblant le TNF (anticorps anti-TNF et TNFR2 soluble), qui ont fait leur preuve dans d'autres pathologies auto-immunes et inflammatoires, sont contre-indiqués du fait d'aggravation de symptômes chez certains patients (1999). A la suite de nos travaux dans le diabète, nous avons émis l'hypothèse qu'une cause sous-jacente à ce phénomène pourrait être la signalisation TNF/TNFR2 induisant un boost des Treg dans les tissus enflammés. En accord avec cette hypothèse, nous avons observé que le blocage du TNF ou du TNFR2 aggrave significativement la sévérité de la maladie tout en réduisant la proportion de Treg dans le système nerveux central.

Nous avons ensuite développé un modèle de souris porteuse d'une invalidation conditionnelle du TNFR2 dans les Treg ($Foxp3^{CRE}TNFR2^{Fllox}$). De façon remarquable, les symptômes de la maladie sont fortement aggravés dans ces souris mutantes, et ceci est associé à un changement du profil transcriptomique des Treg dans le système nerveux mais pas les ganglions lymphatiques. Ces travaux ont permis de tirer différentes conclusions :

- Nous fournissons une explication mécanistique au rôle protecteur du TNF dans l'EAE, via son rôle dans la physiologie des Treg.

- Nous démontrons pour la première fois, que l'inhibition du processus auto-immun par les Treg, a lieu dans les tissus cibles enflammés une fois le processus enclenché, d'avantage que dans les ganglions lymphatiques au cours de l'initiation.

Ces données établissent le que TNF, historiquement décrit comme une cytokine pro-inflammatoire, peut aussi avoir une composante inhibitrice de l'immunité de par son action sur les Treg via sa signalisation par le TNFR2. Ceci a ouvert la voie à différentes perspectives thérapeutiques :

- La stimulation du TNFR2 dans les pathologies auto-immunes, la transplantation ou la maladie du greffon contre l'hôte.

- Le blocage du TNFR2 en immunothérapie du cancer.

2. Travaux de post-doctorat : Le rôle sélectif des facteurs de transcription de la famille NF-kappaB dans la biologie des Treg.

2.1 Introduction : NF-kB, un régulateur crucial de l'immunité

Dans le cadre de mes travaux sur la signalisation TNF/TNFR2, j'ai commencé à m'intéresser aux voies de signalisation et facteurs de transcription impliqués dans les réponses immunitaires, et en particulier à NF-κB. J'ai donc décidé, pour mon stage de post-doctorat, de rejoindre le laboratoire du Pr. Sankar Ghosh, de l'université Columbia, à New York, Etats-Unis. Sankar Ghosh fait partie des pionniers dans l'étude de NF-κB et des voies de signalisation qui lui sont associées.

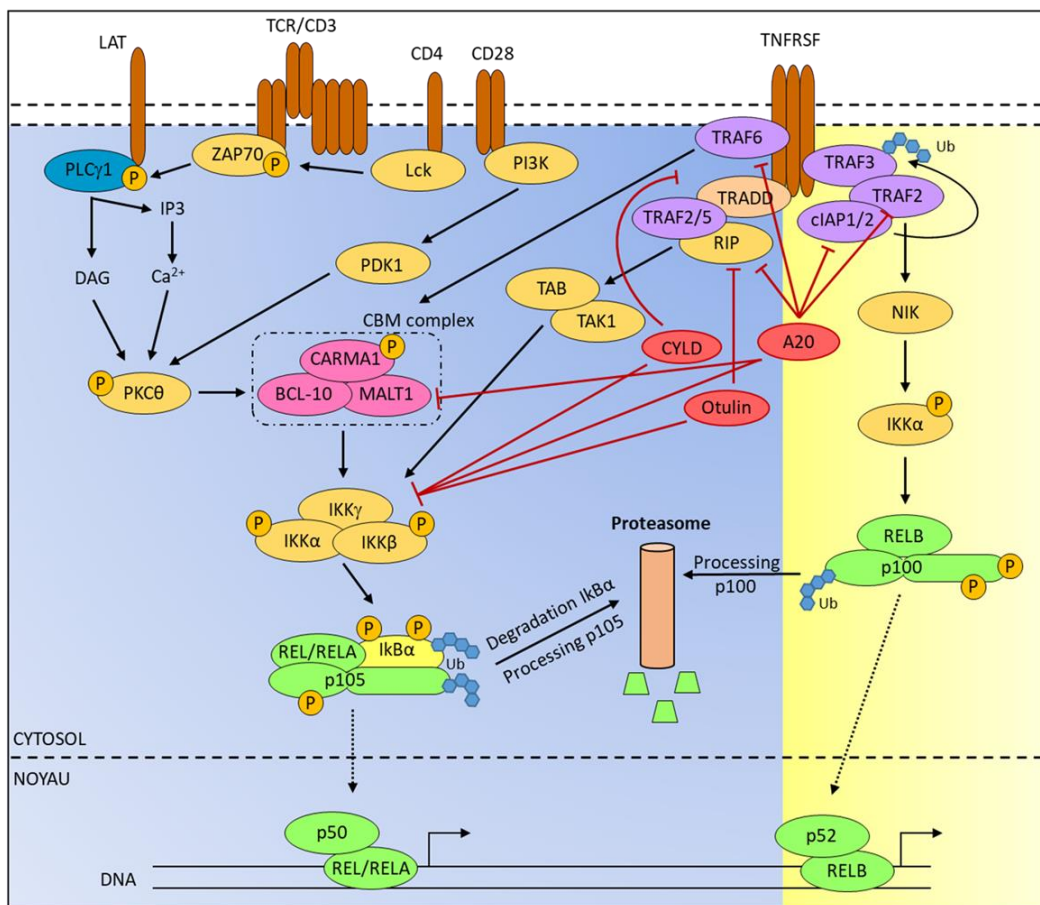


Figure 2. Voies de signalisation associées à NF-κB dans les LT. D'après Voisin et al., IRCMB 2021

Découvert à l'origine dans les cellules B, NF-κB a fait l'objet d'études approfondies dans de nombreux domaines de la biologie. Il est maintenant établi que NF-κB exerce des rôles cruciaux dans la majorité des types cellulaires chez les mammifères, avec diverses fonctions dans la prolifération, la survie, le développement, l'inflammation et l'immunité. NF-κB est en fait une famille de facteurs de transcription composée de cinq sous-unités (SU) qui partagent toutes un domaine d'homologie Rel (RHD) et ont été historiquement divisées en deux voies : RelA (ou p65), c-Rel (codée par *Rel*) et p105/p50 (NF-κB1) appartiennent à la voie canonique, tandis que RelB et p100/p52 (NF-κB2) forment les SU de la voie alternative (Figure 2) (Voisin and Grinberg-Bleyer, 2021). Ces protéines forment des homo- ou hétérodimères pour réguler la transcription des gènes cibles. À l'état basal, les SU NF-κB sont retenues dans le cytosol par les

protéines inhibitrices I κ B, p100 et p105, grâce à leurs domaines ankyrine, qui interagissent avec le RHD des SU. Classiquement, dans les LT, la voie canonique est déclenchée par les signaux du TCR, de CD28 et de certains membres de la famille des récepteurs au TNF (TNFRSF). La voie alternative semble elle activée par l'engagement de certains membres spécifiques de la famille TNFRSF. Toutefois il est notable que ce concept de deux entités séparées a été remis en cause ces dernières années ; d'une part car certains récepteurs membranaires sont capables d'engager les deux voies ; d'autre part car certaines SU des voies canoniques et alternatives sont capables de s'hétéro-dimériser. Plutôt que de parler de « voies », on parlera donc de SU canoniques et alternatives.

NF- κ B fait partie des facteurs de transcription les plus étudiés dans la biologie moderne (plus 113.000 références bibliographiques à ce jour !), et a été largement étudié dans les réponses immunitaires adaptative. Des études publiées dans les années 90 et 2000 ont mis en évidence son rôle dans le développement et la fonction des sous-types de LT et en particulier, sa fonction cruciale dans la différenciation des Treg dans le thymus. Ainsi, l'axe CBM/IKK/RelA-c-Rel est requis pour l'expression de *Foxp3* par les cellules « progénitrices de Treg » CD4⁺Foxp3⁺CD25⁺GITR⁺, comme illustré chez les souris déficientes pour *Carma1*, *Bcl10* ou *Rel*, qui ont toutes un profond déficit en Treg dans le thymus et à la périphérie (Deenick et al., 2010; Isomura et al., 2009; Molinero et al., 2009).

Toutefois, le rôle spécifique de chacune des SU de NF- κ B dans le développement des Treg et dans la fonction des Treg matures, était inconnue jusque récemment, du fait de l'absence de modèles d'études adéquats. En effet, les souris porteuses d'une invalidation de *Rela* dans la lignée germinale, présentent un phénotype de létalité embryonnaire (Beg et al., 1995). De plus, les souris *Relb*^{-/-} développent une auto-immunité systémique létale à partir de 3 semaines après la naissance (Weih et al., 1995). Enfin, le rôle de c-Rel dans la différenciation thymique des Treg prévient son étude dans les cellules matures de la périphérie. Tous ces phénotypes empêchent l'étude des rôles intrinsèques des SU dans les LT.

Pour aborder cette problématique, nous avons profité de la création de modèles de souris porteuses d'allèles *floxés* dans chacune des SU (hormis *Nfkb1*), par notre collaborateur Ulf Klein à Columbia, spécialiste des lymphocytes B (De Silva et al., 2016; Heise et al., 2014). En utilisant des souches CRE permettant de déléter NF- κ B dans les lymphocytes T totaux et les Treg mature, nous avons pu répondre à une question principale : **quelle est la contribution sélective des SU de NF- κ B dans le développement, l'homéostasie et la fonction des Treg ?**

2.2 Les SU de NF- κ B contrôlent l'identité moléculaire des Treg. (Grinberg-Bleyer et al., 2018; Oh et al., 2017)

Nous avons tout d'abord étudié la fonction intrinsèque de NF- κ B dans le développement des Treg, grâce aux souris CD4^{CRE}. Comme suggéré précédemment, l'ablation de *Rel* conduit à une baisse significative à la fois du nombre de progéniteurs CD25⁺GITR⁺Foxp3⁻, et du nombre de Treg Foxp3⁺ dans les thymus et les tissus lymphoïdes secondaires, alors que les autres sous-types de LT sont épargnés. Nous avons détecté un phénomène similaire, dans les souris invalidées pour *Rela*. De façon spectaculaire, les souris double knock-out (KO) *Rel-Rela* présentent une absence quasi complète de progéniteurs et de Treg, suggérant un mécanisme compensatoire partiel dans les souris déficientes pour une seule SU. De plus, c-Rel et RelA sont nécessaires à la transition entre progéniteurs et Treg, c'est-à-dire pour l'expression de

Foxp3 en elle-même. A l'inverse, les souris KO conditionnelles pour *Relb*, *Nfkb2*, ou les deux, présentent un développement normal des Treg dans le thymus. Ces données montrent pour la première fois une implication complémentaire de RelA et c-Rel à différentes étapes de la différenciation thymique des Treg.

Pour analyser les fonctions spécifiques de chaque SU dans les Treg matures et outrepasser le déficit en Treg observé dans les $CD4^{CRE}Rel^{F/F}$ et $Rela^{F/F}$, nous avons ensuite utilisé la souche $Foxp3^{CRE}$. De façon frappante, les souris $Foxp3^{CRE}Rela^{F/F}$ développent un syndrome auto-immun multifocal et létal à partir de 5-6 semaines après la naissance. Ce phénotype n'est pas observé chez les souris $Foxp3^{CRE}Rel^{F/F}$ qui présentent seulement une splénomégalie et une inflammation non létale et tardive (à partir de 8 mois) dans les tissus non lymphoïdes (foie et poumons en particulier). De même, les souris $Foxp3^{CRE}Nfkb2^{F/F}$ présentent un phénotype limité à une inflammation de l'intestin à partir de 10 mois d'âge. Enfin les souris $Foxp3^{CRE}Relb^{F/F}$ sont entièrement épargnées. Ces phénotypes reposent en partie, comme dans le thymus, sur un mécanisme de compensation, puisque les souris double KO $Foxp3^{CRE}Rela^{F/F}Rel^{F/F}$ développent une auto-immunité létale dès 3 semaines après la naissance, similaire au syndrome observé chez les souris *Scurfy* déficientes en Treg. L'analyse transcriptomique de ces différents Treg révèle une implication majeure de la voie canonique dans le maintien de « l'identité » des cellules. En effet, les Treg double KO *Rela/Rel* (et dans une moindre mesure *Rela* et *Rel*KO) perdent l'expression de nombreux marqueurs associés aux Treg, tels que *Foxp3* lui-même, CTLA-4, ou *Helios*. A l'inverse ces cellules acquièrent des propriétés effectrices, avec l'expression de cytokines inflammatoires normalement limitées au compartiment Tconv (IL-2, IFN γ , IL-17A...); à noter que cette expression de marqueurs TH17 est retrouvée dans les Treg déficients pour *Nfkb2*, ce qui explique sans doute l'inflammation intestinale qui se développe chez ces souris. Nos analyses de ChIP-Seq suggèrent que RelA coopère en fait directement avec *Foxp3* pour établir la signature transcriptomique associée aux Treg. **Ces données permettent de tirer une hiérarchie sur la fonction des SU dans les Treg matures, à l'état basal : RelA>c-Rel>NF- κ B2.**

2.3 Le rôle spécifique de la SU c-Rel dans l'immunité anti-tumorale. (Grinberg-Bleyer et al., 2017)

En opposition à leur rôle protecteur dans l'auto-immunité, le rôle délétère des Treg dans l'inhibition de l'immunité anti-tumorale est bien établi. En effet, l'accumulation de Treg dans le sang périphérique et/ou le site tumoral est généralement de mauvais pronostic chez les patients (Whiteside, 2018). Chez la souris, la déplétion des Treg conduit à l'élimination des tumeurs syngéniques transplantées (Onizuka et al., 1999). Ces données font des Treg une excellente cible d'immunothérapie ; d'ailleurs l'effet bénéfique de l'immunothérapie par anti-CTLA4 observé chez les patients atteints de mélanome et de cancer du poumon, pourrait bien reposer sur l'effet de l'anticorps sur les Treg (déplétion ou modulation de la fonction), bien que cela ne soit pas clairement établi chez l'homme (Arce Vargas et al., 2018).

Lorsque nous avons débuté cette étude, les voies de signalisations et les facteurs de transcription impliqués dans l'accumulation et la fonction des Treg dans la tumeur, étaient très mal décrits. Au vu des rôles critiques et spécifiques des SU de NF- κ B dans les Treg, nous nous sommes donc demandés leurs activités pourraient impacter les réponses immunitaires anti-tumorales. De façon remarquable, la croissance de mélanomes transplantés est fortement réduite dans les souris $Foxp3^{CRE}Rel^{F/F}$, mais pas $Foxp3^{CRE}Rela^{F/F}$ ou $Foxp3^{CRE}Nfkb2^{F/F}$. Ceci est associé à une diminution de

l'accumulation des Treg dans la tumeur et une augmentation de l'expression d'IFN γ et de TNF par les Tconv, notamment CD8 $^+$; d'ailleurs la déplétion des LT CD8 $^+$ restaure la pousse tumorale. Ces résultats suggèrent que c-Rel, mais pas RelA, est crucial pour l'homéostasie et/ou la fonction des Treg dans la tumeur. Par conséquent, nous avons étudié le potentiel thérapeutique d'inhibiteurs chimiques de c-Rel pour altérer la fonction Treg, et ainsi promouvoir la réponse anti-tumorale. Nous avons identifié la pentoxifylline (PTXF), précédemment décrite comme un inhibiteur de phosphodiésterases, comme capable d'induire la dégradation de c-Rel sans affecter RelA dans les Treg. Nous avons pu montrer dans différents modèles de tumeurs transplantées, que l'administration de PTXF, seule ou en combinaison avec une immunothérapie par anti-PD-1, était capable de réduire significativement la croissance tumorale, via un mécanisme CD8-dépendant. Sur le plan mécanistique, l'ablation génétique et l'inhibition chimique de c-Rel, conduit à une perte de marqueurs de Treg activés (aTreg), mais surtout à une augmentation de la sécrétion d'IFN γ par ces même Treg. Il est donc possible que ces Treg instables deviennent en fait anti-tumoraux, comme montré dans d'autres modèles (Di Pilato et al., 2019).

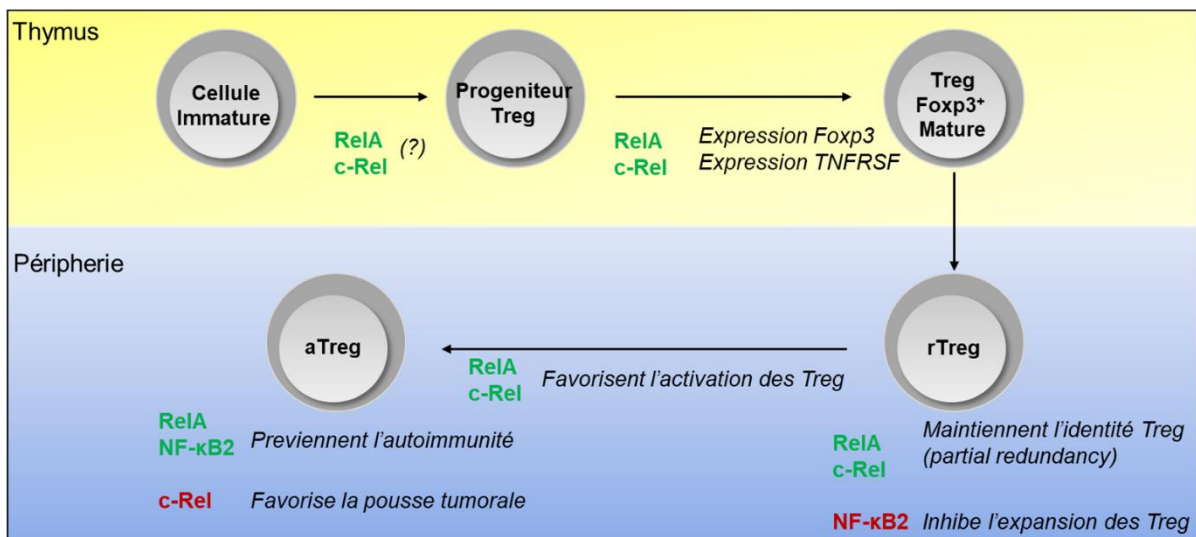


Figure 3. Les sous-unités de NF- κ B dans le développement, l'activation et la fonction des Treg. D'après Voisin et al., IRCMB 2021

2.4 Conclusions

L'ensemble de ces travaux ont permis d'identifier, pour la première fois, une contribution sélective des SU de NF- κ B dans la biologie des Treg, spécifique au contexte (Figure 3). Alors que RelA semble crucial pour le maintien de la signature Treg à l'état basal, c-Rel semble avoir un rôle restreint -mais majeur- à l'immunité tumorale. Il est à noter qu'un rôle potentiel de RelA dans ce contexte n'est pas formellement exclu. En effet, l'auto-immunité spontanée des souris Foxp3^{CRE}Rela^{F/F} pourrait avoir masqué une l'implication de RelA au niveau du site tumoral ; il serait important d'investiguer cette possibilité en utilisant des souris Foxp3^{CRE-ERT2}, dans lesquelles l'ablation de RelA peut être induite à l'âge adulte, par l'administration de tamoxifène.

Quoiqu'il en soit, nos données permettent de proposer une nouvelle classe d'immunothérapies avec des inhibiteurs sélectifs des SU de NF- κ B. Ces inhibiteurs étant plus spécifiques, et donc efficaces et sûrs, que les inhibiteurs d'IKK, qui malgré leur potentiel indéniable dans des modèles murins et *in vitro*, ont presque tous été

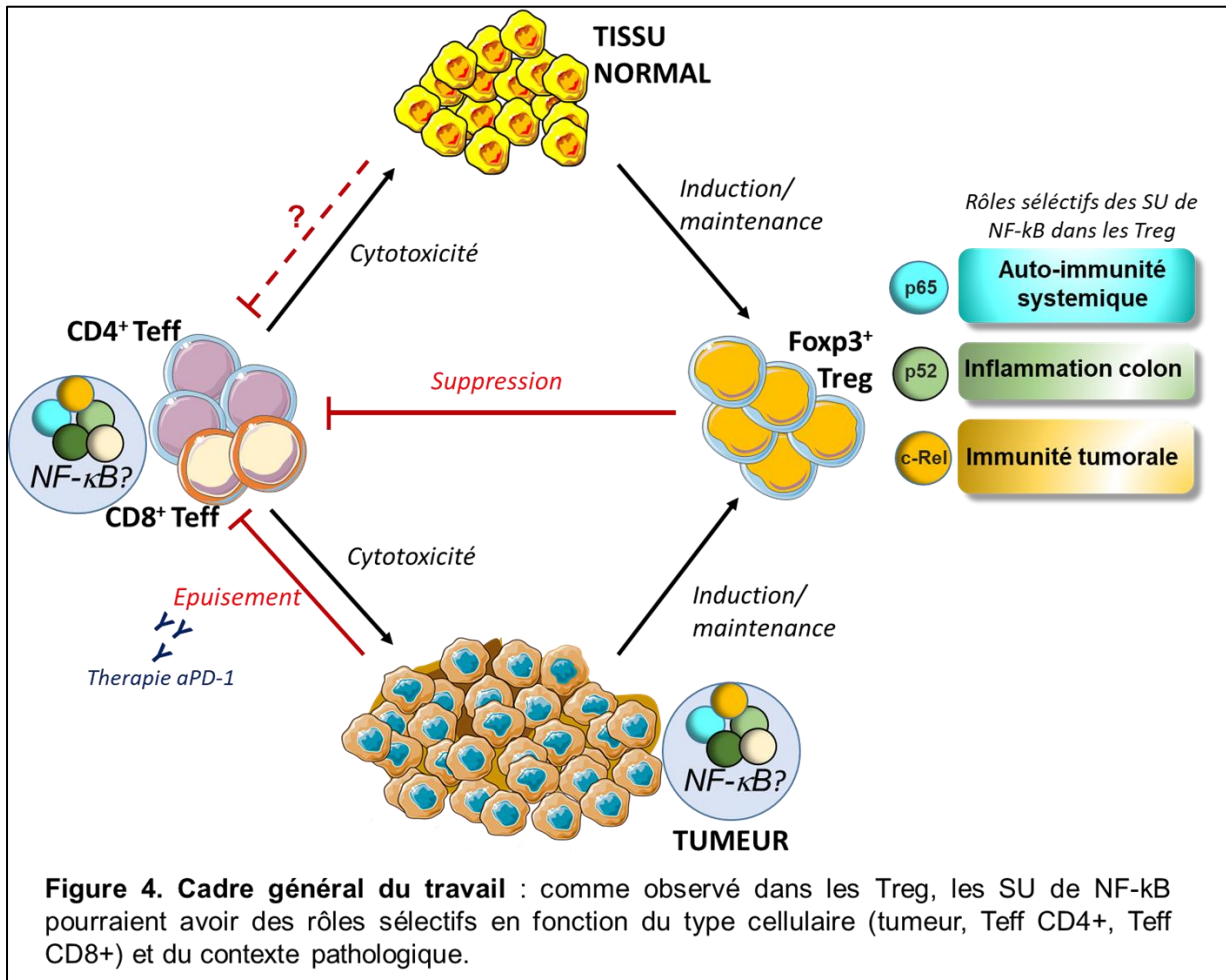
abandonnés en clinique du fait de toxicités sévères ou d'absence d'efficacité (Baud and Karin, 2009). D'autres inhibiteurs de c-Rel ont d'ailleurs été récemment développés et ont montré leur efficacité certaine dans des modèles précliniques de cancers solides, alors qu'aucun effet secondaire détectable n'a été reporté (Li et al., 2020; Shono et al., 2016). Le développement clinique de ces thérapies, en particulier en combinaison avec un anti-PD1, semble donc prometteur, et nous a conduits à déposer un brevet international (WO2017058881 A1).

2e PARTIE

PROJETS

1. Introduction générale

Mes travaux de post-doctorat ont notamment permis d'établir une preuve de concept sur la régulation de la transcription par NF-κB : au sein d'une même cellule, les différents SU ont des rôles spécifiques en fonction du contexte pathologique. Ceci confère un potentiel indéniable au ciblage thérapeutique de ces protéines dans le cadre de l'auto-immunité et du cancer.

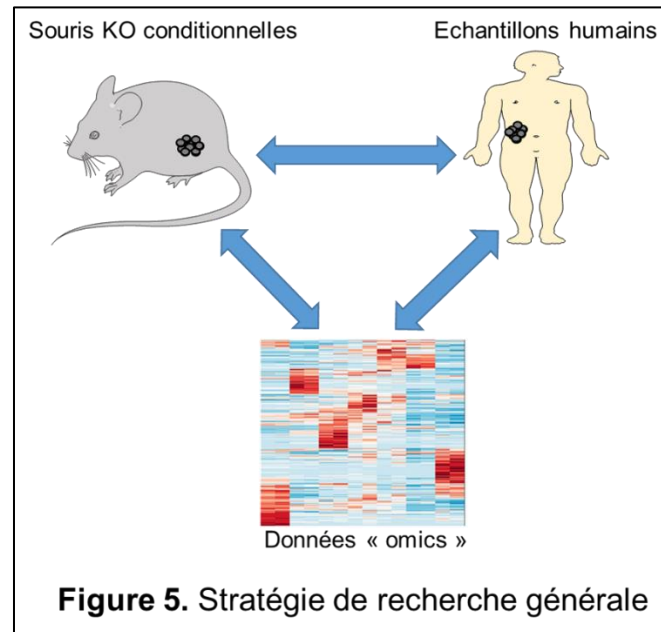


Dans la continuité de ces travaux, j'ai donc émis l'hypothèse que ce phénomène est généralisable à d'autres types de cellules T ainsi qu'aux cellules tumorales elles-mêmes (Figure 4). Spécifiquement, nous travaillons sur 3 axes majeurs :

- la fonction intrinsèque des SU de NF-κB dans l'identité des LT « effecteurs » (Teff) CD4⁺ et CD8⁺ au cours des réponses anti-tumorales et des immunothérapies du cancer.
- leur impact dans le développement des sous-types de LT « helper » CD4⁺ et la physiopathologie des maladies auto-immunes.
- la fonction des SU de NF-κB dans les cellules tumorales, plus spécifiquement leur impact dans la relation cancer-immunité.

D'autre part, dans le cadre d'un projet de recherche translationnelle, nous cherchons à documenter la distribution et la fonction du microenvironnement immunitaire dans les lymphomes T nodaux, avec pour objectif majeur la mise en évidence de certains récepteurs de surface comme potentielles cibles thérapeutiques.

Nos approches reposent sur l'utilisation de souris KO conditionnelles, de LT et de prélèvements tumoraux humains, et d'approche 'omics' (Figure 5). Nous avons un objectif général de type « bench-to-bedside », où nous espérons que la découverte de mécanismes fondamentaux de régulation des réponses immunitaires, pourra conduire à l'intervention thérapeutique.



2. Vers une vision holistique de l'orchestration de la fonction des LT par les SU de NF- κ B (Travaux menés par Guilhem Lalle et Raphaëlle Lautraite, doctorants, et Allison Voisin et Maud Ligier, post-doctorantes)

2.1 NF- κ B comme régulateur central de l'identité des LT CD4 et CD8 chez la souris et l'homme.

Rationnel et hypothèse de travail

De nombreuses publications ont établi NF- κ B comme un régulateur important du développement et de la fonction des LT (voir pour revue (Voisin and Grinberg-Bleyer, 2021)). Par exemple, l'expression transgénique d'une forme non dégradable de la kinase inhibitrice I κ B α , qui conduit à une inhibition globale de la voie canonique, altère profondément la sélection thymique des LT CD8⁺ chez la souris (Jimi et al., 2008). À la périphérie, les LT CD4⁺ de ces animaux présentent une survie réduite. De même, la délétion conditionnelle de *Ikkb* (IKK β) dans les cellules T entraîne une altération de la survie et de l'activation des CD4⁺ et CD8⁺ (Schmidt-Suppryan et al., 2003). La délétion germinale de c-Rel entraîne un phénotype distinct avec un défaut partiel de la sécrétion d'IL-2 et de la polarisation TH17 *in vitro* et *in vivo* (Köntgen et al., 1995; Ruan et al., 2011), tandis que la délétion de *Nfkb1* entraîne des défauts mineurs dans la différenciation TH2 (Das et al., 2001). L'ablation non spécifique de la voie alternative de NF- κ B utilisant des animaux mutants pour *NIK* suggère une implication de RelB ou de NF- κ B2 dans l'activation des cellules T CD4⁺ (Ishimaru et al., 2006).

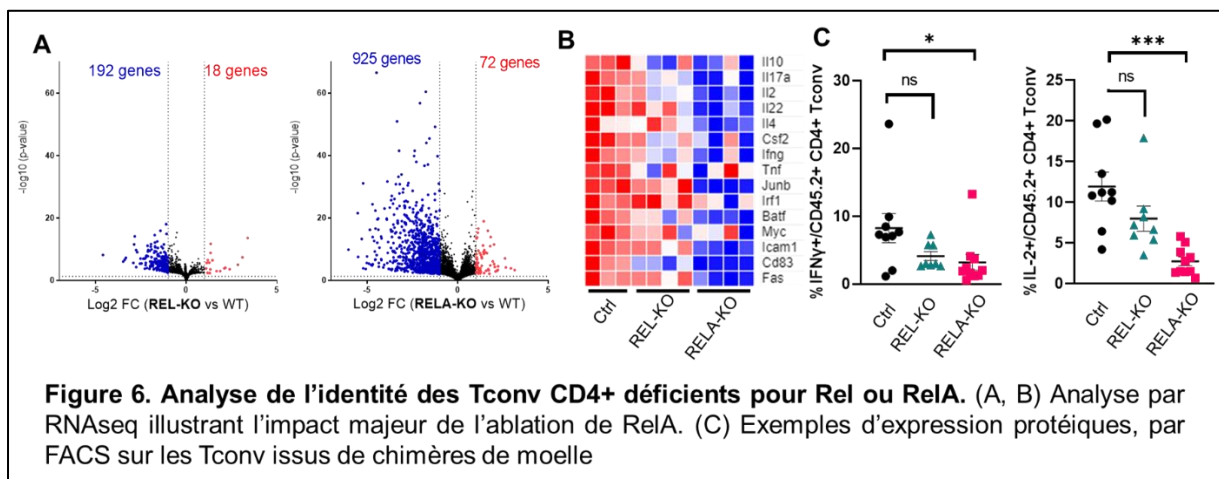
Chez l'homme, les patients porteurs de mutations conduisant à la perte d'expression de IKK β présentent une altération de l'activation des lymphocytes T et de leur expression d'IFN γ (Pannicke et al., 2013). Ces résultats ont été en partie récapitulés chez un patient porteur d'une mutation non-sens dans *REL* (Beaussant-Cohen et al., 2019). Une étude récente de screen par CRISPR/Cas9 suggère un rôle de REL (mais apparemment pas des autres SU) dans la prolifération des LT CD8 $^+$ humains (Shifrut et al., 2018). En revanche, les mutants *NFKB1* et *NFKB2* ne présentent pas de défauts majeurs dans les cellules T circulantes, confirmant les données obtenues chez la souris (Fliegauf et al., 2015; Klemann et al., 2019).

Ces résultats mettent en évidence la complexité de la fonction de NF- κ B dans l'activité des cellules T, et soulignent encore une fois l'absence de consensus sur la fonction spécifique de chaque SU. Nous posons ici l'hypothèse d'une « séparation des tâches » par les SU de NF- κ B dans l'orchestration de l'identité des LT. L'objectif général est d'obtenir une vision holistique récapitulant les différents processus biologiques contrôlés par chaque SU et dans les différents types de LT.

Approches expérimentales

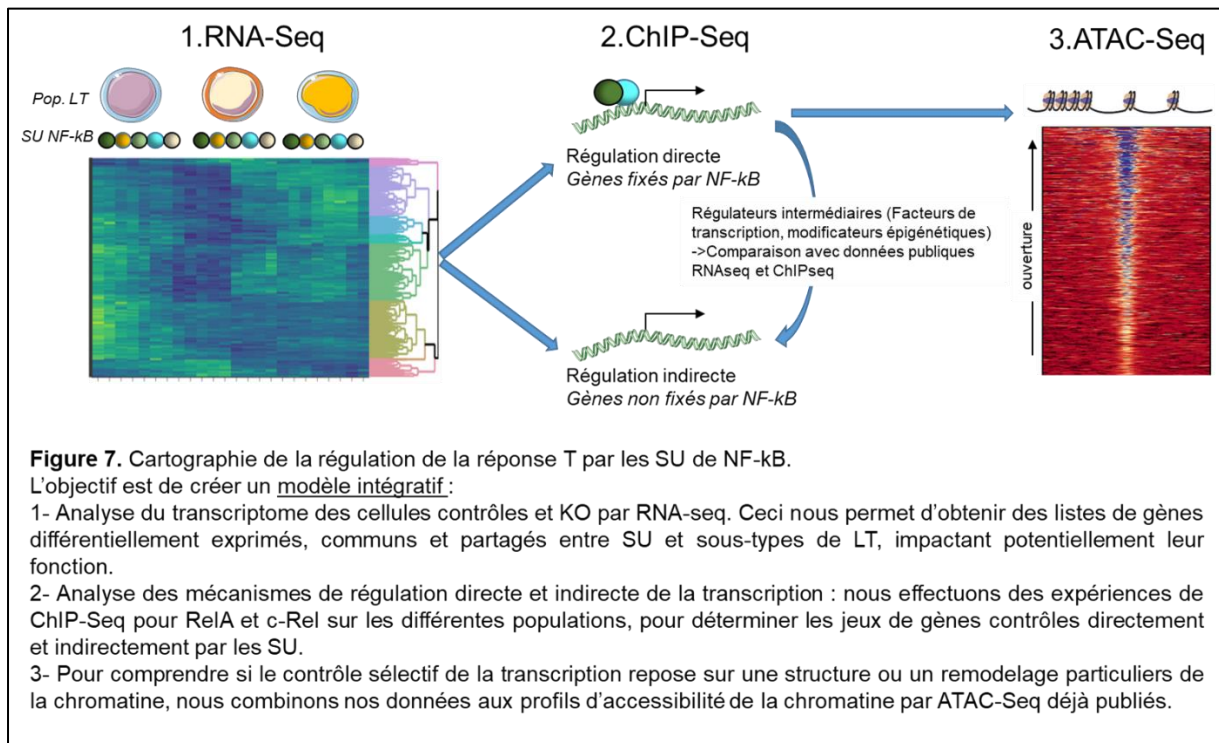
1-Examiner les rôles des SU de NF- κ B dans le développement, le transcriptome et la fonction Teff CD4 et CD8, chez la souris à l'état basal.

Nous utilisons pour cette tâche les souris CD4^{CRE} qui permettent une ablation de chacune des SU dans toutes les populations de LT. Pour investiguer les fonctions intrinsèques des SU dans chaque sous-population, nous utilisons un système de chimères de moelle mixte (transfert de moelle osseuse provenant de souris WT CD45.1⁺ combiné à une moelle KO ou contrôle CD45.2⁺ dans des souris immunodéficientes *RAG2*^{-/-} irradiées). Le phénotype (distribution, activation, expression de cytokines) est ensuite mesuré par cytométrie de flux (FACS). Les Tconv CD4 (CD4⁺CD25⁻) et CD8⁺ sont également triés par FACS et cultivés *in vitro* pour mesurer la capacité de prolifération des cellules.



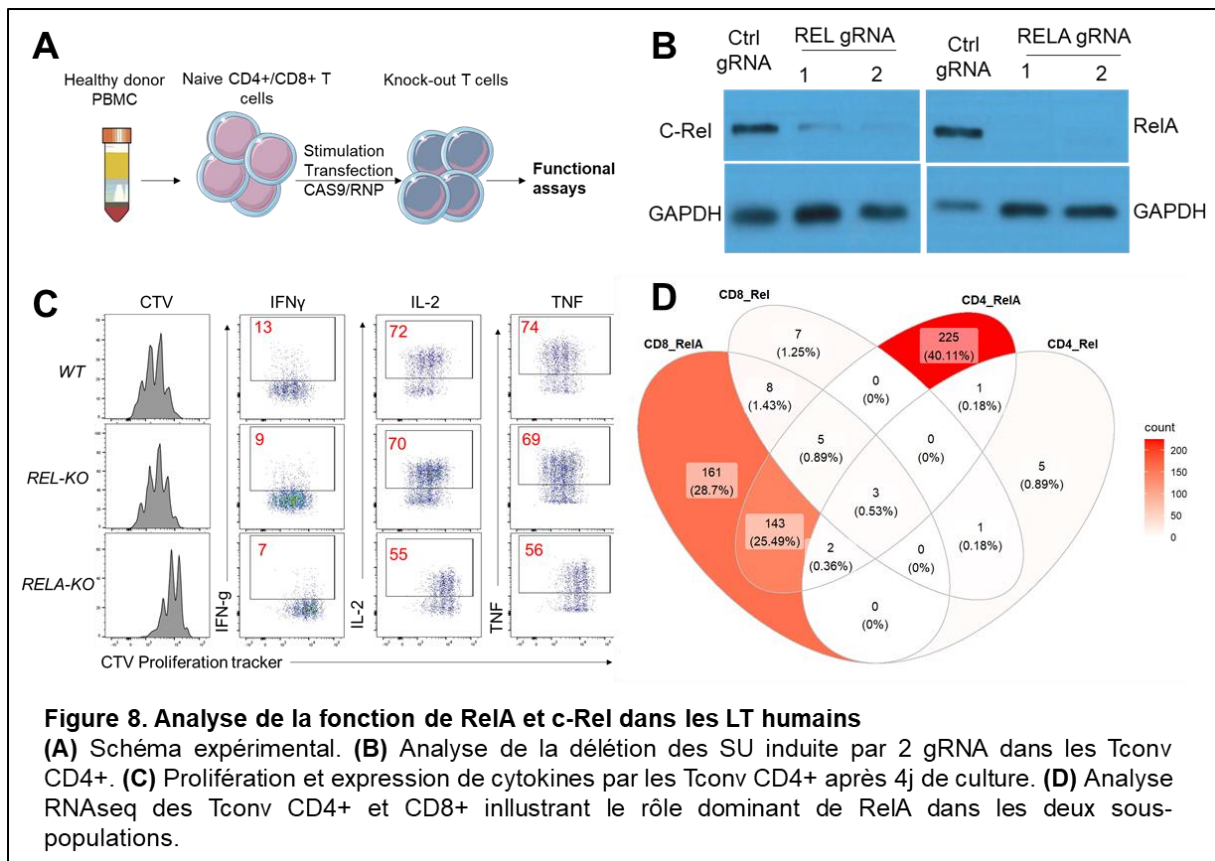
Puisque nous étudions des facteurs de transcription, il est de plus important de comprendre les programmes d'expression génique contrôlés par chaque SU. La première étape est d'obtenir des listes de gènes dont l'expression est modulée par l'absence d'une SU. De façon intéressante, nous avons déjà identifié un rôle prépondérant de RelA, en comparaison de c-Rel, dans les Teff CD4⁺. L'ablation de *Rela* conduit à un changement d'expression de nombreux gènes associés à la fonction

des Teff (récepteurs de surface et cytokines); ceci se traduit notamment par une baisse de l'expression de l'IFN- γ au niveau protéique (Figure 6). Cette analyse du transcriptome est ensuite complétée par des analyses de ChIP-Seq et ATAC-Seq, comme détaillé en figure 7. Au travers de ces différentes analyses, l'objectif est de créer une cartographie complète de la régulation de la biologie des LT par NF- κ B, et ainsi d'identifier des mécanismes fondamentaux des réponses immunitaires.



2-Déterminer la contribution des SU dans les Teff humains

Les rôles des différentes SU de NF- κ B dans les LT humains sont largement inconnus. En complément des analyses fondamentales chez la souris, nous développons des approches de knock-out de c-Rel et RelA dans les Teff primaires humains, via la technologie CRISPR-Cas9. Les LT CD4⁺ et CD8⁺ naïfs isolés du sang de donneurs sains, sont soumis à un protocole de transfection de complexes Cas9/RNP contrôles ou visant un exon de REL ou RELA. Cette méthode permet généralement d'atteindre un niveau de délétion >90%. Comme chez la souris, les cellules sont ensuite soumises à des expériences de culture cellulaire (prolifération, sécrétion de cytokines) ainsi qu'à une analyse du transcriptome par RNA-seq. Validant les données obtenues chez la souris, nos résultats préliminaires indiquent un rôle prédominant de RelA dans le maintien du transcriptome des Teff CD4⁺ et l'expression de cytokines telles que l'IFN γ ou l'IL-2 au niveau protéique, alors que l'effet de l'ablation de *REL* semble intermédiaire (Figure 8). Enfin, pour valider le rôle potentiel de RelA et/ou c-Rel *in vivo*, nous procéderons à des expériences de transfert de Teff CD4 ou CD8⁺ dans des souris lymphopéniques NOD.SCID.gammaC^{-/-} (NSG) (Ehx et al., 2018); le transfert de cellules contrôles induit une xéno-GVHD, c'est-à-dire une inflammation médiée par la xéno-réactivité des LT humains contre les complexes CMH/Peptide de souris. Nous suivrons les signes cliniques de maladie et évaluerons la reconstitution et le phénotype des Teff dans les tissus lymphoïdes et non-lymphoïdes.



2.2 NF- κ B dans la différenciation des LT CD4⁺ helper : implication dans la physiopathologie des maladies auto-immunes

Rationnel et hypothèse de travail

En parallèle de ces études à l'état basal, nous développons une série de projets visant à évaluer le rôle et la portée thérapeutique des SU de NF- κ B en situation pathologique.

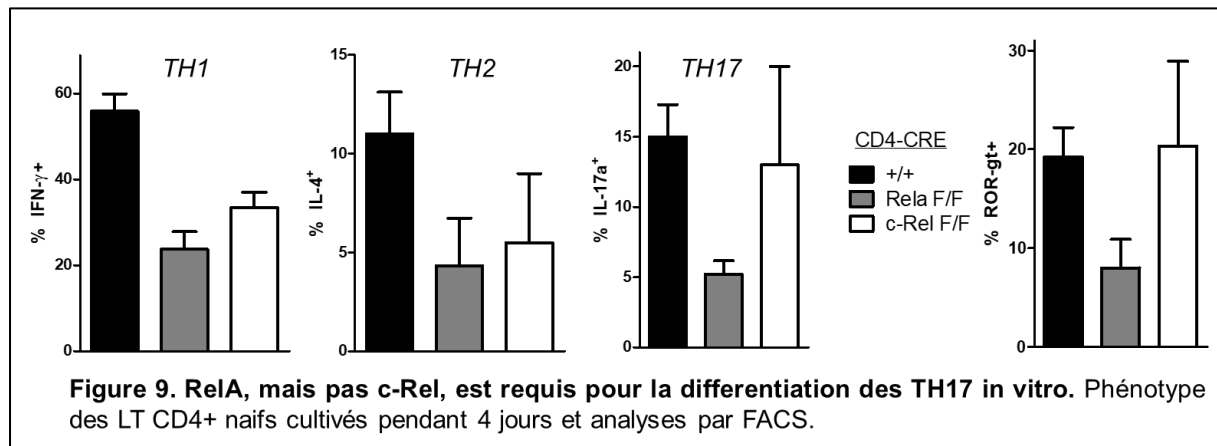
Les Tconv CD4⁺ naïfs peuvent être polarisés, en fonction de la composition du microenvironnement, en différents types de LT dits « helper » (TH1, 2, 9, 17, 22, ainsi que T follicular helper ou TFH et Treg induits ou iTreg) qui ont des fonctions sélectives en réponse à des agents pathogènes spécifiques. Néanmoins, les cellules TH peuvent également être pathogènes en l'absence de mécanismes de rétrocontrôle appropriés. Un certain nombre de maladies auto-immunes sont médiées par différents TH, suite à une rupture centrale ou périphérique de la tolérance. En particulier, les cellules TH17 semblent essentielles dans la pathogenèse de la sclérose en plaques (SEP et son modèle murin, encéphalomyélite auto-immune expérimentale, EAE) (Rostami and Ciric, 2013). NF- κ B est souvent activé de manière constitutive dans les tissus exposés à une inflammation chronique, notamment le cerveau des patients SEP (Rostami and Ciric, 2013). Si c-Rel a été suggéré comme un acteur majeur de la différenciation des TH17 et la physiopathologie de l'EAE (Hilliard et al., 2002), le rôle intrinsèque des autres SU est mal compris, avec notamment des données largement sur les rôles de NF- κ B2 ou RelB (Xiao et al., 2016; Yu et al., 2014).

Grâce à nos modèles de souris, nous avons la **possibilité d'investiguer ces rôles potentiels afin de mettre en évidence de possibles cibles thérapeutiques dans l'auto-immunité.**

Approches expérimentales

1-Mesurer la fonction des SU de NF- κ B dans la polarisation en sous-types de TH *in vitro*

Nous utilisons ici des protocoles établis de polarisation *in vitro* à partir de Tconv naifs isolés des souris CD4^{cre}Rela/Rel/Relb et Nfkb2^{flox}. Nos données suggèrent un rôle majeur de la SU RelA dans l'expression de ROR γ t, le facteur de transcription majeur du lignage TH17, et de l'IL-17A (Figure 9). A l'inverse, et contrairement à une série de papiers utilisant la souris *Rel*^{-/-}, il semble que c-Rel ne soit pas requis intrinsèquement pour la différenciation TH17; nous allons maintenant confirmer ces données en utilisant différents protocoles de stimulation (par exemple anticorps coatés vs feeders, dose-réponses de TGF β , TH17 induits par IL-6, IL-1 β et IL-23 indépendamment du TGF β etc...). Ces données, additionnées à celles obtenues à l'état basal chez la souris et l'homme (figures 6 et 8), nous ont conduits à nous concentrer, dans un premier temps, sur l'étude de RelA dans les contextes pathologiques décrits ci-après.



Sur le plan mécanistique, nous explorons deux axes :

-NF- κ B est-il requis pour l'expression de cytokines TH17 en elle-même ? Les TH17 déficients pour RelA montrent à la fois une baisse de ROR γ t-qui dirige l'expression de l'IL-17 - et de l'IL-17A elle-même. Pour savoir si RelA est directement requis pour l'expression de l'IL-17A et autres cytokines, nous utilisons un système de surexpression de ROR γ t dans les Teff *Rela*^{-/-}, par vecteur rétroviral (Ivanov et al., 2006).

-NF- κ B est-il requis pour la stabilité des TH17 différenciés ? Nous utilisons un modèle de délétion inducible de RelA induite par le tamoxifène (CD4^{cre-ERT2}Rela^{flox}) pour étudier sa fonction potentielle dans la stabilité des TH17 *in vitro*, une fois polarisés.

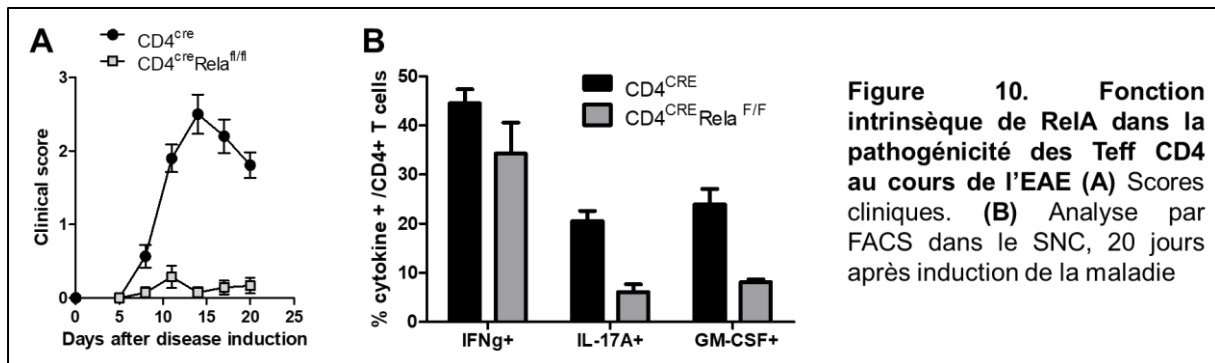
Enfin, nous utiliserons nos Tconv CD4⁺ humains CRISPR-Cas9 pour confirmer le rôle des SU les plus prometteuses chez l'homme.

2-Examiner la fonction intrinsèque des SU de NF- κ B dans la physiopathologie de l'EAE et de la SEP.

Pour modéliser la fonction TH17 *in vivo*, nous utilisons un modèle d'EAE induite par immunisation avec le peptide MOG₃₃₋₅₅, qui conduit à une inflammation du système nerveux central (SNC) médiée par des TH17 pathogéniques sécrétant de GM-CSF. Confirmant nos données *in vitro*, nous avons observé une absence quasi-complète de symptômes chez les souris CD4^{cre}Rela^{flox}, associée à une forte baisse de l'infiltration

dans le SNC et à une perte drastique de l'expression de ROR γ t, IL-17A et GM-CSF (Figure 10). Nous allons maintenant reproduire ces expériences dans le fond CD4^{cre}Rel^{flox}.

D'autre part, nous avons observé une baisse significative des symptômes cliniques chez les souris CD4^{cre}Relb^{flox} (mais néanmoins beaucoup moins forte que chez les souris CD4^{cre}Rela^{flox}), probablement due à une baisse spécifique de l'expression du GM-CSF par les Teff du SNC sans altération de l'expression de ROR γ t ou de l'IL-17A, révélant une facette délétère de l'expression de RelB par les Teff, jusque-là inconnue ; ce travail est en cours de soumission (manuscrit présenté en annexe).



Afin d'explorer les mécanismes pathogéniques médiés par RelA, nous allons également effectuer des expériences de cinétique d'ablation de cette SU. Pour ce faire, l'ablation de RelA sera induite par administration de tamoxifène à des souris CD4^{cre-ERT2}Rela^{flox}, soit avant l'induction de la maladie (pour étudier le rôle de RelA dans le priming des Teff), soit après l'apparition des signes cliniques (rôle dans la sévérité de la maladie). Ces données nous informeront non seulement du mécanisme cellulaire impliquant RelA, mais aussi du timing envisageable pour une intervention thérapeutique par des inhibiteurs spécifiques de RelA (par exemple, traitement de fond avant la poussée de SEP ou traitement 'curatif' pendant les poussées).

A plus long terme, nous prévoyons également d'utiliser des cellules du liquide céphalo-rachidien de patients atteints de SEP progressive. Les cellules seront traitées avec un inhibiteur spécifique de RelA humain récemment décrit (Kanzaki et al., 2021), et l'expression de cytokines inflammatoires sera mesurée par FACS et ELISA.

2.3 La voie canonique de NF- κ B dans la fonction des LT effecteurs CD4⁺ et CD8⁺ : immunité anti-tumorale et réponse aux thérapies par inhibiteurs de checkpoint.

Rationnel et hypothèse de travail

Une abondante littérature a définitivement établi le lien direct entre la qualité de la réponse immunitaire et la croissance tumorale ; ceci a conduit au développement des thérapies par inhibiteurs de checkpoint, ciblant CTLA-4 et PD-1, qui ont révolutionné la prise en charge de certains cancers.

Nous en savons beaucoup sur la nature, le phénotype, la fonction ou la spécificité des cellules T impliquées dans l'immunité anti-tumorale. En revanche, les différentes voies de signalisation et facteurs de transcription impliqués dans la fonction anti-tumorale des Teff, en particulier dans le cadre de la réponse/résistance aux inhibiteurs de

checkpoint, sont mal comprises. Les différentes données indiquant un rôle de NF- κ B dans les Teff à l'état basal, suggèrent que cette voie pourrait être également impliquée dans la réponse au cancer. Confirmant cette hypothèse, l'ablation de *IKK β* -qui empêche l'activation des SU canoniques- dans les LT augmente la croissance de fibrosarcomes et mélanomes transplantés chez la souris ; à l'inverse l'activation constitutive de *IKK β* permet une baisse de la croissance tumorale chez la souris (Barnes et al., 2015; Evaristo et al., 2016). Dans le même ordre d'idée, l'ablation de A20 (*Tnfrsf25*), un puissant inhibiteur de NF- κ B, dans des LT transgéniques spécifiques d'un antigène tumoral, potentialise leur fonction anti-cancéreuse (Giordano et al., 2014). Une étude récente suggère que les dimères p50/p50 inhibent l'expression du Granzyme B par les Teff CD8⁺ ; en conséquence la pousse tumorale est réduite chez les souris *Nfkb1*^{-/-}, bien que le mécanisme sous-jacent ait été incorrectement démontré (Lu et al., 2020).

Il est également notable qu'un défaut d'activation de RelA a été décrite dans les LT du sang périphérique chez des patients atteints de cancer du rein (Li et al., 1994). D'autre part l'engagement du récepteur PD-1 inhibe le « signalosome » du TCR via le recrutement des phosphatases SHP-1/2, suggérant un effet sur l'activation de NF- κ B (Arasanz et al., 2017). L'efficacité de la thérapie par anti-PD1 chez la souris requiert une signalisation intacte par CD28, qui active NF- κ B parmi d'autres voies, et l'expression de l'IFN γ , un gène largement dépendant de NF- κ B dans les LT (Kamphorst et al., 2017; Shi et al., 2016). Enfin, des études transcriptomiques ont fait ressortir un enrichissement massif de gènes dépendants de NF- κ B chez les patients atteints de mélanome ou cancer du poumon métastatique et répondant à l'immunothérapie (Lapiente-Santana et al., 2021; Sade-Feldman et al., 2018; Voabil et al., 2021).

En se basant sur ces observations, **nous émettons l'hypothèse que certaines SU de NF- κ B sont requises pour l'activité anti-tumorale des Teff CD4⁺ et CD8⁺ et que leur activité est requise pour la réponse aux thérapies par inhibiteur de checkpoint.** Nous nous concentrons en particulier sur RelA et c-Rel qui ont un rôle prédominant dans la fonction des Teff à l'état basal.

Approches expérimentales

1- Activation de NF- κ B dans les TILs et impact sur la progression du mélanome chez l'homme. Si l'activation de NF- κ B (surtout RelA) est bien décrite dans les cellules tumorales elles-mêmes, son impact dans le microenvironnement immunitaire est inconnu. Nous avons établi une collaboration avec le Pr. Stéphane Dalle, chef du service d'onco-dermatologie à l'hôpital Lyon-Sud et chercheur au CRCL, afin d'obtenir des échantillons frais et fixés de mélanome avant traitement. Nous utilisons deux approches pour mesurer l'activation de RelA et c-Rel dans les TILs :

-Des marquages FACS sur des biopsies fraîches de mélanome, permettant de mesurer la phosphorylation de RelA dans les sous-types de LT après une restimulation ex-vivo.

-Pour tester cette hypothèse sur une cohorte rétrospective entière, nous développons une approche de marquages par immunofluorescence multiparamétrique sur tissus fixés. Nous rencontrons actuellement des problèmes techniques dus à la spécificité et la sensibilité des anticorps anti-RelA et c-Rel sur lames entières, mais nous espérons à terme obtenir des co-marquages CD4/CD8/Foxp3/RelA/c-Rel/DAPI afin de quantifier la translocation nucléaire

des SU dans chaque sous-population. Ces données seront ensuite confrontées aux données cliniques des patients.

-D'autre part, nos données de transcriptomique (projet 1) seront utilisées pour isoler des signatures de gènes dépendants des SU de NF- κ B, et comparées à des données publiques :

-La base de donnée du TCGA, qui regroupe des données de RNA-seq bulk, sera utilisée pour déterminer les corrélations possibles entre signatures Teff/NF- κ B, la survie globale et la réponse aux traitements.

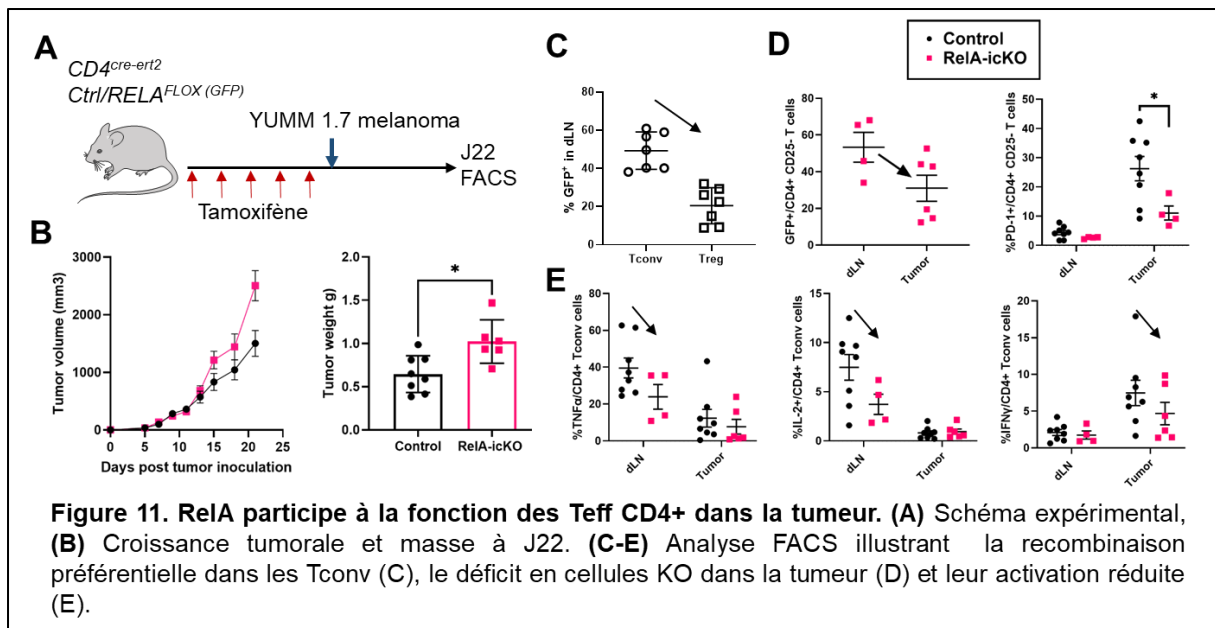
-l'impact de ces signatures sera aussi mesurée aux multiples jeux de données single-cell RNA-seq réalisés sur les LT infiltrant les tumeurs avant et après traitement anti-PD-1, définissant les profils des sous-populations de LT en fonction du statut de réponse/résistance à l'immunothérapie.

-Enfin, nous posons l'hypothèse que l'engagement de récepteurs inhibiteurs tels que PD-1, est une cause sous-jacente à l'absence d'activation de NF- κ B dans les Teff de la tumeur. Nous testons cette hypothèse par tests de biochimie (Western Blot, EMSA) et FACS (phospo-RelA) sur des Teff de donneurs sains, traités avec une molécule engageant PD-1 (PD-L1-Fc) ou un anticorps bloquant PD-1.

2- Rôle des SU de NF- κ B dans les Teff de la tumeur et la réponse aux anti-PD-1 chez la souris.

Nous pouvons également directement tester la fonction intrinsèque des SU de NF- κ B dans les LT, dans le contexte du cancer, en utilisant nos souris KO conditionnelles. Pour interroger le rôle différentiel de NF- κ B dans les Teff CD4⁺ et CD8⁺, deux souches de souris différentes sont utilisées: la lignée CD4^{cre-ERT2} et la lignée E81^{cre} (CD8^{cre}), respectivement. Les souris sont transplantées avec des cellules tumorales : dans un premier temps, l'adénocarcinome de colon MC-38, bien décrit pour son immunogénicité ; puis une lignée de mélanome BRAF600E obtenue via notre collaboratrice Julie Caramel au CRCL; les souris sont traitées avec un anticorps bloquant anti-PD-1 ou son isotype contrôle dès que la tumeur est détectable.

-L'administration de tamoxifène aux souris CD4^{cre-ERT2} permet de déléter de façon inducible l'allèle floxé spécifiquement dans les cellules CD4⁺ de la périphérie (Aghajani et al., 2012). Nous avons même observé que la délétion touche préférentiellement les Tconv en comparaison des Treg, peut-être du fait de leur expression de CD4 légèrement plus haute (Figure 11); ce modèle permet donc d'étudier quasi-spécifiquement l'ablation de NF- κ B dans les Tconv. Nos données récentes montrent que l'ablation de RelA semble permettre l'accélération de la croissance des MC38, associée à des niveaux d'activation moindres dans les ganglions drainants et à une accumulation réduite de Teff dans la tumeur (Figure 11). Nous allons maintenant analyser en détail le phénotype des Teff par FACS et RNA-seq. Pour valider ces données, nous utilisons d'autre part un modèle de transfert adoptif, dans lequel les Tconv contrôle ou KO sont transférés avec des Treg, des LT CD8 et des lymphocytes B contrôle. De façon notable, la réponse à la thérapie anti-PD-1 n'est pas impactée par l'absence des SU de NF- κ B dans les Tconv (donnée non montrée). Ceci suggère (i) soit que l'activation de NF- κ B dans les CD4 n'est pas requise pour la revigoration de la réponse anti-tumorale, (ii) soit qu'il existe une forte redondance entre les 2 SU RelA et c-Rel. Pour élucider ce point, nous créons actuellement des souris



double KO RelA/c-Rel. D'autre part, nous testons le rôle de RelA et c-Rel dans d'autres immunothérapies (blocage de CTLA-4 et activation d'OX-40).

-Pour étudier la fonction intrinsèque de NF- κ B dans les LT CD8⁺ matures, nous utilisons une lignée E81^{cre} (CD8^{cre}) (Maekawa et al., 2008). E81 est un *enhancer* de *Cd8a* actif uniquement dans les LT CD8⁺ matures de la périphérie, et n'induit pas de délétion dans les autres cellules du système immunitaire à l'état basal. Nous venons d'établir cette lignée et n'avons pas encore obtenu de résultats préliminaires. Les modèles tumoraux et méthodes d'analyse seront les mêmes que pour les Teff CD4⁺.

3- Cibler des récepteurs de surface capables d'activer des SU spécifiques pour restaurer l'activité des Teff CD8⁺ dans la tumeur.

Notre hypothèse principale est que l'activité défectueuse de NF- κ B dans les Teff de la tumeur est une cause sous-jacente de leur fonction diminuée. Dans cet axe nous explorons donc la capacité de différents agonistes des récepteurs des familles TNFRSF et CD28, à activer un pattern spécifique de SU de NF- κ B. La plupart de ces molécules sont déjà testées en clinique, et notre objectif est d'apporter un rationnel mécanistique à leur utilisation en immunothérapie. Nous allons mesurer l'engagement spécifique des SU de NF- κ B par EMSA+supershift sur des Teff CD8⁺ de donneurs sains traités *in vitro* avec des agonistes de GITR, 4-1BB, OX-40 et ICOS. Les candidats les plus prometteurs seront ensuite testés *in vivo*, dans nos souris KO conditionnelles (CD8^{cre}) porteuses de tumeurs MC-38.

3. Fonction intrinsèque des SU de NF- κ B dans les cellules de mélanome : rôle dans l'établissement du microenvironnement immunitaire (Travaux menés par Julie Twardowski, ingénieure d'études)

Rationnel et hypothèse de travail

Au-delà de ses rôles dans les réponses T anti-tumorales, NF- κ B a été très largement impliqué dans l'oncogenèse et la progression métastatique. Une activation constitutive de RelA a été détectée dans une variété de tumeurs solides, y compris le mélanome (Ueda and Richmond, 2006). Fait intéressant, les niveaux de protéines RelA, p50 et c-

Rel sont significativement plus élevés dans les mélanomes humains que dans les mélanocytes normaux (McNulty et al., 2001), alors que l'expression de RelB et p52 est augmentée dans les stades métastatiques du mélanome uvéal (Singh et al., 2019). Chez la souris, l'ablation de *IKK β* inhibe le développement du mélanome induit par H-RAS, en favorisant l'apoptose (Yang et al., 2010). *In vitro*, le knock-down de c-Rel, ou l'addition de pentoxifylline, altère le cycle cellulaire dans des lignées cellulaires de mélanome humain et murin, respectivement (Dua et al., 2007; Priebe et al., 2019). L'activité de NF- κ B2 semble inhiber la sénescence du mélanome humain, et son inhibition diminue la pousse tumorale après transfert à des souris immunodéficientes (De Donatis et al., 2016).

De plus, NF- κ B semble impliqué dans la formation du microenvironnement tumoral suppresseur dans le mélanome. Par exemple, il est clair que l'expression de PD-L1 dans les cellules de mélanome dépend de NF- κ B, à la fois au niveau transcriptionnel et en stabilisant la protéine (Gowrishankar et al., 2015; Grinberg-Bleyer and Ghosh, 2016). NF- κ B participe également au « sécrétome » des cellules tumorales, via l'expression de cytokines inflammatoires supportant la transformation, et de chimiokines permettant le recrutement de cellules immunitaires sur le site tumoral (Capece et al., 2018). L'ablation de RelA dans le mélanome murin, apparaît sensibiliser les cellules tumorales à l'action cytotoxique des LT *in vitro* (Lawson et al., 2020), peut-être via une apoptose induite par le TNF (Vredevoogd et al., 2019). Enfin, l'ablation d'intermédiaires de la voie NF- κ B comme *Traf3*, *Traf6* ou *Ripk1*, sensibilise les mélanomes de souris B16F10 à la thérapie par anti-PD1 (Manguso et al., 2017).

A nouveau, l'implication de chaque SU de NF- κ B est très mal comprise dans ces mécanismes. **Nous émettons ici l'hypothèse que NF- κ B, via ses différentes SU, participe à l'établissement du microenvironnement immunitaire dans le mélanome.** De telles informations semblent indispensables au développement de thérapies du cancer basées sur l'inhibition ou l'activation spécifique de SU de NF- κ B.

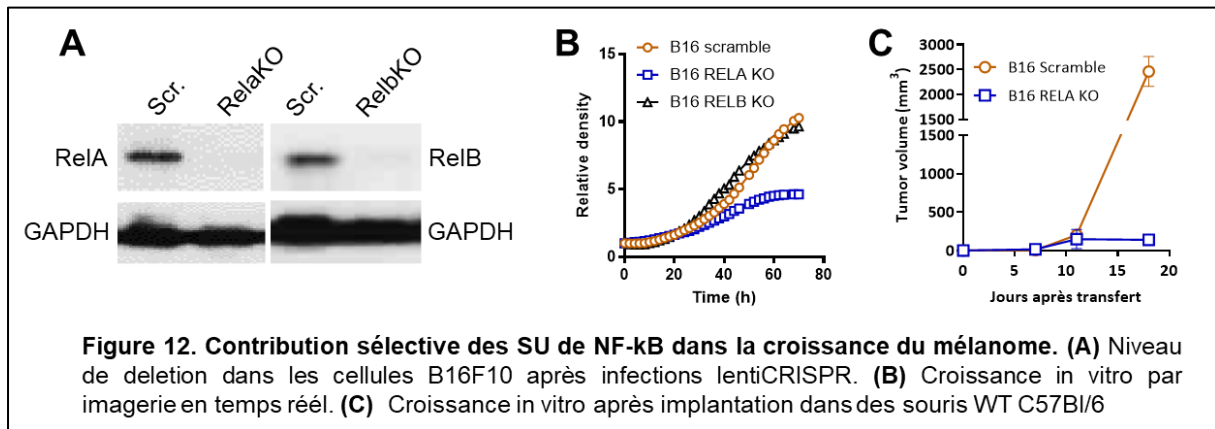
Approches expérimentales

Pour interroger systématiquement le rôle de chaque SU, nous utilisons une stratégie d'ablation des gènes par infection avec des lentivirus Cas9/gRNA dans une lignée de mélanome de souris B16F10, qui est naturellement résistante à l'immunothérapie par anti-PD-1. Nous avons d'ores et déjà créé des lignées déficientes pour *Rela* et *Relb* ; nous procédons désormais au clonage de ces cellules pour s'assurer de l'ablation complète et spécifique des gènes. De plus la contribution des SU les plus intéressantes sera confirmée dans un système de 'rescue' par surexpression de la version humaine de chaque gène, qui ne sera pas touchée par le CRISPR et permettra de s'assurer que les phénotypes ne reposent pas sur un effet « off-target » dû à l'expression constitutive de la Cas9 par les cellules.

-Nous mesurons tout d'abord les propriétés de prolifération et survie *in vitro* sous différents conditions (normales, réduction du taux de sérum, addition de TNF), par « live imaging » (microscope Incucyte). Dans une série d'expériences préliminaires, nous avons observé une diminution de la pousse des cellules déficientes pour *Rela* mais pas pour *Relb* en condition 'normales' (Figure 12), suggérant une première ligne de spécificité fonctionnelle des SU.

-Les cellules sont ensuite transférées par voie sous-cutanée dans des souris contrôles (WT). La pousse tumorale est mesurée et le microenvironnement tumoral est évalué par cytométrie de flux multiparamétrique (distribution, phénotype et expression de

cytokines par les cellules myéloïdes et lymphoïdes). Nous avons observé une forte diminution de la croissance des cellules *Rela*-KO en comparaison du contrôle (Figure 12C) et allons maintenant détailler l'infiltrat immunitaire. D'autre part nous mesurerons l'effet de l'ablation des SU sur la réponse à l'anti-PD-1.



-Pour évaluer le rôle du système immunitaire dans ces phénotypes, nous transférerons des cellules contrôles et KO dans des souris *RAG2*^{-/-} (dépourvues de LT et LB) ou des souris NSG (qui ont en plus de profonds défauts en cellules myéloïdes), en fonction des résultats de cytométrie obtenus ci-dessus.

-Pour comprendre les mécanismes précis conduisant à la modulation de la pousse tumorale, nous prévoyons d'effectuer une analyse du transcriptome des cellules tumorales *in vivo* par RNA-seq, après isolation par tri FACS. Ceci pourrait nous permettre d'identifier les gènes cibles de NF-κB possiblement impliqués dans le phénotype. La modulation des gènes les plus pertinents sera confirmée au niveau protéique par FACS, Western Blot ou ELISA. Enfin la fonction du (des) candidat(s) le(s) plus prometteur(s) sera confirmée par surexpression ou knock-out.

-Enfin, la valeur translationnelle de ces données sera validée dans des cellules de mélanome humaines (lignée WM115, *BRAF*^{V600E}), dans le cadre d'expériences *in vitro* comme décrit ci-dessus.

4. Analyse du microenvironnement immunitaire dans les lymphomes T périphériques nodaux : impact pronostic et ciblage thérapeutique (Travaux menés par Pierre Stéphan, doctorant)

Rationnel et hypothèse de travail

En complément de ces travaux fondamentaux sur la régulation transcriptionnelle de l'immunité anti-tumorale, j'ai souhaité mettre en place un projet de recherche pouvant avoir un impact thérapeutique plus direct. De très nombreux travaux des dernières années ont documenté le lien fort entre la distribution et le phénotype des cellules immunitaires sur le site tumoral, et la progression du cancer et la réponse aux thérapies. En particulier, l'expression de récepteurs de surface activateurs ou inhibiteurs, témoignant de l'état d'activité ou d'épuisement des LT, sont souvent corrélés au contrôle et à l'échappement des tumeurs solides. Cette observation a

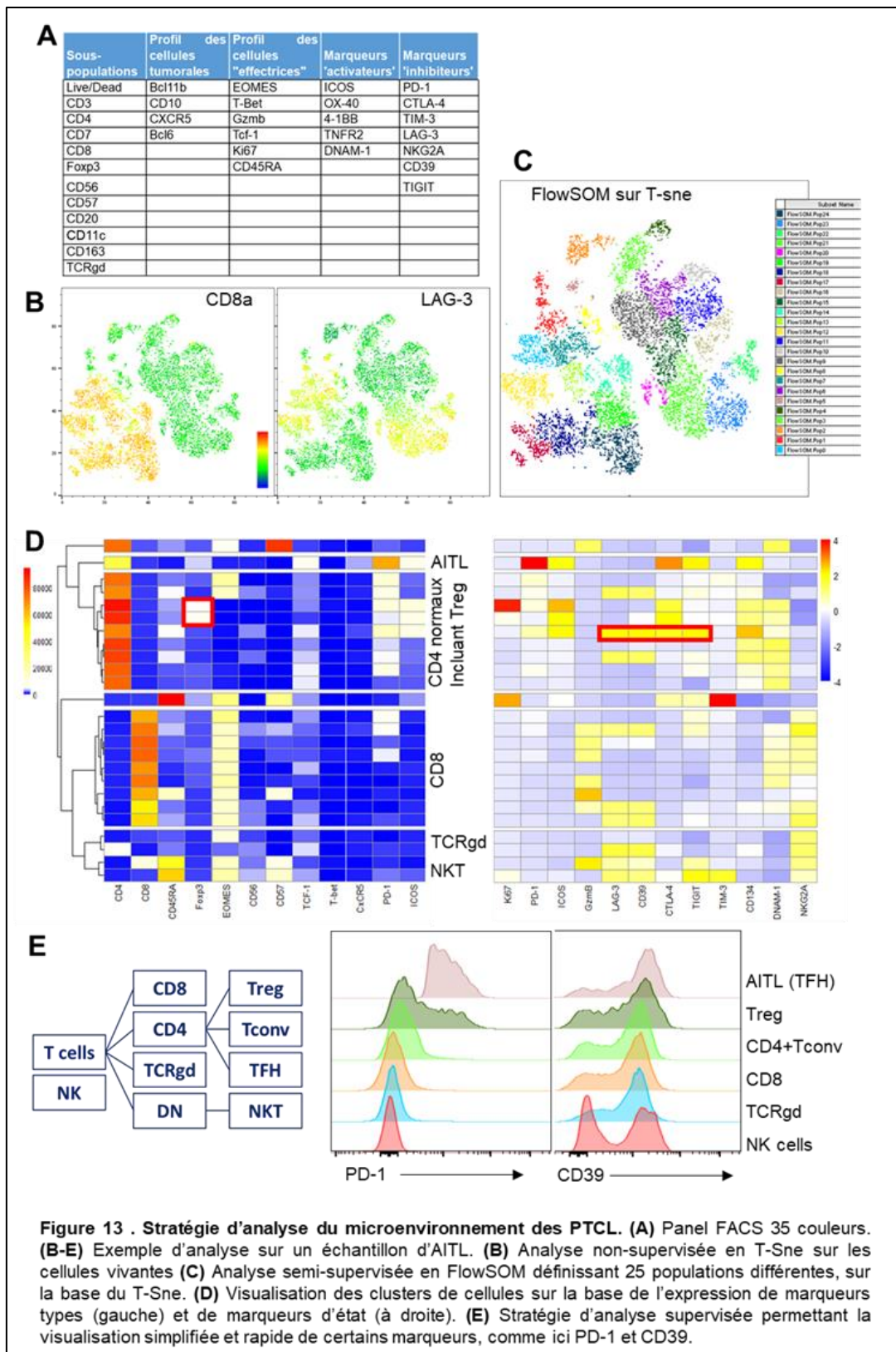
conduit à une myriade d'essais pré-cliniques et cliniques visant à cibler ces récepteurs de surface (à commencer par PD-1 et CTLA-4).

En revanche, la relation entre le microenvironnement immunitaire « sain » et la progression des lymphomes T est encore très peu étudiée. Les lymphomes T périphériques (PTCL) sont des néoplasmes dérivés de LT ou NK matures. Ils représentent 10 à 15% des lymphomes non hodgkiniens (1997). Il s'agit de maladies très hétérogènes en terme de cellules d'origine, de mutations et pronostics. Les lymphomes T nodaux sont les plus fréquents. Ils regroupent :

- Les PTCL angio-immunoblastiques (AITL), les PTCL folliculaires, et les PTCL nodaux avec phénotype folliculaire, qui ont tous les 3 pour origine la cellule CD4⁺ T helper folliculaire (TFH),
- Les PTCL anaplasiques à grandes cellules (ALCL), constitués de cellules T blastoïdes CD30⁺, exprimant ou non la protéine de fusion ALK.
- Les PTCL sans autre spécificité (PTCL-NOS, les plus fréquents), catégorie très hétérogène. Les cellules tumorales sont généralement CD3⁺ et peuvent être CD4⁺ ou CD8⁺ ou avoir perdu l'expression de ces 2 marqueurs.

La polychimiothérapie est utilisée en première ligne dans la majorité des PTCL mais les résultats sont limités. En cas de rechute, de hautes doses de chimiothérapie avec greffe de cellules souches hématopoïétique autologue ou allo-génique peut être envisagée. Cependant il s'agit de thérapies lourdes avec des complications importantes. A l'heure actuelle seule une stratégie d'immunothérapie présente des résultats intéressants : le Brentuximab vedotin (anticorps Anti-CD30 couplé monométhylauristatin E) (Horwitz et al., 2019). D'autres immunothérapies, tel que l'Alemtuzumab (anti-CD52) ont été testées mais sans résultats probant (Wulf et al., 2021).

La nécessité de mieux caractériser les PTCL nodaux est évidente afin de trouver des nouveaux facteurs pronostics et prédictifs de réponse au traitement ainsi que de définir de nouvelles cibles et de nouveaux médicaments améliorant la prise en charge thérapeutique. Une accumulation de Treg a été observée dans les PTCL, mais leur valeur pronostique est débattue (Lundberg et al., 2019). L'accumulation de macrophages 'suppresseurs' semble être de mauvais pronostic dans les PTCL-NOS (Sugio et al., 2018). La suppression de la réponse anti-tumorale pourrait également reposer sur l'expression de cytokines ou de récepteurs inhibiteurs par les cellules malignes ; ainsi les cellules tumorales de PTCL-NOS et AITL expriment généralement PD-L1, suggérant un mécanisme actif de suppression des T_H1 et cellules NK (Wilcox et al., 2009). A l'inverse la forte expression de PD-1 par les cellules tumorales semble freiner leur expansion (Wartewig and Ruland, 2019). En accord avec cette hypothèse, un récent essai clinique visant à traiter des patients atteints d'AITL et de PTCL-NOS par anti-PD-1 a dû être arrêté à la suite d'hyper-progressions (Neuwelt et al., 2020). Le domaine de la thérapie par stimulation ou inhibition des checkpoint immunitaires reste donc très peu exploré. De plus, à ce jour, aucune étude ne s'est directement intéressée au phénotype différentiel détaillé des cellules malignes et normales dans les PTCL nodaux. **Dans ce contexte, il semble crucial d'obtenir une cartographie à large spectre, afin de révéler un potentiel impact du système immunitaire sur la progression de la maladie, et pour mettre en évidence de nouvelles cibles thérapeutiques d'immuno-modulation.**



Approches expérimentales

- Etude de la distribution et du phénotype des LT et cellules NK dans le microenvironnement des PTCL nodaux. Cet axe vise à documenter le phénotype des cellules normales et malignes dans les ganglions envahis de PTCL. Nous avons établi des collaborations avec l'équipe de Laurent Genestier à l'hôpital Lyon-Sud, avec

l'Institut Carnot CALYM dédié aux lymphomes, pour obtenir des échantillons en suspensions d'une vingtaine de patients au diagnostic. Nous proposons une analyse à haut débit, par cytométrie spectrale, pour analyser 35 différents marqueurs de lymphocytes T et NK. Cette technologie permet une analyse multi-paramétrique sur un nombre réduit de cellules, et avec une résolution globalement plus fine que la cytométrie de masse (CytOF) (Figure 13). Nous procédons à des analyses non-supervisées et supervisées avec deux objectifs principaux :

- Associer des phénotypes détaillés de cellules immunitaires saines avec des types de PTCL précis (AITL, PTCL-NOS) voire de raffiner les PTCL-NOS en sous-classe en fonction de leur phénotype ou de celui du micro-environnement.

- Identifier un ou plusieurs marqueurs d'activation membranaires, exprimé soit par les cellules « pathogènes (tumoraux et/ou Treg) mais pas par les cellules saines à potentiel anti-tumoral (CD8, NK...), soit l'inverse, constituant ainsi une banque de cibles possibles d'immuno-modulation. Comme contrôles, 5 échantillons d'amygdales sont utilisés.

-Validation des marqueurs potentiels in situ et étude de leur valeur pronostique à large échelle. Afin d'étudier la valeur pronostique des marqueurs d'activation identifiés ci-dessus, il est nécessaire d'étudier leur niveau d'expression ainsi que leur impact, sur une cohorte rétrospective à large échelle. Nous utiliserons ici des techniques d'immunofluorescence multiparamétrique de dernière génération sur des échantillons fixés au formol et inclus en paraffine (FFPE). Nous utiliserons deux lames de Tissue Micro-Array (TMA), regroupant au total 60 échantillons de patients de ganglions de patients atteints d'AITL ou de PTCL-NOS, obtenus via notre collaboratrice le Professeur Alexandra Traverse-Glehen, chef du service d'anatomopathologie du CHU Lyon-Sud. La distribution et le nombre des cellules immunitaires, ainsi que leur expression des marqueurs d'intérêt, sera ensuite comparée avec les données cliniques (survie, réponse au traitement) anatomopathologiques, et génétiques (quand disponible). Nous effectuerons une analyse multivariée, en collaboration avec les biostatisticiens de la plateforme Gilles Thomas au CRCL, pour déterminer les potentielles corrélations entre expression des marqueurs d'intérêt, réponse aux traitements et survie.

-Validation fonctionnelle des cibles d'intérêt. Si nous réussissons à mettre en évidence un phénotype associé à un type de lymphome et/ou à la survie des patients, il sera ensuite important de disséquer la pertinence de ces observations en terme de fonction anti-tumorale. Pour ce faire nous avons établi système de co-culture *in vitro*. Des cellules de patients et une lignée de lymphome T humain marquée au CFSE, seront stimulées en présence d'anticorps agonistes ou antagonistes commerciaux. Après 24h le profil d'activation des LT et la proportion de cellules tumorales CFSE⁺ vivantes et apoptotiques seront mesurés. Ceci nous permettra fournir un rationnel pré-clinique au ciblage de ces récepteurs

5. Discussion générale

L'objectif des travaux décrits ci-dessus est d'impacter à la fois la recherche fondamentale en immunologie et le développement de nouvelles immunothérapies. Spécifiquement, nos travaux pourraient permettre :

-De mettre en évidence des mécanismes fondamentaux régissant l'activité des LT, permettant d'attribuer des rôles sélectifs aux SU de NF- κ B dans les processus d'immunité et de tolérance. Globalement, nous souhaitons participer à l'évolution du dogme considérant NF- κ B comme une entité unique, vers une vision plus détaillée considérant les fonctions uniques et partagées, des différentes SU. A terme, l'objectif serait bien entendu de cibler l'activité spécifique de certaines SU-ou dimères- pour moduler les réponses T dans le contexte de l'auto-immunité et du cancer.

-D'améliorer la compréhension des mécanismes régissant la réponse/résistance aux immunothérapies par inhibiteurs de checkpoint dans les tumeurs solides, à la fois dans les LT et dans les tumeurs.

-De mieux définir le profil de la réponse immunitaire dans les lymphomes T et de mettre en évidence des vulnérabilités (expression de marqueurs de surface spécifique) permettant un ciblage thérapeutique.

Ciblage de NF- κ B en immunothérapie: inhibition ou activation ?

Nos résultats préliminaires suggérant un rôle de RelA et c-Rel dans les sous-types de Teff complètent nos connaissances sur leurs fonctions dans la biologie des Treg. Se pose donc une question majeure : doit-on inhiber ou activer NF- κ B en immunothérapie ? La réponse tient probablement dans la spécificité du ciblage en termes de pathologie, de SU et de cellule-cible.

-Dans les modèles précliniques de cancer, il est probable que les inhibiteurs de NF- κ B sont efficaces car ils ciblent principalement les cellules suppressives (Treg, MDSC) qui s'accumulent généralement dans l'environnement tumoral, et les cellules malignes elles-mêmes (via une inhibition de la prolifération et de l'EMT, une induction de l'apoptose, l'augmentation de l'immunogénicité...). Cependant, NF- κ B semblant également être un régulateur de la fonction des Teff, la durée, la posologie et la spécificité de ces traitements doivent être étroitement contrôlées pour éviter les effets délétères. Par exemple, Heuser et al. ont montré que l'administration de doses élevées de KINK-1, un inhibiteur d'IKK, altère la fonction des lymphocytes T CD8, tandis que des doses plus faibles ont des effets thérapeutiques dans le mélanome (Heuser et al., 2017).

D'autre part, malgré les résultats encourageants obtenus avec des molécules inhibant entièrement la voie canonique dans des modèles pré-cliniques, leur application clinique a été largement prévenue par des effets toxiques sévères, qui reposent probablement en partie sur leur absence de sélectivité. Les progrès récents de l'ingénierie des médicaments peuvent aider au développement de médicaments plus spécifiques, donc plus puissants et plus sûrs. Par exemple, des conjugués anticorps-médicament, permettant le ciblage de composés à des types cellulaires définis, pourraient être utilisés pour administrer des inhibiteurs de NF- κ B à des cellules tumorales ou à des cellules immunosuppressives. L'efficacité thérapeutique de conjugués curcumine-anticorps anti-cancer, dans des modèles murins de mélanome et de glioblastome, est une bonne preuve de concept (Langone et al., 2012; Langone et al., 2014).

Sur le moyen terme, on peut envisager différentes stratégies pour le développement d'inhibiteurs spécifiques de SU : (i) des petites molécules induisant la dégradation spécifique d'une SU sans toucher les autres (par exemple via un screening de bibliothèques

sur des cellules exprimant des molécules de fusion RelA-GFP, c-Rel-RFP, RelB-BFP) ; (ii) des inhibiteurs bloquant une activité transcriptionnelle spécifique (utilisant des cellules reportrices kB-Luc/GFP et sur-exprimant toutes les SU non ciblées) (Shono et al., 2014) ; (iii) des études in silico permettant le design de molécules basées sur les structures 3D spécifiques des SU (Shiroma et al., 2020). Un autre type de stratégie, plus spécifique et donc entraînant moins d'effets secondaires, mais peut-être aussi moins efficace, serait de cibler des protéines dont l'expression est dirigée spécifiquement par une SU (cytokine, chimiokine, autre facteur de transcription). Pour ce faire, notre collection de données de séquençage sera d'un apport considérable.

-A l'inverse, la stimulation spécifique de certaines SU de NF- κ B dans les Teff, pourrait être induite par stimulation de certains membres des familles CD28 et TNFRSF, et améliorer leur activité anti-tumorale. La spécificité de ce ciblage est le sujet du 1^{er} projet de recherche décrit ci-dessus. Une autre stratégie repose sur le développement de thérapies cellulaires (CAR-T cells, TCR-T cells) modifiées pour activer spécifiquement une ou plusieurs SU, via CRISPR par exemple.

-Le problème opposé se pose pour la thérapie de l'auto-immunité, dans laquelle les inhibiteurs d'IKK ont fait leurs preuves dans des modèles pré-cliniques, probablement via un mécanisme d'inhibition de la fonction des Teff pathogènes.

Quels sont les mécanismes moléculaires conférant leur spécificité fonctionnelle aux SU de NF- κ B ?

La littérature des dernières années a établi de façon équivoque la sélectivité des SU de NF- κ B, dans le contrôle des programmes d'expression et des fonctions biologiques d'un même type cellulaire. Reste encore à comprendre comment cela est possible! Est-ce une question de composition des dimères de SU, de cinétique d'activation, de site de reconnaissance de l'ADN légèrement différent ? Différentes approches peuvent être développées pour aborder ce concept :

-Une analyse transcriptomique des sous-types de LT déficients pour les SU, à différents temps de stimulation (nous nous concentrons à l'heure actuelle sur des temps courts -4 et 16 heures- pour des raisons budgétaires et de simplification, mais nous perdons sans doute un certain nombre d'informations).

-Les analyses par ChIP-Seq décrites dans le 1^{er} projet. Nous avons commencé par RelA, mais notre ambition est à terme d'effectuer des analyses différentielles pour chacune des SU, à nouveau à différents temps de stimulation. Ceci permettra de déterminer des séquences consensus spécifiques, de savoir si les SU se fixent de façon préférentielle à des promoteurs, enhancers ou super-enhancers, et d'aborder l'aspect 'cinétique' de fixation à l'ADN.

-A plus long terme, nous voudrions investiguer la formation/désassemblage de super-complexes, via des approches d'immuno-précipitation/spectrométrie de masse pour établir une cartographie des interactions entre SU et avec d'autres kinases ou modificateurs transcriptionnels.

Conclusion

Il peut sembler ambitieux d'attribuer un rôle à 5 protéines différentes, aux multiples sous-populations de lymphocytes T dans différents contextes pathologiques. Cependant, cela semble être une étape nécessaire si nous voulons développer des thérapies ciblées pour améliorer ou atténuer les réponses immunitaires d'une manière spécifique. Grâce aux technologies de séquençage à haut débit, aux nouveaux modèles murins et aux patients porteurs de mutations dans les voies NF- κ B, nous avons en main les stratégies pour surmonter cette complexité. À l'ère des immunothérapies, NF- κ B pourrait être la clé pour libérer ou contrôler pleinement le pouvoir des cellules T dans le cancer, l'auto-immunité et les infections.

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ANNEXES

NF- κ B c-Rel Is Crucial for the Regulatory T Cell Immune Checkpoint in Cancer

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SUMMARY

Regulatory T cells (Tregs) play a pivotal role in the inhibition of anti-tumor immune responses. Understanding the mechanisms governing Treg homeostasis may therefore be important for development of effective tumor immunotherapy. We have recently demonstrated a key role for the canonical nuclear factor κ B (NF- κ B) subunits, p65 and c-Rel, in Treg identity and function. In this report, we show that NF- κ B c-Rel ablation specifically impairs the generation and maintenance of the activated Treg (aTreg) subset, which is known to be enriched at sites of tumors. Using mouse models, we demonstrate that melanoma growth is drastically reduced in mice lacking c-Rel, but not p65, in Tregs. Moreover, chemical inhibition of c-Rel function delayed melanoma growth by impairing aTreg-mediated immunosuppression and potentiated the effects of anti-PD-1 immunotherapy. Our studies therefore establish inhibition of NF- κ B c-Rel as a viable therapeutic approach for enhancing checkpoint-targeting immunotherapy protocols.

INTRODUCTION

Tumors employ a wide variety of strategies to grow, metastasize, and avoid recognition and elimination by the immune system. One of the major mechanisms of immune evasion by tumors is through the engagement of immunosuppressive receptors, such as PD-1 and CTLA-4, on effector T cells by their ligands expressed in the tumor microenvironment. Emerging cancer immunotherapy approaches are aimed toward overcoming such tolerance mechanisms and promoting a productive anti-tumor immune response. Although these immune checkpoint blockade therapies are revolutionizing cancer care (Sharma and Allison, 2015), there are a significant number of tumors that do not benefit from such approaches alone. One possible explanation

for such resistance is that other immunosuppressive mechanisms are engaged in such tumors. For example, CD4⁺Foxp3⁺ regulatory T cells (Tregs) suppress anti-tumor responses (Nishikawa and Sakaguchi, 2010; Tang and Bluestone, 2008). Tregs are highly enriched in patient melanomas (Jandus et al., 2008; Ouyang et al., 2016) and their presence in tumors correlates with poor prognosis (Baumgartner et al., 2009).

Tregs, that either develop in the thymus (nTreg) or are induced in the periphery (iTreg), normally represent 5%–15% of the CD4⁺ T cell population. They can inhibit immune responses through diverse mechanisms that include secretion of inhibitory cytokines, direct cytotoxicity, disruption of metabolic pathways in target cells, or inhibition of antigen-presenting cells (Tang and Bluestone, 2008). The expression of the forkhead-box transcription factor Foxp3 is crucial for the acquisition of the Treg suppressive program and maintenance of Treg identity (Samstein et al., 2012). Tregs may exist as specialized subsets that have distinct functions. In mice, the resting Treg population (rTreg) that resides in lymphoid tissues acts to prevent lymphoproliferation and autoimmunity and is maintained by the transcription factor Foxo1 (Huehn et al., 2004; Luo et al., 2016). In contrast, the “effector-memory like” activated Treg subset (aTreg) migrates to inflamed tissues and tumors and has been shown to act as a potent inhibitor of anti-tumor responses (Darrasse-Jèze et al., 2009; Levine et al., 2014; Luo et al., 2016). It was recently proposed that IRF-4 and Myb could help maintain aTreg homeostasis (Levine et al., 2014; Dias et al., 2017). However, the regulators of aTreg differentiation and function are poorly characterized. Hence, understanding the molecular signals that drive Treg development and homeostasis remains of great interest.

Several studies have shown that the transcription factor NF- κ B, in particular the c-Rel subunit, is crucial for the expression of FoxP3 and the thymic development of Tregs (Isomura et al., 2009; Long et al., 2009; Ruan et al., 2009). Activation of nuclear factor κ B (NF- κ B) occurs through two pathways, the canonical and the non-canonical pathway. The canonical pathway leads to activation of NF- κ B heterodimers consisting of p50 and p65, or p50 and c-Rel, and is primarily involved in immune activation and cell survival. The non-canonical pathway leads to nuclear translocation of p52-RelB heterodimers and is mainly involved

in lymphoid organogenesis. NF- κ B complexes containing either p65 or c-Rel can perform distinct biological roles. While p65 containing NF- κ B complexes are mainly responsible for cellular activation responses, c-Rel containing NF- κ B complexes play more specialized roles in the immune response and lymphoid development. Germline deletion of p65 leads to embryonic lethality, and cells lacking p65 show broad defects in survival, proliferation, and function (Beg et al., 1995). In contrast, mice lacking c-Rel are viable and despite strong c-Rel expression in lymphocytes, show limited immunological defects (Köntgen et al., 1995). Hence, availability of inhibitors that could target only NF- κ B c-Rel would likely avoid the undesirable side effects that have halted advancement of NF- κ B-inhibitors for therapeutic applications in inflammatory diseases and cancer (DiDonato et al., 2012).

With our discovery that NF- κ B c-Rel and p65 have divergent roles in the maintenance of Treg function (Oh et al., 2017), we tested the hypothesis that inhibiting c-Rel might be a viable method of selectively modulating Treg activity in cancer. We first observed that c-Rel, but not p65, was an important regulator of aTreg maintenance. Using genetic models with specific deletion of either p65 or c-Rel in Tregs, we found that c-Rel was selectively required for Treg-mediated melanoma tolerance. We have confirmed that Pentoxifylline (PTXF), an FDA-approved drug, can cause selective degradation of c-Rel, without affecting p65. Inhibition of c-Rel using PTXF delays tumor growth by altering Treg identity and function. Finally, we show that PTXF administration, along with PD-1-checkpoint blockade, has an additive effect in treating established melanoma in mice. These results confirm recent work suggesting that prevention of tumor tolerance and suppression of lymphoproliferation and autoimmunity are the result of distinct Treg functions (Luo et al., 2016; Sugiyama et al., 2013) and provide preclinical evidence for the use of c-Rel inhibitors in conjunction with other immune checkpoint inhibitors.

RESULTS

NF- κ B c-Rel Regulates Differentiation and Gene Expression of the Activated Treg Population

In the accompanying study (Oh et al., 2017), we show that mice lacking p65 in Tregs develop a lethal autoimmune syndrome by 6–12 weeks, whereas deletion of c-Rel leads to mild inflammation, but only after 20 weeks of age. These phenotypes were associated with distinct transcriptional programs governed by p65 and c-Rel and suggested a more prominent role for p65 in Tregs for the maintenance of peripheral tolerance and prevention of autoimmunity. We wondered whether the NF- κ B subunits played distinct roles in the different Treg subsets and therefore analyzed gene expression profiles of WT aTreg (CD44^{hi}CD62L^{low}) and rTreg (CD44^{low}CD62L^{high}) by RNA sequencing (RNA-seq) and compared them to the gene expression profiles from wild-type (WT), as well as p65 and c-Rel-deficient total Tregs (Oh et al., 2017). We first identified gene sets enriched in aTreg (841 genes) and rTreg (320 genes) populations. Ablation of p65 only marginally affected aTreg and rTreg gene signatures (Figure 1A). However, in the absence of c-Rel, the numbers of affected genes increased dramatically (Figure 1B). Hallmarks of aTregs, such as *Itgae*, *Tigit*, *Klrg1*, *Il1r2*, or *Tnfrsf8*, were downre-

gulated in c-Rel-deficient Tregs, while their expression was unaffected in p65-deficient Tregs (Figure 1C). The majority of the affected genes have been suggested to regulate Treg homeostasis and/or function, especially during cancer. We wanted to know whether this effect on aTreg genes was due to a reduction in aTreg numbers or rather due to an intrinsic defect in gene expression. Fluorescence-activated cell sorting (FACS) analysis of splenic T cells from *Foxp3*^{CRE}*p65*^{F/F} animals showed a slight increase in the proportion of aTreg among total Tregs compared to controls; in contrast, we observed a profound decrease of aTregs in *Foxp3*^{CRE}*c-Rel*^{F/F} mice (Figures 1D and 1E), whereas rTreg numbers were unchanged (Figure 1F). Similarly, the proportion of Tregs expressing CD103 and Ki-67 was reduced, whereas the Ki-67⁺ and Ly6C⁺ population was expanded in these mice (Figures 1G, S1A, and S1B). Therefore, c-Rel, but not p65, appears to be important for the maintenance of aTreg numbers. We next assessed whether NF- κ B was required for establishment of the aTreg transcriptional landscape in already differentiated aTregs, by performing RNA-seq on sorted aTreg and rTreg from p65- and c-Rel-deficient mice. Interestingly, deletion of either subunit led to impairment of the aTreg gene signature, as 103 or 104 genes, respectively, out of the 841 genes that characterize aTregs, were significantly downregulated (Figures 1H, S1C, and S1D). However, careful examination of these genes revealed a more important role for c-Rel in the expression of aTreg hallmark genes such as *Tnfrsf8*, *Klrg1*, *Il1r2*, *Tigit*, or *Ccr8* that were all downregulated in c-Rel knockout (KO) aTregs, but affected to a lesser extent in p65-deficient aTregs (Figure 1H). A significant decrease in *Klrg1* and PD-1 protein expression in c-Rel-deficient, but not p65-deficient aTreg was also detected by FACS (Figures S1G and S1H). Finally, analysis of the rTreg transcriptome did not reveal a critical involvement of either p65 or c-Rel (Figures S1E and S1F), suggesting that other transcription factors, such as Foxo 1, might be more important for the maintenance of the rTreg population. Taken together, these results demonstrate that c-Rel plays a central role in aTreg biology and suggests that c-Rel deletion might have effects on anti-tumor responses.

Tregs Specifically Require c-Rel to Inhibit Anti-tumor Effector Responses

To test the role of p65 and c-Rel in Tregs during anti-tumor responses, we compared the growth of B16F1 melanoma cells in WT mice versus mice lacking either NF- κ B subunit. We observed exponential melanoma growth in control *Foxp3*^{CRE} transgenic animals (Figure 2A), and despite the functional defects in Tregs lacking p65 (Oh et al., 2017), tumor growth was unaltered in *Foxp3*^{CRE}*p65*^{F/F} mice (Figure 2A). In contrast, only 50% of the *Foxp3*^{CRE}*c-Rel*^{F/F} animals exhibited detectable tumors after 2 weeks, and these tumors were significantly smaller than those seen in control littermates (Figure 2A). We next measured T cell infiltration 2 weeks after tumor inoculation and observed an equal increase of CD8⁺ T cells in p65- and c-Rel-deficient mice in tumor infiltrating lymphocytes (TILs). CD4⁺ T cells, however, were only increased in *Foxp3*^{CRE}*c-Rel*^{F/F} mice (Figures 2B and 2C), but the intrinsic expression of IFN- γ by T cells remained unchanged between strains (Figure 2D). We observed an equivalent reduction in the proportion of TIL Tregs in both c-Rel- and

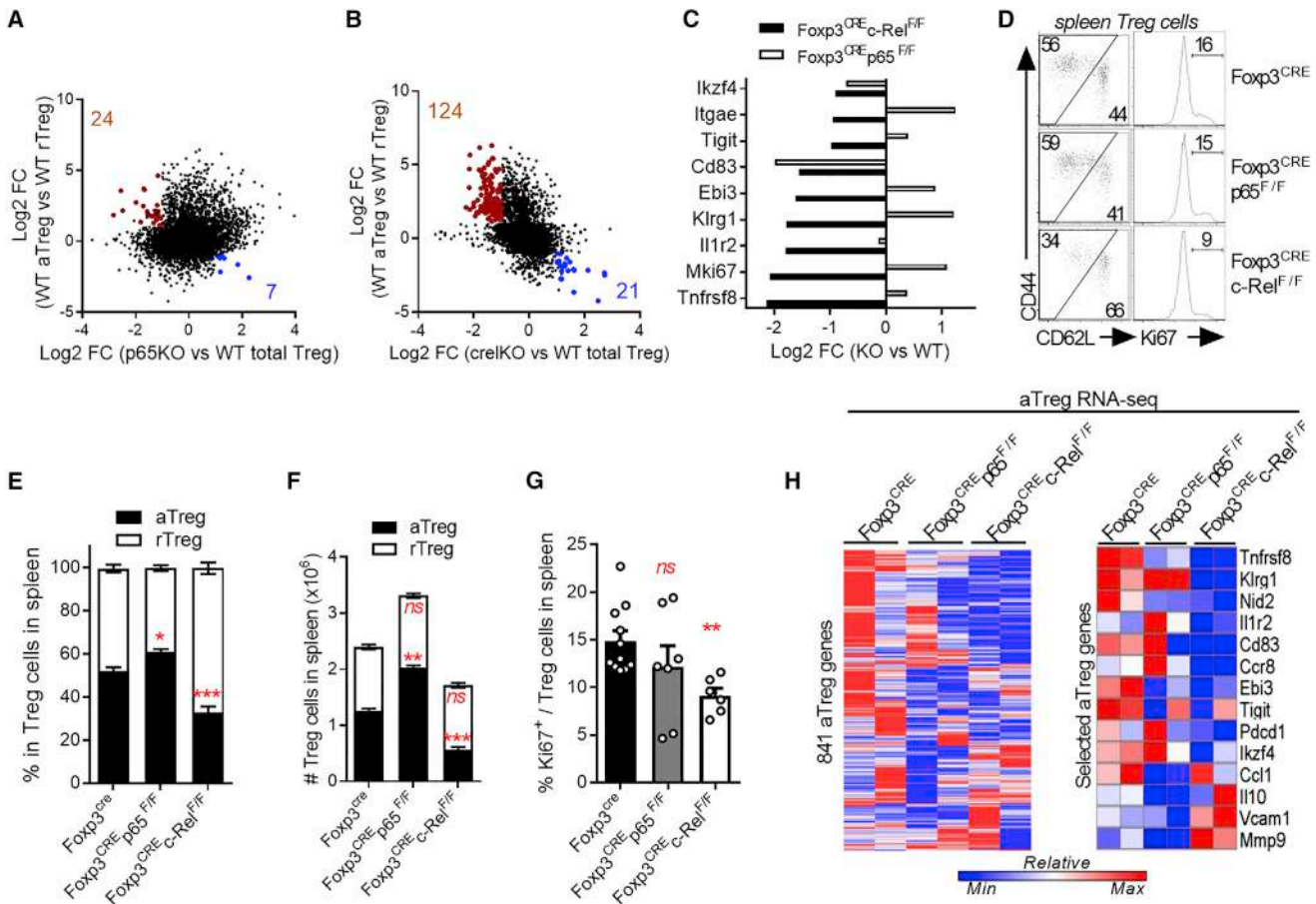


Figure 1. NF-κB c-Rel Regulates the Activated-Treg Differentiation and Gene Expression

(A–C) CD62L^{low} CD44^{high} aTreg and CD62L^{high} CD44^{low} rTreg were sorted from *Foxp3^{CRE}-YFP* (WT) mice and submitted to RNA-seq analysis. Gene expression was compared to that in stimulated total *Foxp3^{cre}p65^{F/F}* (p65KO) and *Foxp3^{cre}c-Rel^{F/F}* (c-RelKO) Treg (Oh et al., 2017). (A and B) Gene expression changes in WT aTreg versus WT rTreg were plotted against those in p65KO versus WT total Tregs (A) and c-RelKO versus WT total Tregs (B). Numbers and colored dots indicate genes upregulated in aTreg while downregulated in KO Treg (red) and downregulated in aTreg while upregulated in KO Treg (blue) (fold change >2, p < 0.01). (C) Expression of selected aTreg genes. (D) Representative FACS profiles in spleen Tregs from 5- to 7-week-old *Foxp3^{cre}*, *Foxp3^{cre}p65^{F/F}*, and *Foxp3^{cre}c-Rel^{F/F}* mice. (E and F) Cumulative % (E) and absolute numbers (F) of rTreg and aTreg in spleen Tregs. (G) Cumulative % of Ki67⁺ in spleen Tregs. (H) RNA-seq analysis of aTregs sorted from *Foxp3^{cre}*, *Foxp3^{cre}p65^{F/F}*, and *Foxp3^{cre}c-Rel^{F/F}*. Left heatmap shows expression of all 841 aTreg genes (fold change >2, p < 0.01 in WT aTreg versus WT rTreg) in each genotype. Right heatmap shows expression of selected aTreg genes. All RNA-seq data come from 2 independent experiments. FACS data is shown as mean ± SEM of 3 experiments with >6 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001; ns, non-significant. See also Figure S1.

p65-deficient animals when compared to WT littermates (Figure 2F). Therefore, the difference in tumor growth between the p65 and c-Rel KO strains reflects divergence in the functional capability of Tregs. This fits our initial observation on the important role of c-Rel in the homeostasis of the aTreg population, which was suggested to specifically control tumor immunity (Figure 1).

Among the 688 genes whose expression was modified by the absence of c-Rel but not p65, we observed a striking loss of Treg signature genes. For example, the expression of *Ikzf2* (Helios) or *Ppar-γ*, which are important regulators of Treg homeostasis, were significantly decreased; while expression of *Pde3b*, which is deleterious for *Foxp3* stability, was increased (Fig-

ure S2A). On the other hand, the expression of effector transcription factors and cytokines, such as *Eomes*, *Tbx21*, *Il2*, or *Ifng*, were strongly increased in Tregs lacking c-Rel, but not p65 (Figure S2A). The modulation of Helios and IFN-γ expression in Tregs was also specifically observed by flow cytometry in the TILs of *Foxp3^{CRE}c-Rel^{F/F}* mice, upon melanoma transplantation (Figures 2E, 2G, and 2H). This strongly suggests that the molecular program driven by c-Rel, in contrast to p65, allows Tregs to inhibit anti-tumor responses at early stages following tumor implantation and raises the possibility that Tregs lacking c-Rel could actually promote tumor immunity through effector cytokine production.

To better understand how c-Rel deficiency in Tregs affects early anti-tumor responses, we assessed T cell phenotype

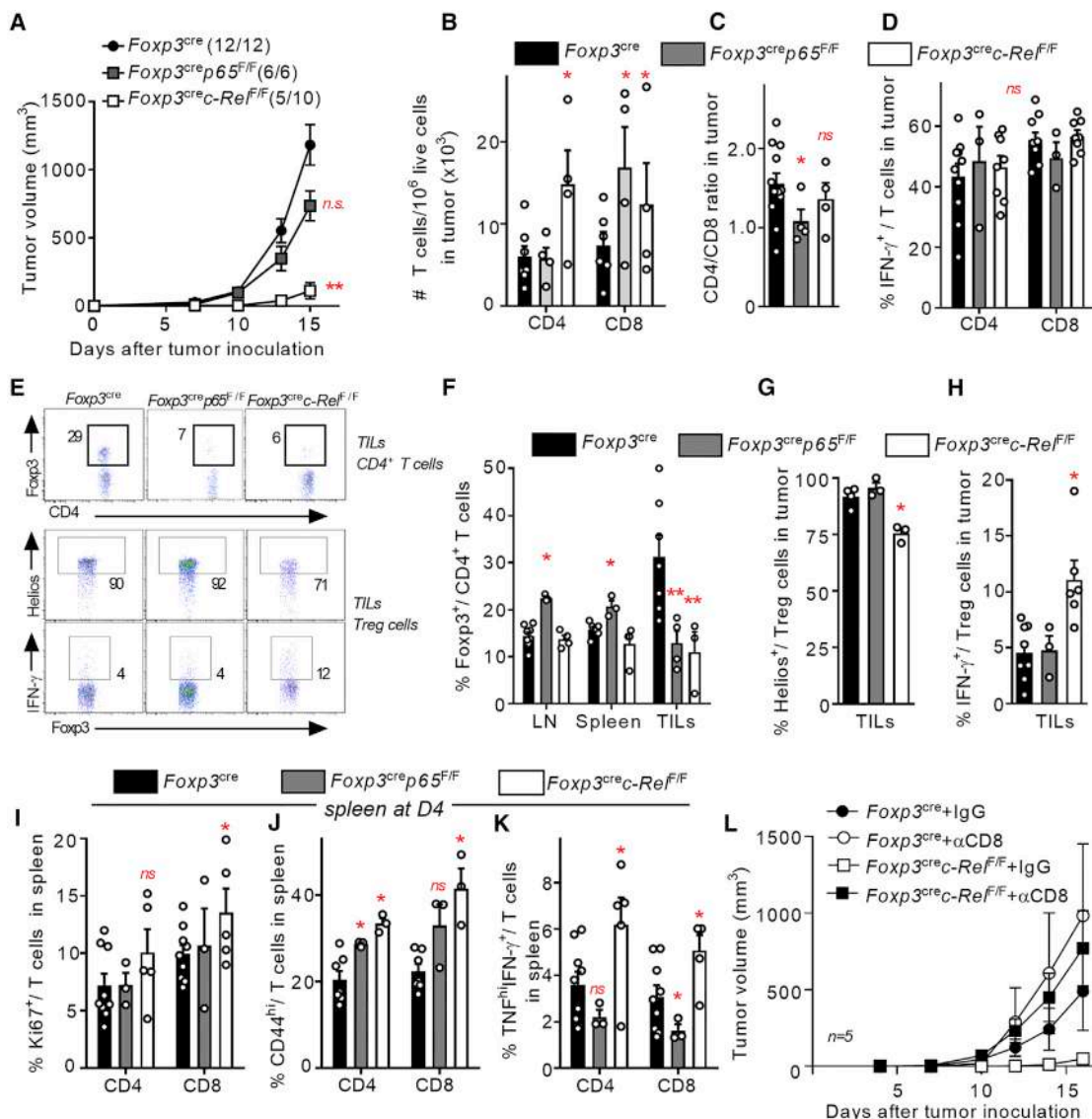


Figure 2. c-Rel Expression in Tregs Restricts Anti-tumor Immune Responses

Five- to 7-week-old *Foxp3^{cre}*, *Foxp3^{cre}p65^{F/F}*, and *Foxp3^{cre}c-Rel^{F/F}* were transplanted subcutaneously with B16F1 cells.

(A) Tumor growth over time. Numbers indicate the number of mice with detectable tumors at the end of the experiment.

(B–H) Flow cytometry analysis 16 days after tumor challenge, with (B–D, G, and H) or without (E–G) PMA-ionomycin re-stimulation. (B) Numbers of T cells among 10⁶ live cells. (C) CD4/CD8 T cells ratio. (D) Percent of IFN- γ ⁺ in gated CD4⁺*Foxp3*[−] (CD4) and CD8⁺ (CD8) live T cells. (E) Representative FACS profiles in TILs. Numbers indicate % in gate. (F) Percent of *Foxp3*⁺ cells among CD4⁺ T cells. (G and H) Percent of Helios⁺ and IFN- γ ⁺ among Tregs in TILs.

(I–K) Splenocytes were stained immediately (I and J) or after PMA-ionomycin re-stimulation, 4 days after tumor challenge. Proportion of Ki67⁺ (I), CD44^{high} (J), and TNF^{high}IFN- γ ⁺ (K) cells is shown.

(L) Mice were transplanted as in (A) and were injected at D0 and D3 with anti-CD8 or IgG isotype control. Tumor growth over time is shown. All data are represented as mean \pm SEM of at least 3 experiments. *p < 0.05, **p < 0.01; n.s., non-significant.

See also Figure S2.

4 days after melanoma inoculation. We observed enhanced expression of Ki67 and CD44 in splenic CD8 T cells of animals with conditional deletion of c-Rel in Tregs, compared to WT littermates (Figures 2I and 2J). This was associated with increased levels of polyfunctional (IFN- γ TNF^{high}) T cells upon polyclonal restimulation ex vivo (Figure 2K). These parameters remained mostly unchanged in p65-deficient mice. This may explain

the different outcomes in tumor growth between c-Rel- and p65-deficient animals. Enhanced CD8 T cell activation in *Foxp3^{cre}c-Rel^{F/F}* mice was observed following transplantation of the tumor, but not in unmanipulated mice (Figure S2B). Moreover, we observed increased secretion of IFN- γ by CD8 T cells upon gp100/p-mel restimulation (Figure S2C). In line with these results, CD8 depletion restored normal tumor growth in all the

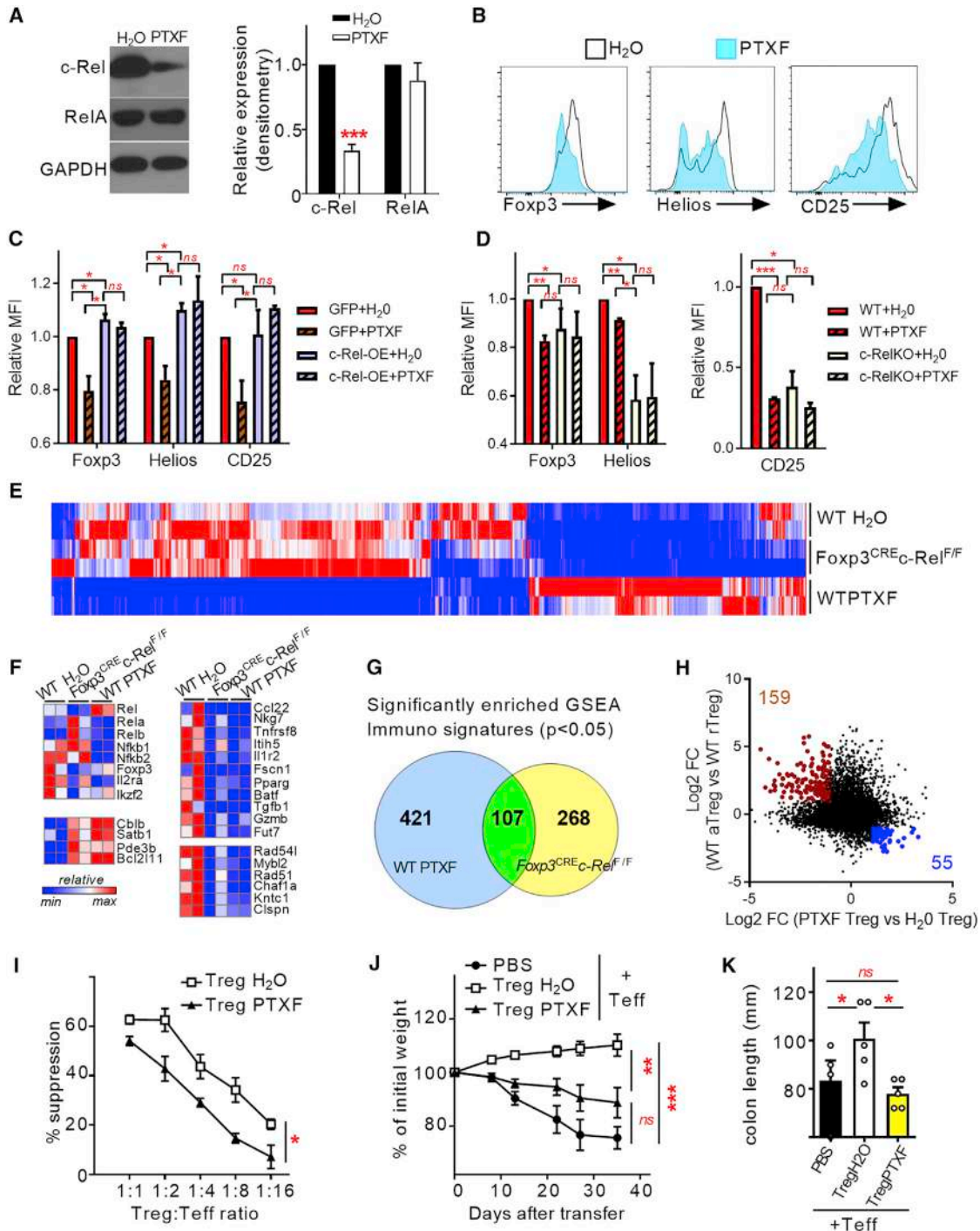


Figure 3. Chemical c-Rel Inhibition Impairs Treg Identity

(A and B) CD4⁺GFP⁺ Tregs were sorted from Foxp3^{GFP} spleens and stimulated for 16 hr with anti-CD3/CD28 and mIL-2 in the presence of 500 μg/mL PTXF or H₂O. (A) Western Blot on total cell lysates and cumulative expression of c-Rel and RelA across 3 experiments. (B) Representative FACS profiles in gated live CD4⁺ cells.

(C) Tregs sorted from Foxp3^{RFP} mice were infected with GFP or c-Rel-GFP-encoding retroviruses and treated with PTXF or H₂O as in (A). Data is shown as MFI relative to the GFP+H₂O sample.

(D) Tregs sorted from Foxp3^{cre} (WT) and Foxp3^{cre}c-Rel^{F/F} (c-RelKO) were treated as in (A) and analyzed by FACS. Data is shown as MFI relative to the WT+H₂O sample.

(E–H) CD4⁺YFP⁺Tregs sorted from Foxp3^{cre} (WT) and Foxp3^{cre}c-Rel^{F/F} were activated as in (B) and submitted to RNA-seq analysis. (E) Heatmap of differentially expressed genes (changed in at least one condition, using a fold-change cut-off >2 and a p value < 0.05 when compared to the WT+H₂O samples). Gene (legend continued on next page)

Foxp3^{CRE}*c-Rel*^{F/F} animals (Figure 2L). We confirmed these findings with 2 additional melanoma models. In mice injected intravenously with B16F10 melanoma cells, examination of the lungs showed significantly reduced number of metastatic foci in *c-Rel*-deficient animals compared to controls (Figure S2D). We also observed decreased growth when we transplanted subcutaneous *BRAF*^{CA}*Pten*^{-/-} melanomas into *c-Rel*-deficient animals (Figure S2E). Taken together, our results demonstrate that *c-Rel* controls a specific genetic program in Tregs that is required for inhibition of the anti-melanoma protective immune response mediated by CD8 T cells.

c-Rel Inhibition Impairs Treg Identity

Although canonical NF- κ B signaling is important for conventional T cell responses, the role of *c-Rel* in cellular immunity *in vivo* is not well established (Köntgen et al., 1995). Therefore, we hypothesized that inhibition of *c-Rel* would selectively block Treg-mediated tumor tolerance with minimal inhibition of anti-tumor responses. Pentoxifylline (PTXF) is a xanthine derivative that has been approved by FDA for clinical use for a wide variety of clinical conditions over the past 30 years. PTXF has been proposed to act through multiple targets *in vivo*, including as a potent inhibitor of *c-Rel* expression and activation in T cells (Neo et al., 2014; Wang et al., 1997). In our hands, treatment of Tregs with PTXF led to a significant reduction of *c-Rel* protein, whereas levels of p65 were unaffected (Figure 3A). We observed significantly decreased expression of Treg markers such as *Foxp3*, *CD25*, and *Helios* (Figure 3B), genes that were also reduced in Tregs upon conditional deletion of *c-Rel* (Figure S2A). To assess whether the decrease of these Treg markers was due to the effect of PTXF on *c-Rel*, we overexpressed *c-Rel* in Tregs using retroviral transduction. Interestingly, *c-Rel* overexpression alone increased steady-state expression of *Foxp3* and *Helios* (Figure 3C). Whereas PTXF treatment led to a significant decrease in *Foxp3*, *Helios* and *CD25* expression in GFP-transduced cells, it did not in *c-Rel*-transduced Tregs. We next used the converse approach of treating WT and *c-Rel*-deficient Tregs with PTXF. As expected, the levels of *Foxp3*, *Helios* and *CD25* were reduced in *c-Rel*-deficient Tregs compared to WT; also, PTXF failed to further decrease the expression of these proteins (Figure 3D). This suggested that PTXF induced a perturbation of the Treg-associated signature through its effect on *c-Rel*. To determine the extent to which PTXF inhibited the *c-Rel*-mediated transcriptional program in Tregs, we next compared the transcriptomes of control, PTXF-treated, and *c-Rel*-deficient Tregs. PTXF treatment led to a more profound alteration of the Treg transcriptome than genetic ablation of *c-Rel* (Figures 3E, S3A, and S3B), which was not surprising, given that PTXF likely affects additional signaling

pathways (Deree et al., 2007; Kamran and Gude, 2013; Pinzani et al., 1996). Of note, the expression of the *c-Rel* mRNA itself was unaffected by PTXF, suggesting that PTXF was affecting *c-Rel* through a post-translational mechanism (Figure 3F). A number of genes described to maintain Treg function and homeostasis were altered upon both chemical and genetic inhibition of *c-Rel* (Figures 3F, S3C, and S3D), such as *Gzmb* and *Tgfb1*, both involved in Treg function during cancer. Moreover, known NF- κ B target genes, such as *Tnfrsf8*, *Tnaip2*, or *Pde3b*, also displayed altered expression in both PTXF-treated and *c-Rel*-deficient Tregs. In contrast, *Cbl-b* and *Satb1*, which destabilize Tregs, were upregulated in both conditions. Confirmation of gene expression by qPCR showed that these genes were, in fact, affected to a comparable extent by both chemical and genetic inhibition of NF- κ B (Figure S3D). Interestingly, we observed only a minor overlap between the WT Treg+PTXF and *Foxp3*^{CRE}*p65*^{F/F} transcriptomes, reinforcing the idea that PTXF affects *c-Rel* but not p65 (Figure S3B). Gene set enrichment analysis (GSEA) indicated that, in addition to unique signatures found in the PTXF and *c-Rel* KO samples, 107 gene signatures overlapped between the 2 samples (Figure 3G). Among them, we detected a loss of Treg-associated genes and an impairment of the T cell activation processes and memory phenotype (Figure S3E). Ingenuity Pathway Analysis revealed that inhibition of the NF- κ B pathway was a key feature of the *c-Rel*-deficiency and PTXF-induced phenotype (Figure S3F). We next assessed whether PTXF treatment also affected the aTreg/rTreg signature. A total of 159 aTreg genes were significantly downregulated in PTXF-Tregs (Figure 3H), including *Tnfrsf8*, *Il1r2*, or *Nid2*. In contrast, 55 rTreg genes were enhanced, such as *Klfl2* or *S1pr1*. This indicates an enrichment of rTreg genes at the expense of aTreg genes. Thus, PTXF induced a substantial shift in Treg transcriptional program that is consistent with the change seen upon loss of *c-Rel* activity.

Tregs lacking NF- κ B *c-Rel* exhibit little change in suppressive ability in *in vitro* T cell suppression assays, despite a complete loss of function in *in vivo* assays (Oh et al., 2017). In an *in vitro* suppression assay, PTXF-treated Tregs exhibited modest, but significant, reduction in suppression of effector T cell proliferation when compared to control Tregs (Figure 3E) suggesting that PTXF likely affected some additional targets besides those regulated by *c-Rel*. However, in an *in vivo* suppression assay, PTXF-treated Treg completely failed to prevent colitis (Figures 3F, 3G, and S3H) similar to *c-Rel*-deleted Tregs (Oh et al., 2017). In both experiments, PTXF did not affect Treg survival but rather impaired their intrinsic function (Figures S3G and S3I). Taken together, these results show that PTXF treatment impacts the homeostasis and function of Tregs similar to deletion of *c-Rel*.

expression in normalized for each row. (F) Expression of selected genes in each condition. (G) RNA-seq datasets were analyzed for signature enrichment using the C7 GSEA Collection (ImmunoSigDB). The proportion of unique and overlapping signatures (p value < 0.05) is shown. (H) Gene expression in WT aTreg versus WT rTreg (see Figure 1) was plotted against that in PTXF-treated Tregs versus H₂O-treated Tregs. Numbers and colored dots indicate genes upregulated in aTreg while downregulated in PTXF Treg (red) and downregulated in aTreg while upregulated in PTXF-Treg (blue) (fold change >2, p < 0.01).

(I–K) CD4⁺GFP⁺ Tregs were stimulated as in (B) and subsequently tested for *in vitro* suppression (I) and *in vivo* colitis assays (J and K). (I) Suppression of responder T cells proliferation. (J) Weight changes upon cell transfer, shown as % of the initial weight at D0. (K) Total colon length at D35 after transfer. In (A)–(D) and (I)–(K), data are shown as mean \pm SEM and are cumulative of 3–4 experiments. In (E)–(H), RNA-seq data is from 2 independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001; n.s., non-significant.

See also Figure S3.

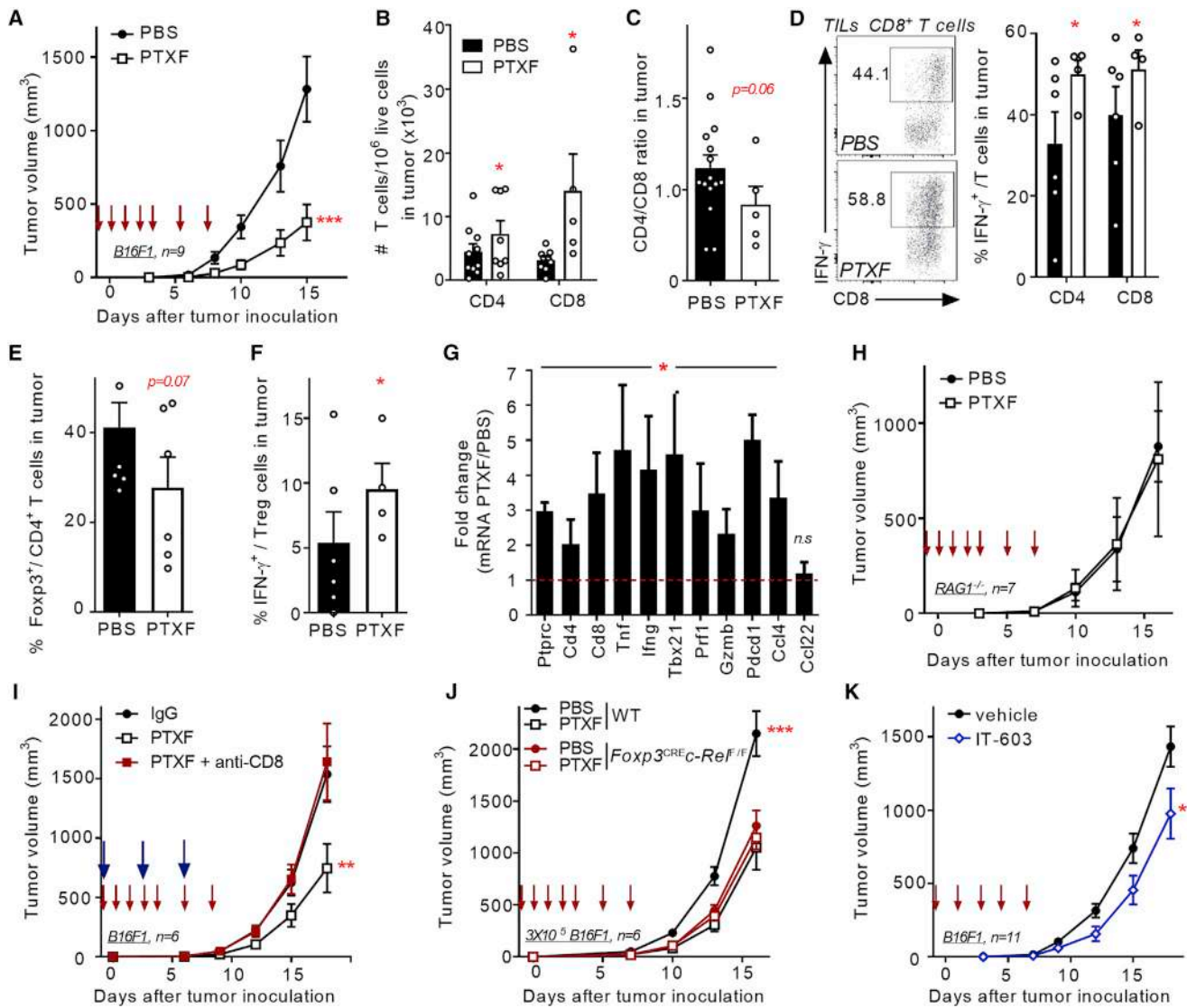


Figure 4. c-Rel Inhibition by PTXF Suppresses Melanoma Growth

(A–G) WT C57BL/6J mice were transplanted subcutaneously with B16F1 cells and treated from D–1 to D7 with PTXF or PBS. (A) Tumor growth over time. Arrows indicate the days of PTXF or PBS injection. (B–F) TILs were restimulated with PMA and ionomycin 16 days after tumor inoculation. (B) Cumulative numbers of T cells among 10^6 live cells. (C) CD4/CD8 T cells ratio. (D) Representative expression in CD8⁺ T cells (left) and cumulative % (right) of IFN- γ in T cells. (E) Percent of Foxp3⁺ Tregs in CD4⁺ T cells. (F) Percent of IFN- γ ⁺ in Tregs. (G) qPCR analysis of total tumor RNA at D16.

(H) RAG1^{-/-} mice were transplanted and treated as in (A). Tumor growth over time is shown.

(I) WT C57BL/6J mice were transplanted and treated as in (A) with or without anti-CD8 administration (blue arrows).

(J) Foxp3^{cre} and Foxp3^{cre}-c-Rel^{F/F} were transplanted subcutaneously with 3×10^5 B16F1 cells and treated as in (A).

(K) WT C57BL/6J mice were transplanted subcutaneously with B16F1 cells and treated from D–1 to D7 with IT-603 or vehicle (arrows). Data are represented as mean \pm SEM of 3 experiments. * $p < 0.05$, *** $p < 0.001$.

See also Figure S4.

c-Rel Inhibition Suppresses Tumor Growth

We predicted that administration of a c-Rel-inhibiting compound like PTXF would break Treg-mediated tumor immune tolerance and augment anti-tumor responses, similar to c-Rel deletion in Tregs. WT mice were transplanted with B16F1 melanoma cells and received daily injections of 50 mg/kg PTXF or PBS starting 1 day before tumor cell inoculation. Consistent with previous reports (Dua et al., 2007), we observed a significant reduction in the

rate of tumor growth in PTXF-treated animals as compared to control animals (Figure 4A). No observable differences in the size or composition of lymphoid tissues were detected, and no adverse events were observed (data not shown). As it has been reported that PTXF can induce tumor cell apoptosis in vitro (Edward and MacKie, 1991; Ratheesh et al., 2007), we wanted to determine whether the effects of PTXF on tumor growth were tumor intrinsic or related to improved immune

responses. Analysis of tumor infiltrates at D16 revealed an increased infiltration of total T cells, as well as IFN- γ producing CD4 and CD8 T cells (Figures 4B–4D). Additionally, there was a trend toward a decreased proportion of Tregs in the tumors, which also displayed an altered phenotype (Figures 4E, 4F, and S4A). As observed in the *Foxp3^{CRE}c-Rel^{F/F}* model, tumor-infiltrating Tregs exhibited increased IFN- γ production (Figure 4F). Moreover, qPCR analysis of total tumors revealed an enhanced inflammatory response, including increased expression of activated T cell markers and cytokines (Figure 4G). This suggests that immune cells, especially T cells, may mediate the protective effect of PTXF on melanoma growth. As previously reported (Ratheesh et al., 2007), we also detected a decrease in the expression of certain integrins, such as *Itga2b* and *Itgb3* (Figure S4B), suggesting that PTXF might act through the inhibition of tumor adhesion. To test whether there were direct effects on tumor growth independent of the changes in T cell responses, we transplanted immunodeficient *RAG1^{-/-}* mice with B16F1 cells and treated them with PBS or PTXF. In this background, PTXF had no effect on tumor growth (Figure 4G). Therefore, lymphocytes are required for the anti-tumor effects of PTXF. Moreover, CD8-depletion in PTXF-treated mice entirely restored tumor growth (Figure 4I). We further confirmed these results in the settings of B16F10 metastatic melanoma. Again, in immunocompetent mice, early PTXF treatment significantly decreased the total number of metastatic foci in lungs (Figure S4C).

To assess whether the beneficial effect of PTXF relied on its inhibition of c-Rel in Tregs, we next measured the influence of PTXF administration in *Foxp3^{CRE}c-Rel^{F/F}* mice. To ensure B16F1 melanoma growth in KO animals, we transplanted 3×10^5 cells instead of 5×10^4 cells. Melanoma growth was accelerated in these settings, but PTXF was still able to significantly reduce tumor growth in WT mice (Figure 4J). Moreover, all *Foxp3^{CRE}c-Rel^{F/F}* mice harbored a visible tumor by D10, even though the tumors were smaller than those in control mice. Interestingly, PTXF was unable to further decrease melanoma growth. This suggested that PTXF acted, at least partially, by inhibiting Treg function through the suppression of c-Rel. Finally, to confirm that the observed effects on melanoma growth can be attributed to c-Rel inhibition, we tested whether an unrelated chemical c-Rel inhibitor could recapitulate the effects of PTXF. We used IT-603, a commercially available compound that inhibits c-Rel-mediated transcription and reduces lymphoid leukemia severity in mouse models (Shono et al., 2014). Administration of 200 μ g IT-603 every other day, starting at D-1, reduced B16F1 growth modestly but significantly (Figure 4K). Increased T cell infiltration, IFN- γ production, and reduced Tregs were observed in the tumors of IT-603-treated mice (Figure S4D). These results demonstrate that chemical c-Rel inhibition can inhibit Treg function and prevent Treg-mediated tumor tolerance.

PTXF Treatment Potentiates the Effects of Checkpoint Blockade Therapies to Inhibit Melanoma Growth

The emergence of checkpoint blockade therapies has been a major step forward in the field of oncology. Antibody-mediated blockade of the PD-1/PD-L1 is believed to work by relieving exhaustion of CD8 T cells, thereby allowing the re-activation of

anti-tumor effector cells (Francisco et al., 2010). However, anti-PD-1 monotherapy is ineffective for the treatment of many tumors, including animal models (e.g., in B16-induced melanoma in mice) (Chen et al., 2015). We therefore wondered whether targeting of Tregs with PTXF could potentiate the beneficial effect of PD-1-blockade. Mice were first transplanted with B16F1 melanoma cells and treated with vehicle, PTXF, and/or anti-PD-1 mAb when the tumors reached a volume $>2 \text{ mm}^3$ (D5 to D6). PTXF or anti-PD-1 monotherapies did not change tumor growth compared to PBS controls. However, the combination of PTXF and anti-PD-1 significantly flattened the growth curves (Figure 5A). Co-administration of PTXF and anti-PD-1 increased T cell infiltration and expression of IFN- γ by CD4 and CD8 T cells, while the CD4/CD8 ratio was decreased (Figures 5B–5D). The proportion of Tregs was reduced while Treg IFN- γ expression increased (Figures 5E and 5F). Of note, curative PTXF monotherapy did not induce dramatic changes in the tumor infiltrate, underscoring the importance of early therapeutic intervention. RNA expression analysis demonstrated a cumulative effect of PTXF and anti-PD-1 treatments, with inflammatory cytokines and activation markers being strongly upregulated in the tumors of mice receiving the combination therapy (Figure 5G). Finally, CD8 depletion with a monoclonal antibody (mAb) fully restored tumor growth in PTXF+anti-PD-1-treated animals (Figure 5H) demonstrating a requirement for CD8 T cells for improved tumor clearance. We confirmed that these findings were not specific to the anti-PD-1 mAb by blocking the ligand of PD-1, PD-L1. Again, the combination of both PTXF and anti-PDL1 had an additive effect in decreasing and delaying melanoma growth (Figure 5I). We also assessed the effect of PTXF/anti-PD-1 therapy in a different genetic background. BALB/c mice were transplanted with CT-26 colon carcinoma and subsequently treated with PTXF and suboptimal doses of anti-PD-1 (because high doses of anti-PD-1 induced a significant decrease in CT-26 growth, data not shown). Again, only the combination of both drugs led to a significant reduction of tumor growth (Figure 5G). To test whether the inhibition of Treg c-Rel was sufficient to potentiate the curative effect of anti-PD-1 therapy, we next treated melanoma-bearing *Foxp3^{CRE}c-Rel^{F/F}* mice with anti-PD-1. Strikingly, unlike WT animals, tumor size was significantly reduced upon checkpoint-blockade therapy in mice whose Tregs lacked c-Rel (Figure S5A). Finally, curative combinatorial therapy with the c-Rel inhibitor IT-603 and anti-PD-1 led to reduced melanoma growth and increased T cell infiltration and activation (Figures 5K and S5B). Taken together, our data highlight the therapeutic potential of c-Rel inhibition in addition to checkpoint-blockade antibodies.

DISCUSSION

NF- κ B c-Rel Controls the Homeostasis of the aTreg Subset that Suppresses Anti-tumor Responses

Multiple lines of evidence demonstrate that the Treg pool can be separated into subsets with differing phenotypes and function. In both mice and humans, Tregs can be split into resting and activated populations (Huehn et al., 2004; Miyara et al., 2009). In mice, it was suggested that transcription factor Foxo1 maintains the CD62L^{high}CD44^{low} rTreg pool (Luo et al., 2016). These rTregs

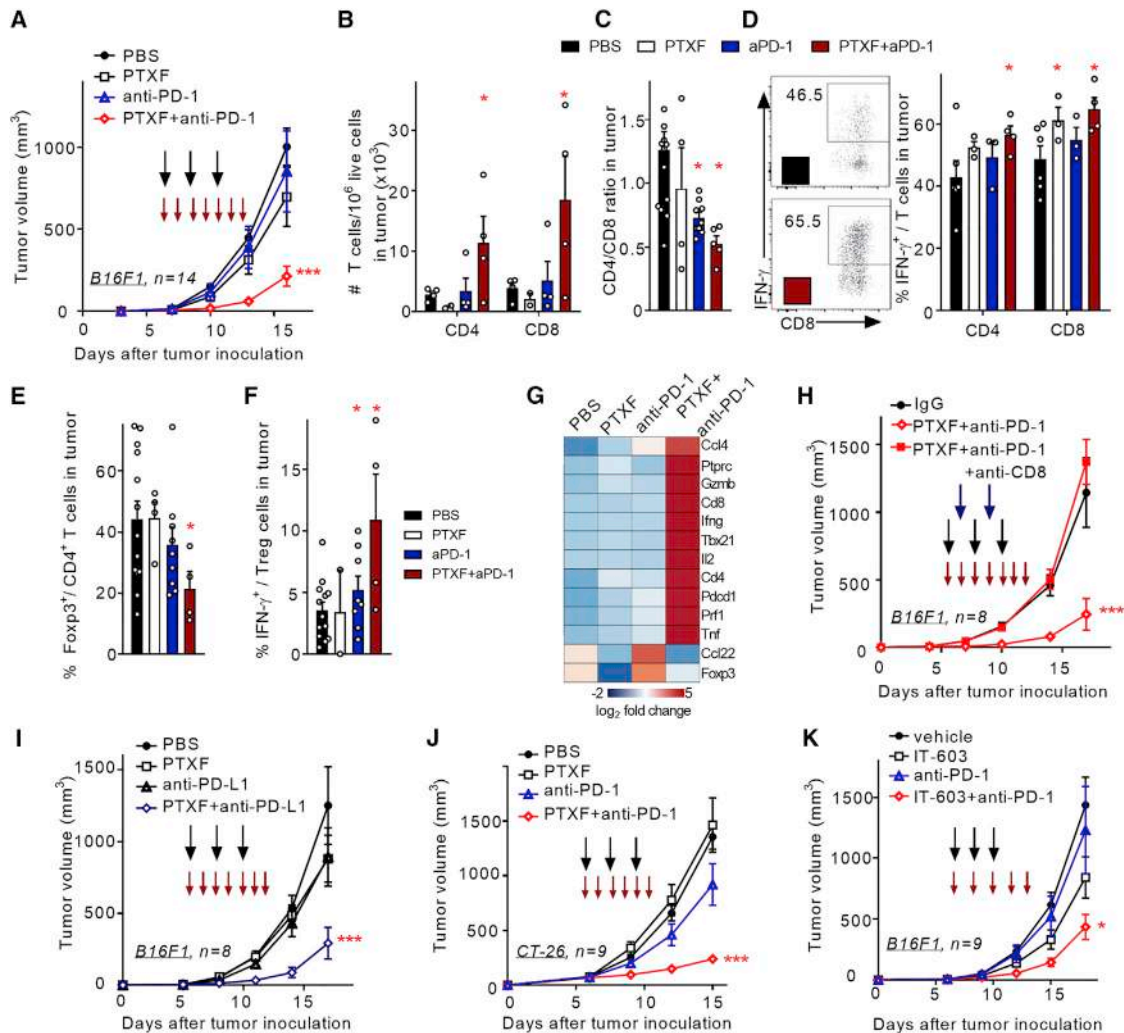


Figure 5. PTXF and PD-1-Blockade Have Additive Inhibitory Effects on Growth of Established Melanoma

(A–G) WT C57BL/6J mice were transplanted subcutaneously with B16F1 cells and treated from D6 with PTXF or anti-PD-1 mAb or PBS.

(A) Tumor growth over time. Red arrows, PTXF injections; black arrows, anti-PD-1 injections.

(B–F) TILs were restimulated with PMA and ionomycin 16 days after tumor inoculation. (B) Cumulative numbers of T cells among 10⁶ live cells. (C) CD4/CD8 T cells ratio. (D) Representative expression in CD8⁺ T cells (left) and cumulative % (right) of IFN- γ in T cells. (E) Percent of Foxp3⁺ Tregs in CD4⁺ T cells. (F) Percent of IFN- γ ⁺ in Tregs.

(G) qPCR analysis of total tumor RNA at D16.

(H) Mice were treated as in (A) and were injected with anti-CD8 mAb or IgG at D7 and D9 (blue arrows).

(I) Mice were treated as in (A) but with anti-PD-L1 mAb.

(J) WT Balb/C mice were transplanted subcutaneously with CT-26 cells and treated from D6 with PTXF or anti-PD-1 mAb (100 μ g) or PBS.

(K) WT C57BL/6J mice were transplanted subcutaneously with B16F1 cells and treated from with IT-603, and/or anti-PD-1, or vehicle. Data are represented as mean \pm SEM of 3 to 4 experiments. **p* < 0.05, ****p* < 0.001.

See also Figure S5.

are retained in lymphoid tissues through expression of high levels of CD62L, CCR7, and S1PR1; and it is believed that they are crucial for the prevention of lymphoproliferative disease, as illustrated by the early splenomegaly and lymphadenopathy observed in Treg-restricted Foxo1 conditional mutants (Ouyang et al., 2012). In contrast, CD62L^{low}CD44^{high} aTregs, which differentiate upon TCR-stimulation, acquire the ability to migrate to inflamed tissues through expression of homing receptors (Huehn et al., 2004; Rosenblum et al., 2011). Therefore, they are more

specialized in the suppression of local tissue inflammation. Recently, Tregs have emerged as crucial regulators of cancer progression (Darrasse-Jèze et al., 2009; Luo et al., 2016). It has been suggested that the expression of aTreg-specific molecules, such as CD103, CCR8, Klrp1, or Tigit, are critical for tumor homing of Tregs and suppression of effector T cells, thereby providing a novel specialized function to the aTreg subset (De Simone et al., 2016). However, the precise molecular mechanisms that regulate aTreg differentiation and function are poorly

described. It was proposed that IRF4 and Egr2 may be important for aTreg differentiation, and signaling through the TCR was absolutely required for their maintenance (Levine et al., 2014; Vahl et al., 2014). In a recent report (Messina et al., 2016), it was suggested that p65 may be involved in effector Treg development in the competitive environment of fetal liver recipients. However, the authors only observed a mild decrease in aTreg in the lymph nodes, but not the spleen, of *Foxp3^{CRE}p65^{F/F}* mice. Although we observed some effects of p65 deletion on the expression of genes enriched in aTregs, we did not observe a decrease in the aTreg population in vivo. Instead, we show that c-Rel was required to maintain a normal pool of aTregs and a normal aTreg transcriptome. As NF- κ B activation is a key downstream event of TCR/CD28 signaling, this observation is in agreement with previous reports. As p65 was mostly dispensable for aTreg, this reinforces the idea that c-Rel and p65 control separate gene targets and exert different functions in Tregs (Oh et al., 2017), possibly because the activation kinetics are different. A number of genes that are crucial for aTreg homing and homeostasis in inflamed tissues, such as CD83, PD-1, and Klr1 were impaired in c-Rel-deficient Tregs and also in TCR^{-/-} aTreg and in *Foxo1^{CA}*, but not in p65-deficient Tregs (Levine et al., 2014; Luo et al., 2016) (Figure 1). Thus, our data suggests that c-Rel is specialized in the maintenance of the aTreg population, while p65 controls crucial suppressive properties of Tregs in both the resting and the activated subsets. Hence, this explains the early lethal lymphoproliferation observed in *Foxp3^{CRE}p65^{F/F}* mice and the relative absence of autoimmunity in *Foxp3^{CRE}c-Rel^{F/F}* animals.

These data suggested that c-Rel deletion might have a specific effect on tumor tolerance. Indeed, the ablation of c-Rel, but not p65, in mature Tregs strongly impaired melanoma growth in a CD8 T cell-dependent manner and destabilized Treg-associated gene expression. Expression of Helios, which was recently implicated in the maintenance of Treg homeostasis and the suppression of tumor immunity, was reduced in the absence of c-Rel (Nakagawa et al., 2016). Although the immunological abnormalities present in the *Foxp3^{Cre}p65^{ff}* mice (Oh et al., 2017) complicate the analysis of tumor growth, it is notable that we did not observe any increase in anti-tumor immune response in Treg p65-deficient mice. In WT mice, Tregs efficiently inhibited anti-tumor effector CD8 T cell responses, thereby allowing uncontrolled cancer cell proliferation. In *Foxp3^{CRE}c-Rel^{F/F}* animals, however, the impaired Treg function allowed increased activation of effector cells and enhanced tumor cell killing. This, combined with the lack of evidence of a role for c-Rel in prevention of autoimmunity (Oh et al., 2017), and in effector CD8 responses, suggested that the therapeutic targeting of c-Rel could selectively ablate Treg-mediated tumor tolerance.

c-Rel Inhibition as a Novel Cancer Immunotherapy

An extensive body of literature describes the roles of NF- κ B in the initiation, proliferation, and propagation of tumors. This has led multiple groups to test whether global inhibition of NF- κ B could have an effect on tumor growth (DiDonato et al., 2012). For instance, inhibition of IKK activity by thalidomide, by the chemical BAY11-7082, or by the small inhibitory Nemo-Binding Domain (NBD) peptide, decrease severity of lymphomas (re-

viewed in Kim et al., 2006). Bortezomib (Velcade), which prevents degradation of the NF- κ B inhibitor I κ B α , is now a Food and Drug Administration (FDA)-approved drug and is used for the treatment of multiple myeloma (Mulligan et al., 2007). Despite these promising results, the use of such non-specific NF- κ B inhibitors has been complicated by the observation of multiple adverse effects, such as systemic inflammation through interleukin (IL)-1 β overexpression or non-immune-related complications (Greten et al., 2007). This could be a consequence of (1) the inhibition of non-NF- κ B-related pathways upon inhibition of IKK (Oeckinghaus et al., 2011), or (2) the inhibition of the p65 subunit of NF- κ B that is well known to exert central roles in organogenesis and inflammation. Our results using conditional mutant mice suggested that c-Rel, whose biological function is mainly restricted to the adaptive immune system, could be a specific target for the treatment of cancer through its role in Tregs. In vitro, we observed that PTXF reduced the level of c-Rel protein in Tregs, while impairing the molecular identity of Tregs and Treg suppressive function. In agreement with previously published results, PTXF affected c-Rel, but not other NF- κ B subunits (Wang et al., 1997). RNA-seq revealed a profound effect of PTXF on Treg-associated gene expression that was partially overlapping with changes observed in c-Rel-deficient Tregs. Genes required for optimal Treg function and immunosuppression in the tumor microenvironment, such as Tgfb1 or Gzmb (Boissonnas et al., 2010; Donkor et al., 2011), as well as numerous aTreg genes, were directly repressed by PTXF treatment, similar to that seen upon genetic deletion of c-Rel. Thus, PTXF affects Treg identity both directly by modifying c-Rel-dependent transcription and indirectly by forcing the expression of genes that promote anti-tumor responses.

PTXF has previously been described as an anti-cancer drug. Early PTXF administration reduced growth of subcutaneous B16F10 melanomas and decreased their metastatic potential (Dua et al., 2007). When injected together with the alkylating agent thiotepea, PTXF diminished growth of human bladder and breast cancer xenografts (Fingert et al., 1988). This has led to several ongoing clinical trials testing the effect of PTXF, in combination with existing treatments, in leukemia, glioblastoma, and non-small cell lung cancer (<https://clinicaltrials.gov>). It has been proposed that the anti-cancer properties of PTXF were attributable to reduced expression of adhesion molecules or induction of apoptosis in cancer cells (Bravo-Cuellar et al., 2013; Edward and MacKie, 1991). The effects of PTXF on T cells are still debated, as the drug seems to downregulate T cell effector functions in vitro but has an opposite effect in vivo (Jimenez et al., 2001; Suresh et al., 2002). In this study, we show that PTXF improves effector cells anti-tumor responses likely through inhibition of Treg function.

However, PTXF alone failed to reduce the growth of established tumors. This is because B16F1 melanoma has long been described to be highly aggressive and resistant to most monotherapies, including anti-PD-1/PD-L1 (Kleffel et al., 2015). It has been proposed that such treatments could be improved by the co-administration of two drugs. For instance, anti-CTLA-4 mAbs that are believed to directly target Tregs, and anti-PD-1 mAbs that relieve the exhaustion of effector T cells, could have a synergistic effect in monotherapy-resistant cancers (Curran

et al., 2010). Although promising preliminary results have been obtained from clinical trials, one major pitfall has been the frequent (>70%) and multiple adverse effects induced by anti-CTLA-4 mAbs, such as cutaneous inflammation or colitis (Bertrand et al., 2015). In contrast, PTXF is well-tolerated in patients and mice (<https://www.fda.gov/Safety/MedWatch/SafetyInformation/ucm314605.htm>). Here, we show that PTXF potentiates the effect of PD-1-blockade in our melanoma model without any adverse effects. Thus, we propose that this multi-therapy could be used as a safe novel treatment for solid cancers.

Understanding how Treg homeostasis is controlled during tumor growth may reveal new targets for the development of novel anti-cancer therapies. In this study, we have demonstrated that activation of the c-Rel subunit of NF- κ B in Tregs is central to the prevention of protective anti-tumor responses. Hence our findings provide a new rationale for the use of selective NF- κ B inhibitors, especially those targeting c-Rel, for the treatment of cancer.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENTS AND RESOURCES SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Animals
 - Cells
- METHOD DETAILS
 - Tumor transfer and treatments
 - Flow cytometry
 - In vitro PTXF treatment
 - Suppression assays
 - Retroviral transduction
 - Western Blotting
 - RT-qPCR and RNA-sequencing
- QUANTITATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2017.08.004>.

AUTHOR CONTRIBUTIONS

Y.G.-B. and S.G. conceived the experiments. Y.G.-B., M.S.H., and S.G. wrote the paper. Y.G.-B., H.O., and R.C., performed experiments. Y.G.-B., A.D., D.B., T.C., M.S.H., and S.G. analyzed the data. U.K. and R.S. provided mice and reagents. S.G. secured funding.

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An NF- κ B Transcription-Factor-Dependent Lineage-Specific Transcriptional Program Promotes Regulatory T Cell Identity and Function

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SUMMARY

Both conventional T (Tconv) cells and regulatory T (Treg) cells are activated through ligation of the T cell receptor (TCR) complex, leading to the induction of the transcription factor NF- κ B. In Tconv cells, NF- κ B regulates expression of genes essential for T cell activation, proliferation, and function. However the role of NF- κ B in Treg function remains unclear. We conditionally deleted canonical NF- κ B members p65 and c-Rel in developing and mature Treg cells and found they have unique but partially redundant roles. c-Rel was critical for thymic Treg development while p65 was essential for mature Treg identity and maintenance of immune tolerance. Transcriptome and NF- κ B p65 binding analyses demonstrated a lineage specific, NF- κ B-dependent transcriptional program, enabled by enhanced chromatin accessibility. These dual roles of canonical NF- κ B in Tconv and Treg cells highlight the functional plasticity of the NF- κ B signaling pathway and underscores the need for more selective strategies to therapeutically target NF- κ B.

INTRODUCTION

Homeostasis of the immune system is maintained by a delicate balance between activation and suppression. Disruption of this balance can lead to autoimmune diseases and immune dysfunction. Immune tolerance is maintained through central and peripheral mechanisms, including specialized cell subsets. Among these, CD4⁺FoxP3⁺ regulatory T cells (Treg cells) play a pivotal

role in the inhibition of immune responses. Treg cells, which develop in the thymus or in the periphery, are characterized by the expression of the transcription factor FoxP3, and by their ability to suppress the activation and function of conventional T cells (Tconv), and other immune cells, to maintain immune homeostasis. Thus, although they arise from the same progenitors in the thymus, Tconv and Treg cells have completely opposed biological roles. Remarkably, it is still unclear how engagement of the same T cell receptor on these two related cell types induces such different biological outcomes, despite the fact that many of the same signaling molecules and transcription factors are activated (Levine et al., 2014).

The NF- κ B transcription factor family consists of five members, p65 (RelA), c-Rel (encoded by *Rela* and *Rel*, respectively), p105/p50, p100/p52, and RelB. NF- κ B is activated in response to a variety of signals through two distinct pathways that are known as the canonical and noncanonical pathways. The canonical pathway leads to activation of NF- κ B heterodimers containing primarily p65 or c-Rel, bound to p50, whereas the noncanonical pathway leads to activation of NF- κ B heterodimers consisting of RelB and p52. Stimulation of the TCR together with costimulation of CD28 leads to activation of canonical NF- κ B complexes, which translocate to the nucleus, where they bind to specific sites in promoters and enhancers of target genes. Thus, in Tconv cells, NF- κ B induces expression of genes responsible for T cell activation, proliferation and production of inflammatory cytokines. Given the well-documented role for NF- κ B in activation of a variety of immune effector cells, it was unclear what role NF- κ B might play in Treg cells, whose biological function is to suppress the immune response.

We, along with other groups, have shown previously that NF- κ B c-Rel plays an essential role in the thymic development of Treg cells (Isomura et al., 2009; Long et al., 2009; Ruan et al., 2009). In Treg precursors, c-Rel binds directly to the CNS3 enhancer region and promotes the expression of FoxP3.

Treg development is impaired in mice lacking c-Rel and hence the role of NF- κ B in Treg function has remained unexplored. To determine how NF- κ B contributes to mature Treg function we have now conditionally deleted floxed alleles of NF- κ B *Rela* and *Rel* in Tregs. We found that both c-Rel and p65 played important, but only partly redundant roles in Treg function, and only deletion of both c-Rel and p65 led to completely non-functional Tregs and lethal autoimmunity, similar to that seen in mice lacking Tregs. Analysis of the gene-expression program in these cells revealed that expression of several key genes that are known to be critical for Treg identity and function was NF- κ B dependent. This suggested that NF- κ B was able to access different target genes in Treg and Tconv cells. Genome-wide p65 ChIP-seq revealed a large number of lineage specific target genes in Treg cells, associated with an enhanced open chromatin conformation in Tregs. Therefore, our results suggested that an altered global chromatin state in Tregs allows NF- κ B induced by the TCR to access lineage-specific binding sites and establish Treg identity and suppressive function. In summary, the studies presented here reveal the plasticity of a key transcription factor in regulating the diametrically opposed biological functions of two highly related cell types.

RESULTS

Canonical NF- κ B Signaling Is Crucial for Treg Development

We explored the specific roles of the canonical NF- κ B subunits c-Rel and p65 in natural (n)Treg and induced (i)Treg development by crossing mice with floxed *Rel* and *Rela* alleles with a *Cd4^{cre}* deleter strain resulting in loss of p65 and c-Rel expression (Figure S1A). We did not observe changes in development of CD4⁺ and CD8⁺ T cells in the thymus (data not shown). However, we observed a dramatic decrease in the proportion and number of CD4⁺CD8⁻FoxP3⁻CD25⁺GITR⁺ Treg precursors in mice lacking p65 and c-Rel (Figures 1A and 1B and S1B and S1C). There was a graded reduction in the frequency and number of FoxP3⁺ Treg cells upon deletion of *Rel*, *Rela*, or both. This demonstrated unique and partially redundant functions of both canonical NF- κ B subunits in the development of Treg progenitors.

To assess whether NF- κ B activation was required for the transition of Treg progenitors to FoxP3⁺ Treg cells, we isolated Treg progenitors and induced deletion of NF- κ B subunits in vitro using TAT-CRE protein (Joshi et al., 2002; Lio and Hsieh, 2008). We observed a 3-fold reduction in Treg frequency in cells lacking *Rel*, or both *Rela* and *Rel* (Figure 1C and data not shown). Hence these results suggested an intrinsic, specific, and non-redundant role for canonical NF- κ B subunits in the specification of FoxP3⁻ Treg precursors and in the expression of FoxP3. Moreover, deletion of *Rela* alone led to a modest, but statistically significant, decrease in the proportion and numbers of Treg cells in both spleen and lymph nodes (LN), but not in other tissues (Figures 1D–1F and S1D). Mice lacking *Rel* exhibited a dramatic decrease in Treg frequency in all tissues. This was further amplified by the deletion of both p65 and c-Rel, demonstrating a partially redundant role of both NF- κ B subunits in homeostasis of peripheral Treg cells.

Finally, we assessed the potential role of each NF- κ B subunit in iTreg induction in vitro. Upon stimulation with interleukin-2

(IL-2) and transforming growth factor- β (TGF- β), cells lacking *Rela* gave rise to normal proportions of FoxP3⁺ cells (Figure 1G). Naive T cells lacking *Rel* exhibited a partial defect in iTreg induction that was rescued by increasing doses of TGF- β . However, full ablation of the NF- κ B canonical pathway, by deletion of both *Rela* and *Rel*, almost completely abolished the in vitro differentiation of naive T cells into iTreg cells. These results suggested that, although p65 and c-Rel partially compensated for one another, they also played discrete roles in multiple steps of both nTreg and iTreg development.

Treg-Specific Deletion of c-Rel Leads to a Late and Mild Inflammatory Phenotype

To bypass the block imposed by loss of NF- κ B on Treg development and assess the role of NF- κ B subunits in the homeostasis and function of mature Treg cells, we deleted *Rel* in Tregs, but not Tconv cells, using the *Foxp3^{YFP-cre}* deleter strain (Figure S2A). We did not observe early lethality or systemic inflammation (Figure 2A and data not shown) in *Foxp3^{YFP-cre}Rel^{fl/fl}* mice. Although 6- to 8-week-old *Foxp3^{YFP-cre}Rel^{fl/fl}* mice did not show any overt signs of illness, lung and liver histology revealed a modest increase in lymphocyte infiltration relative to the WT controls (Figure 2B). Moreover, the cellularity of the peripheral LN, but not the spleen, was significantly increased (Figure 2C). The percentage and number of activated CD4⁺ and CD8⁺ T cells were similar between WT and *Foxp3^{YFP-cre}Rel^{fl/fl}* mice in spleen (Figures 2D–2F), but there was a mild increase in CD8⁺ T cell activation in LN (Figure S2B). We observed a trend toward increased production of IL-17 and interferon (IFN)- γ by splenic CD4⁺ T cells in *Foxp3^{YFP-cre}Rel^{fl/fl}* mice (Figures 2G and 2H). Also, production of IFN- γ , but not IL-17, by CD4⁺ T cells in the colon lamina propria and skin was increased in *Rel*-deficient mice (Figures S2E and S2F). The proportion and number of Treg cells were unaffected by the absence of c-Rel (Figures 2I and 2J and S2C). This data showed that c-Rel in Tregs was mostly dispensable for the maintenance of immune tolerance despite its crucial role in thymic Treg development.

Although aged *Foxp3^{YFP-cre}Rel^{fl/fl}* mice did not exhibit any obvious morbidity, 8-month-old mutant mice showed overt splenomegaly and lymphadenopathy (data not shown), and liver and lung histology revealed increased lymphocytic infiltrates (Figure S2G). We also observed an increase in the frequency of effector T cells with a skewing toward IFN- γ secreting T helper 1 (Th1) cells in spleen and LN (Figure S2H). Finally, we tested the suppressive function of c-Rel deficient Treg in T cell transfer-induced colitis, in which naive Tconv cells were transferred to *Rag1^{-/-}* recipients alone or with WT or mutant Tregs. In contrast with WT Tregs, *Rel*-deficient cells were completely unable to protect the recipient animals from weight loss or intestinal pathology (Figures 2K and 2L) suggesting a role for c-Rel in Treg suppressive function. Taken together, this data showed that c-Rel was largely dispensable for maintenance of immune tolerance but was important for regulation of Treg function in lymphopenic hosts.

Mice with Treg Specific Deletion of NF- κ B p65 Develop a Severe Inflammatory Phenotype

To assess the potential role of p65 in Tregs, we crossed *Rela*-floxed mice with *Foxp3^{YFP-cre}* mice, which resulted in selective

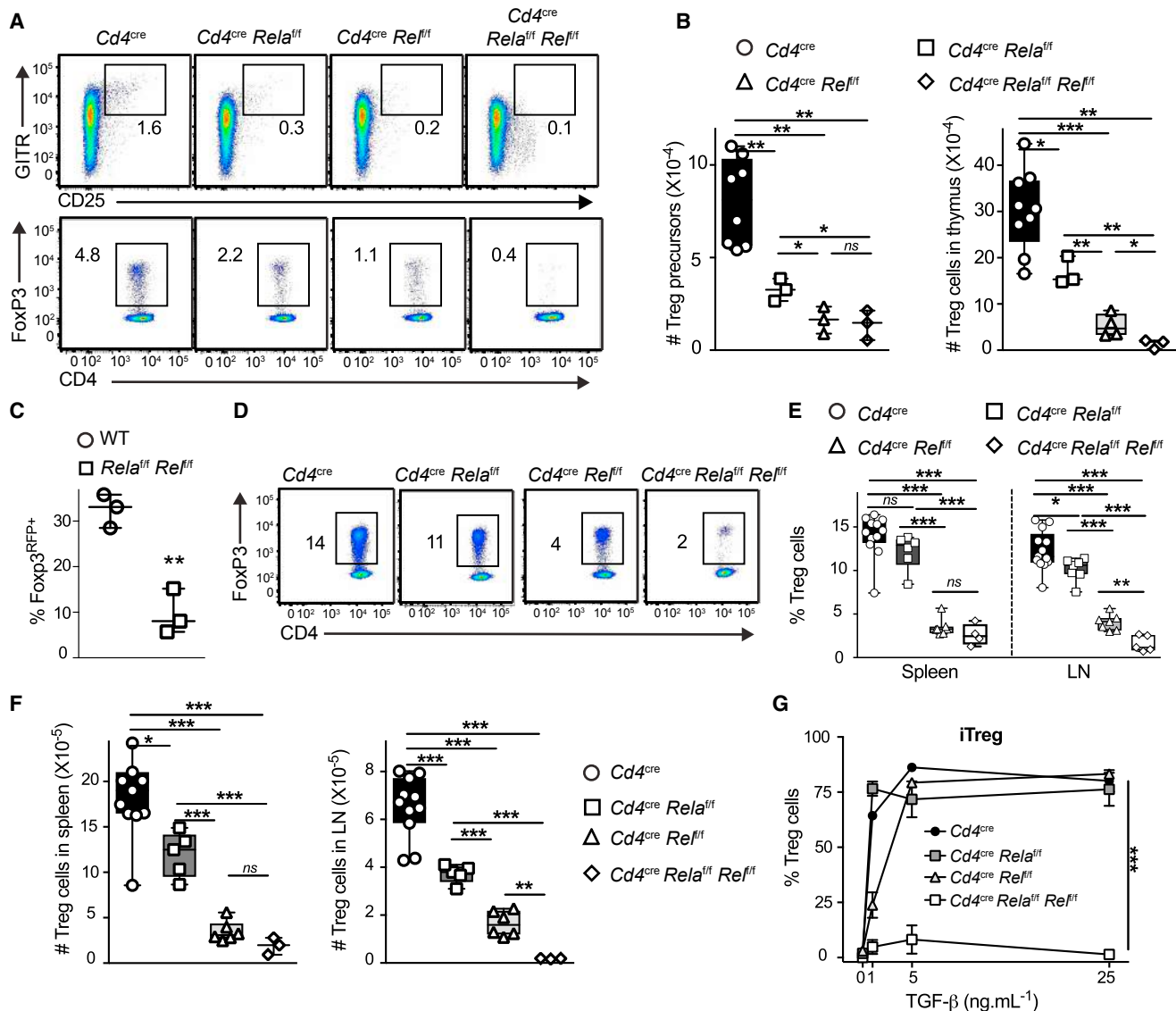


Figure 1. Discrete NF- κ B Subunits Control Sequential Steps of Treg Cell Development

(A and B) Thymii from 5- to 7-week-old *Cd4^{cre}* mice crossed to mice bearing floxed alleles of *Rela* and *Rel*, were analyzed by flow cytometry. (A) Representative dot plots of gated live $CD4^+CD8^-$ FoxP3⁻ (top) and $CD4^+CD8^-$ cells (bottom). Numbers indicate the percentage of cells in each gate. (B) Absolute numbers of $CD4^+CD8^-$ FoxP3⁻CD25⁺GITR⁺ Treg precursors (left) and $CD4^+CD8^-$ FoxP3⁺ Treg cells.

(C) $CD4^+CD8^-$ FoxP3^{RFP}CD25⁺GITR⁺ cells were FACS-sorted from *Foxp3^{RFP}* (WT) and *Foxp3^{RFP}Rela^{fl/fl}Rel^{fl/fl}* thymii, treated with TAT-CRE and cultured with IL-2 for 2 days before analysis. Graph shows the percentage of FoxP3⁺CD25⁺ Treg in gated live cells.

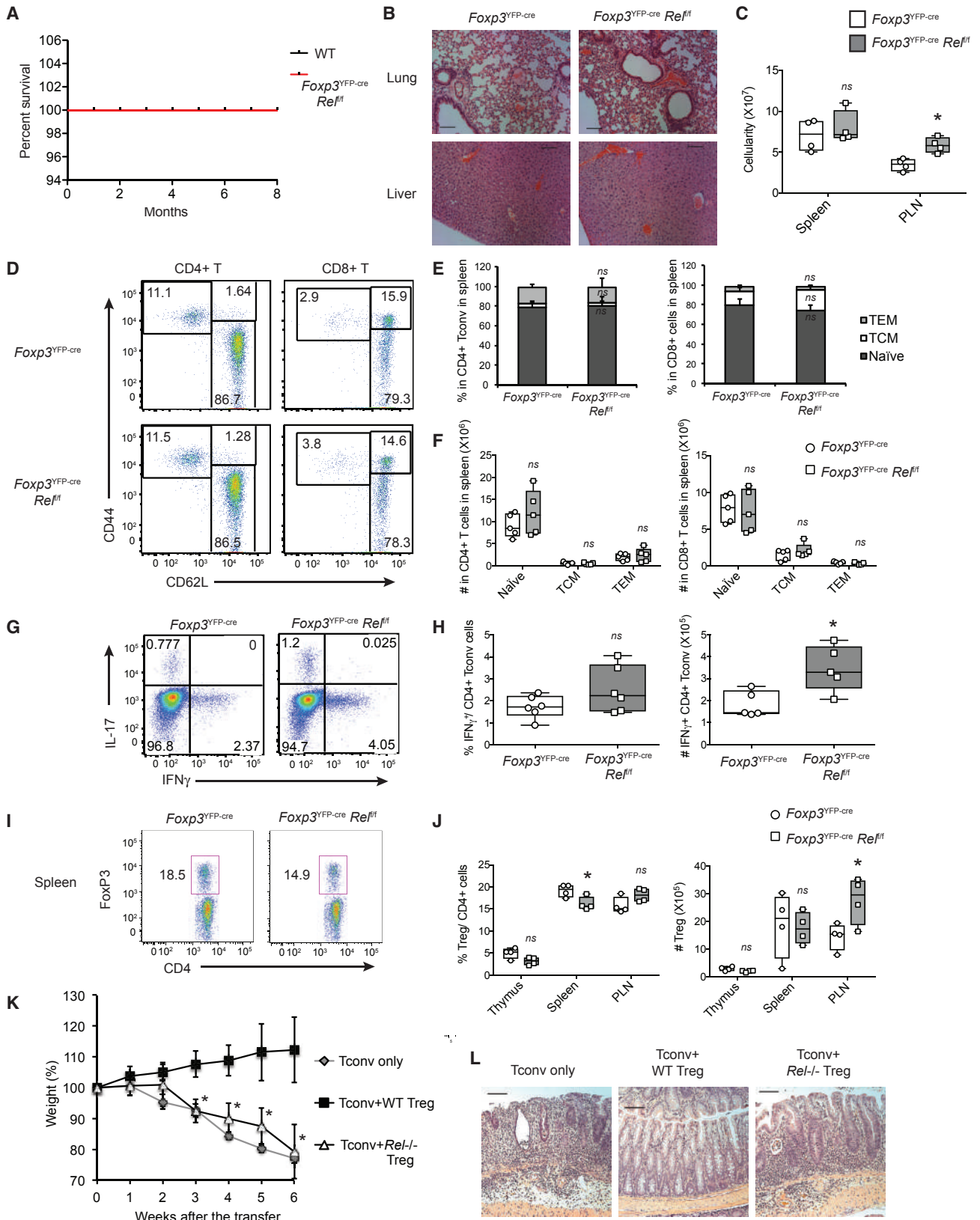
(D–F) FACS analysis of lymphoid tissues of mice described in (A). (D) Representative FoxP3 expression in gated live TCR β^+ CD4⁺CD8⁻. Numbers indicate the percentage in the gate. (E and F) Percentage (E) and absolute numbers of TCR β^+ CD4⁺CD8⁻ FoxP3⁺ Treg cells (F).

(G) $CD4^+CD25^-$ CD44^{low} naive T cells were sorted from the LN of indicated mice and stimulated with grading doses of TGF- β . Graph shows the percentage of FoxP3⁺ among CD4⁺ cells after 4 days. In (B), (C), (E), and (F), data is represented as mean \pm SEM of at least 3 experiments ($n = 4$ –10 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. non-significant. See also Figure S1.

loss of p65 in Treg, but not Tconv cells (Figure S3A). *Foxp3^{YFP-cre}Rela^{fl/fl}* mice were smaller than their WT littermates (data not shown) and showed accelerated mortality, with all mice dying between 5–15 weeks after birth (Figure 3A). This was associated with lymphoproliferative disease including splenomegaly and lymphadenopathy, as well as lymphocytic infiltration of lung and liver (Figures 3B and 3C and data not shown). Dorsal skin sections from *Foxp3^{YFP-cre}Rela^{fl/fl}* mice re-

vealed hyperplastic epidermis and a robust immune cell infiltration with significant epidermal thickening (Figures S3B and S3C). Moreover, there was a dramatic increase in frequencies and numbers of CD4⁺ and CD8⁺ T cells secreting IFN- γ and with an activated/memory phenotype (Figures 3D–3H and S3D).

Despite the lymphoproliferative disease, the proportion and number of Treg cells were in fact increased in secondary lymphoid tissues (Figures 3I and 3J and S3E). p65-deficient



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Tregs displayed normal expression of CD25 and Helios, which are important factors for Treg homeostasis (Figure S3F). This suggested a defect in the suppressive function of Tregs lacking p65. Tregs from *Foxp3*^{YFP-cre}*Rela*^{f/f} mice were able to suppress proliferation of Tconv cells in vitro (Figure S3G). However, *Rela*^{-/-} Treg cells were completely unable to rescue recipient mice from colitis in an in vivo suppression assay (Figures 3K and 3L). This, together with the increased production of IFN γ and IL-17 by CD4⁺ T cells in multiple tissues of *Foxp3*^{YFP-cre}*Rela*^{f/f} mice (Figures S3H and S3I), demonstrated that p65 is essential for Treg-dependent maintenance of immune tolerance.

Complete Ablation of Canonical NF- κ B in Tregs Leads to a Scurfy-like Phenotype

Our results (Figure 1) suggested that c-Rel and p65 exhibited partially redundant functions, and therefore to test this prediction we generated *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice (Figure S4A). Consistent with our hypothesis, these mice showed a profound Scurfy-like phenotype with robust cutaneous inflammation and severe runting (data not shown). *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice died between 2 and 4 weeks after birth (Figure 4A) and demonstrated pronounced lymphocytic infiltration in lungs and liver (Figure 4B), as well as severe splenomegaly and lymphadenopathy (Figure 4C). *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice had significantly higher numbers and frequencies of CD44^{high} central and effector memory CD4⁺ and CD8⁺ T cells (Figure 4D–4F and S4B). There was also an increase in the number and frequency of CD4⁺ Tconv cells from mutant mice that produced IFN- γ (Figures 4G and 4H). In addition, we also detected an increase in antibodies recognizing double stranded DNA (anti-dsDNA antibodies) in *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice (Figure S4E), similar to observations in Scurfy mice (Aschermann et al., 2013). Thus, canonical NF- κ B in Treg cells is required for cellular and humoral tolerance.

This phenotype observed in *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice suggested a profound loss of Treg homeostasis. *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice had decreased Treg frequency in all examined tissues (Figures 4I and 4J and S4C). Also, the mean fluorescence intensity (MFI) of Helios and CD25 was decreased in NF- κ B-deficient Treg cells (Figure S4D). We next investigated whether those Treg cells were functional in vivo. In contrast to WT cells, *Rela*^{-/-}*Rel*^{-/-} Tregs were completely unable to rescue *Rag1*^{-/-} mice from colitis induced by transfer of WT CD4⁺ Tconv cells (Figures 4K and 4L). Increased production of inflammatory cytokines in multiple tissues also confirmed the inability of *Rela*-*Rel*-deficient Treg cells to suppress effector and systemic inflammatory responses (Figures S4F and S4G). Thus, complete ablation of canonical NF- κ B signaling impairs Treg homeostasis and function.

This highlighted the crucial role of the canonical NF- κ B pathway in the maintenance of immune tolerance.

Canonical NF- κ B Maintains the Function and Identity of Mature Treg Cells

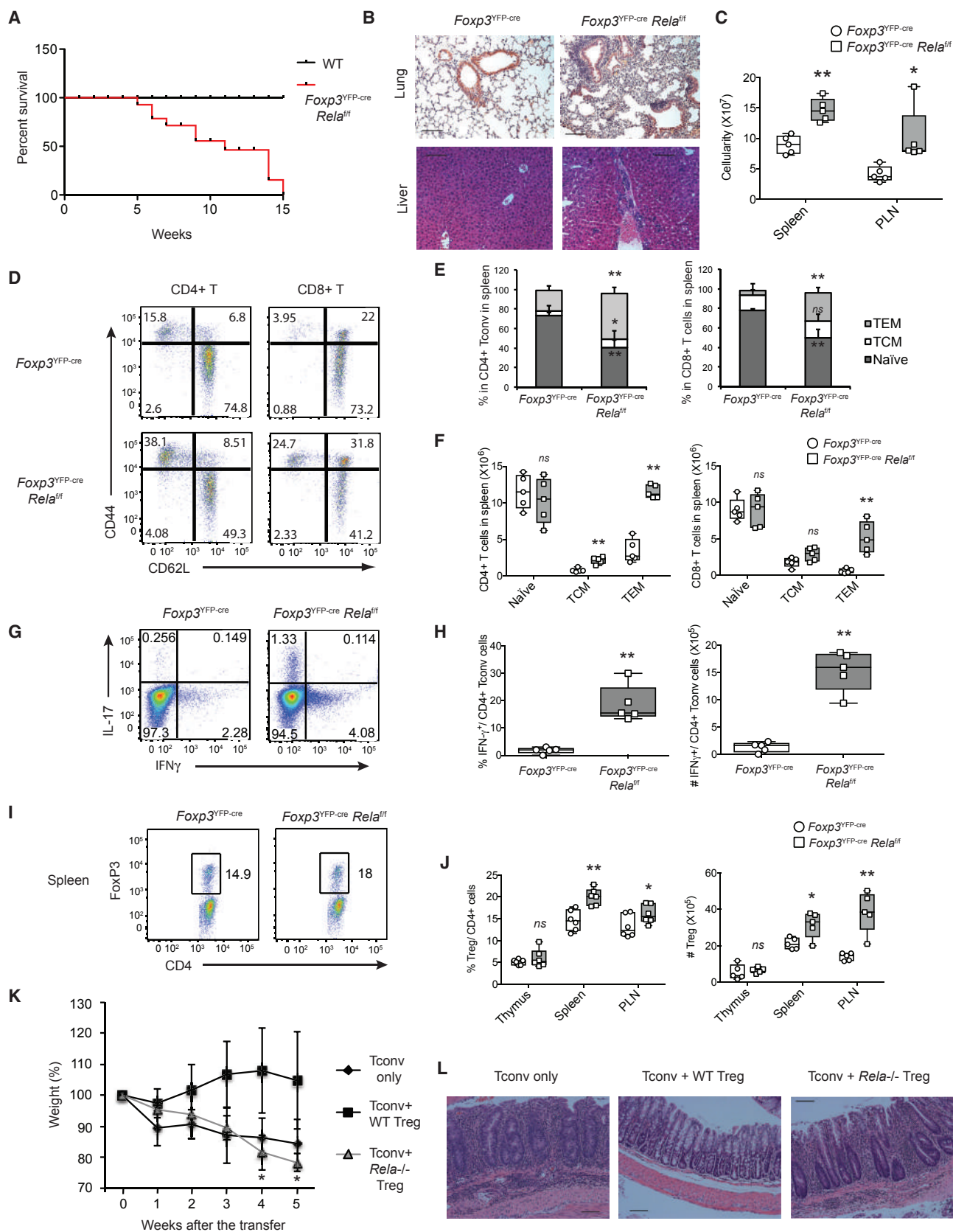
We next investigated whether NF- κ B was continuously required for mature Treg function. We induced conditional deletion of p65 and c-Rel in peripheral Tregs by administering tamoxifen to *Rela*- and *Rel*-floxed mice crossed with *Foxp3*^{eGFP-Cre-ERT2}*xRosa26*^{stop-eYFP} mice (Rubtsov et al., 2010) (Figure 5A). In these mice, all Tregs express green fluorescent protein (GFP), but tamoxifen treatment induces expression of Cre and yellow fluorescent protein (YFP), allowing isolation of NF- κ B deleted Treg cells by flow cytometry. We assessed Treg frequency 7 days after initiating tamoxifen treatment. The percentages of FoxP3⁺ Tregs were significantly reduced upon deletion of *Rel* and *Rel+Rela* (Figure 5B), suggesting that NF- κ B played a role in the maintenance of Tregs or Treg identity. Moreover, the MFI of staining for FoxP3, as well as CD25 and GITR, was decreased in Tregs following inducible deletion of *Rel* or both *Rel* and *Rela*, but not in cells in which only *Rela* was deleted (Figure 5C). We also observed a loss of *Foxp3* mRNA expression in *Rel+Rela* deleted cells (Figure 5D). These data suggested that canonical NF- κ B activity and, in particular c-Rel, was essential for the maintenance of the mature Treg population. We next tested the in vivo suppressive function of Treg cells with inducible deletion of *Rel* and *Rela* (induced-double knockout, iDKO Treg). Similar to *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} Tregs, iDKO (*TMX-Foxp3*^{eGFP-Cre-ERT2}*Rosa26*^{stop-eYFP}*Rela*^{f/f}*Rel*^{f/f}) Tregs failed to prevent development of colitis in *Rag1*^{-/-} mice (Figures 5E and 5F). This data established an essential role for NF- κ B in Treg function in vivo and suggested the canonical NF- κ B pathway might play an important role in the maintenance of Treg identity.

Canonical NF- κ B Maintains the Treg Transcriptome

The finding that NF- κ B was required for Treg suppressive function is consistent with NF- κ B being a key mediator of transcriptional events downstream of TCR ligation. However, it is unclear how NF- κ B fulfills this role in both Treg and Tconv cells. Our finding that loss of canonical NF- κ B in Tregs resulted in a loss of Treg function led us to hypothesize that NF- κ B controls a distinct, lineage specific, transcriptional program in Treg cells. To delineate the NF- κ B-dependent transcriptomes in Tregs, we used high-throughput RNA-sequencing (RNA-seq) analysis to compare the global gene-expression patterns between Tregs of various genotypes and WT Tconv cells stimulated or not with

Figure 2. c-Rel Is Mainly Dispensable for Treg Homeostasis at Steady-State

(A) Survival curves of WT (*Foxp3*^{YFP-cre}*Rel*^{f/f}) and *Foxp3*^{YFP-cre}*Rel*^{f/f} littermates. (B) H/E staining of lung and liver sections from 6-week-old mice. Scale bars represent 100 μ m; original magnification: 100X. (C–J) Lymphoid tissues of 6-week-old mice were analyzed by flow cytometry. (C) Total cell count in spleen and LN. (D) Representative CD44 and CD62L expression in spleen T cells. (E and F) Percentage and numbers of naive (CD44^{low}CD62L^{hi}), TCM (CD44^{hi}CD62L^{hi}), and TEM (CD44^{hi}CD62L^{low}) in spleen T cells. (G) Representative IL-17A and IFN- γ expression in spleen CD4⁺ T cells. Numbers indicate the percentage in quadrants (H) percentage and numbers of IFN- γ ⁺CD4⁺ T cells in spleen. (I) Representative FoxP3 expression in spleen CD4⁺ T cells. Numbers indicate the percentage in the gate. (J) Cumulative percentage and number of Treg cells in spleen. (K–L) In vivo colitis suppression assay. (K) Weight curves, shown as percentage of original weight. (L) Representative colon histology 6 weeks after transfer. Scale bars represent 100 μ m; original magnification: 100X. In (C) and (F)–(J), mean \pm SEM is shown. Data is representative or cumulative of five mice per group from two experiments. *p < 0.05, **p < 0.005. See also Figure S2.



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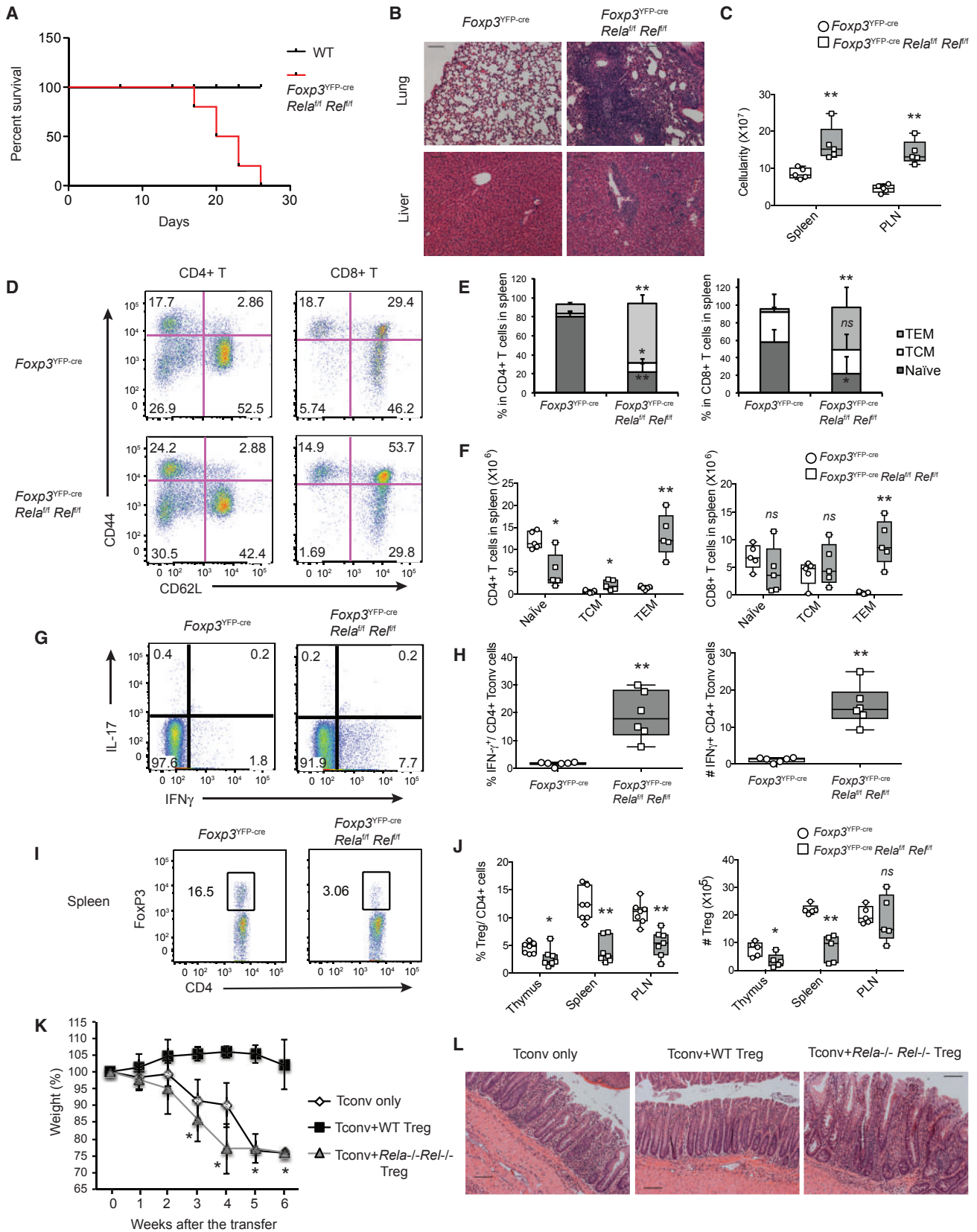
anti-CD3 and anti-CD28. Unsupervised hierarchical clustering (Figure 6A) of the data by genotype indicated a profound effect of NF- κ B on the Treg transcriptome. As expected, based on the disease phenotype observed, single deletions of *Rela* or *Rel* induced largely distinct perturbations in gene expression. *Rela*^{-/-}*Rel*^{-/-} Tregs were distinct from WT Tregs, *Rela*^{-/-}, and *Rel*^{-/-} Tregs, clustering as an outlier group. Furthermore, we found that stimulated and unstimulated Tregs clustered together, segregating by genotype rather than by presence or absence of stimulation. When additional published Treg and Tconv datasets were included in clustering (Wakamatsu et al., 2013), *Rela*^{-/-}*Rel*^{-/-} Tregs clustered together with WT Tconv cells, emphasizing the loss of Treg signature gene expression upon NF- κ B deletion (Figure S5A). The distinct set of genes in *Rela*^{-/-}*Rel*^{-/-} containing loss of Treg signature genes was also observed in the clustering of our RNA-seq dataset without public data, using the genes with ANOVA fold change higher than 1 ($p < 0.05$) (Figure S5B). Principal component (PC) analysis confirmed that *Rela*^{-/-}*Rel*^{-/-} Tregs exhibit a distinct transcriptome in comparison to either single knockout or WT Tregs (Figure S5C). While the first PC, accounting for 32.9% of the variance, established *Rela*^{-/-}*Rel*^{-/-} Tregs as having a divergent transcriptional profile, PC2 and PC3, together accounting for 31.2% of the variance, emphasized the transcriptional similarity between *Rela*^{-/-}*Rel*^{-/-} Treg and Tconv cells (Figure S5C). Consistent with both the PC analysis and clustering, we found that gene expression was equally impaired in unstimulated and stimulated *Rela*^{-/-}*Rel*^{-/-} Tregs (Figure 6B).

Although NF- κ B can both activate and repress gene expression, c-Rel and p65 are generally considered positive regulators of transcription. However, almost equal numbers of genes were up- and downregulated upon loss of these NF- κ B subunits in Tregs (Figure 6C). We also found that the majority of genes differentially expressed in *Rela*^{-/-}*Rel*^{-/-} versus WT Tregs cells were not significantly altered in either *Rel* or *Rela*-deficient Tregs (Figure 6C). This, together with the partial phenotypic overlap resulting from Treg specific gene deletion (Figures 2 and 3), suggested considerable compensation between p65 and c-Rel. We found that numerous Treg signature genes were downregulated, and conversely expression of inflammatory cytokine transcripts increased, in *Rela*^{-/-}*Rel*^{-/-} Tregs compared to WT Tregs in both stimulated and unstimulated cells (Figures 6B and 6D). In particular, the expression of *Ifng*, *Il17a*, *Il2*, *Il4*, and *Il13* was increased at the mRNA and protein levels in *Rela*^{-/-}*Rel*^{-/-} Tregs (Figure 6E, S5F). Gene set enrichment analysis (GSEA) revealed significant enrichment of Treg genes as well as chemokine, cytokine, and transcription factor genes (Figure S5E). Within the Treg

gene set, expression of *Foxp3*, as well as two transcription factors that act cooperatively with FoxP3, *Ikzf2* (Helios), and *Ikzf4* (Eos) were reduced in Tregs from *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice (Figure 6D, left panel, and S5F). Similarly, the Treg signature genes, *Cd83*, *Gpr83*, and *Lrrc32* (GARP), were also downregulated in *Rela*^{-/-}*Rel*^{-/-} Tregs. Additional genes implicated in Treg homeostasis that were significantly altered in *Rela*^{-/-}*Rel*^{-/-} Tregs included chemokines and chemokine receptors, e.g., *Ccr7* and *Ccr6* (Figure S5E). These results suggested that deficiencies in Treg localization and recruitment might potentially explain why the defect in function of Treg lacking NF- κ B manifested in vivo, but not in vitro. We also noted that several cytokines such as *Il22*, *Il4*, and *Il13*, which are not expressed in WT Treg cells, were upregulated in *Rela*^{-/-}*Rel*^{-/-} Tregs and further increased upon TCR stimulation (Figures 6B and 6D and Figures S5D–S5F). As expression of *Foxp3* was downregulated in *Rela*^{-/-}*Rel*^{-/-} Treg cells, we also assessed whether the impairment of *Rela*^{-/-}*Rel*^{-/-} Treg transcriptome could be the consequence of FoxP3 loss rather than a direct effect of NF- κ B ablation. Comparison of FoxP3-dependent genes (Gavin et al., 2007) with *Rela*^{-/-}*Rel*^{-/-}-affected genes revealed only a partial overlap (Figure S5G). While some prototypic Treg genes with decreased expression in *Rela*^{-/-}*Rel*^{-/-} Tregs, such as *Ctla4* or *Ikzf2*, were FoxP3-dependent, GSEA of the 2653 *Rela*^{-/-}*Rel*^{-/-}-affected FoxP3-independent genes also highlighted a loss of the Treg signature (Figure S5H). Thus, ablation of NF- κ B led to a loss of Treg identity not only indirectly through the downregulation of FoxP3 expression but also by directly affecting expression of NF- κ B-dependent genes. As *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice develop an early systemic inflammatory syndrome, we then asked whether the altered gene expression in *Rela*^{-/-}*Rel*^{-/-} Tregs was due to the intrinsic absence of NF- κ B in the cells, or due to inflammatory cytokines in the local environment. The modest but significant increase of the expression of *Ifng*, *Il5*, and *Il22* in *Rel*-deficient Tregs was a first indication that loss of NF- κ B subunits led toward a Teff-like phenotype independent of local inflammation as young *Foxp3*^{YFP-cre}*Rel*^{f/f} mice do not display any autoimmune phenotype (Figure 2). To further analyze the effect of NF- κ B-ablation independently of the local milieu, we deleted floxed *Rela* and *Rel* in vitro, using TAT-Cre. Gene-expression patterns of control and iDKO Tregs were then compared by RNA-seq. As observed upon in vivo deletion of NF- κ B, iDKO Tregs exhibited increased expression of various inflammatory cytokines such as *Il17a*, *Il22*, *Ifng*, and *Il4* (Figure 6F). Moreover, some hallmarks of Treg identity, such as *Foxp3*, *Cd83*, and *Id3*, were also downregulated. This demonstrated that NF- κ B exerts a cell-intrinsic effect to maintain Treg identity

Figure 3. Mice Lacking NF- κ B p65 in Treg Cells Develop Lethal Autoimmune Syndrome

(A) Survival curves of WT (*Foxp3*^{YFP-cre}*Rela*^{+/+}) and *Foxp3*^{YFP-cre}*Rela*^{f/f} littermates. (B) H/E staining of lung and liver sections from 4- to 6-week-old mice. Scale bars represent 100 μ m; original magnification: 100X. (C–I) Lymphoid tissues of 4–6 week-old mice were analyzed by flow cytometry. (C) Total cell count in spleen and LN. (D) Representative CD44 and CD62L expression in spleen T cells. (E and F) Percentage and numbers of naive (CD44^{low}CD62L^{hi}), TCM (CD44^{hi}CD62L^{hi}), and TEM (CD44^{hi}CD62L^{low}) in spleen T cells. (G) Representative IL-17A and IFN- γ expression in spleen CD4⁺ T cells. Numbers indicate the percentage in quadrants. (H) Percentage and numbers of IFN- γ ⁺CD4⁺ T cells in spleen. (I) Representative FoxP3 expression in spleen CD4⁺ T cells. Numbers indicate the percentage in the gate. (J) Cumulative percentage and number of Treg cells in spleen. (K–L) In vivo colitis suppression assay. (K) Weight curves, shown as percentage of original weight. (L) Representative colon histology 5 weeks after transfer. Scale bars represent 100 μ m; original magnification: 100X. In (C) and (F)–(J), mean \pm SEM is shown. Data is representative or cumulative of five mice per group from three experiments. * $p < 0.05$, ** $p < 0.005$. See also Figure S3.



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and prevent the expression of Tconv-specific genes. Taken together, this analysis suggested that NF- κ B regulates distinct transcriptional programs in Treg and Tconv cells and demonstrated an important role for NF- κ B in maintenance of a lineage-specific transcriptome in Tregs.

NF- κ B p65 Directly Coordinated the Expression of Treg-Signature Genes

To characterize lineage specific, differential DNA binding patterns by NF- κ B in Treg and Tconv cells, we used chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq). After identifying putative p65 occupancy sites using the irreproducible discovery rate (IDR) framework (Li, 2011) as outlined by ENCODE, we compared the number of peaks between populations. A fraction of peaks were detected both with and without stimulation, and these were mostly overlapping between Treg and Tconv (Figure 7A). To define a more rigorous set of overlapping regions putatively bound by p65 across all four conditions, we identified peaks in stimulated Treg cells that were also observed in stimulated Tconv cells, as well as in both unstimulated Treg and Tconv cells. We named this subset of 1,571 peaks associated with 1,297 genes “constitutive” because of the relatively high enrichment compared to input across all 4 samples (Figure 7B). GSEA on these “constitutive” p65-bound genes showed genes such as cell cycle-related genes (e.g., *Cdk4* and *Birc5*) (Figure S6A). Interestingly, at a number of peaks, binding intensity was strongly increased upon stimulation in both Tconv and Treg similarly, confirming that NF- κ B activation and binding to chromatin is induced upon TCR+CD28 signaling (Figures 7A and 7B). We defined a set of shared peaks in both Treg and Tconv stimulated cells that were also differentially enriched in stimulated versus unstimulated Treg cells, but not significantly differentially occupied between Treg and Tconv (see STAR Methods). We named this subset “Treg-Tcon shared” and found that this gene set corresponding to 3552 genes was enriched for genes such as cytokines and cytokine receptors, e.g. *Tnf*, as well as known NF- κ B-induced genes such as *Nfkbia* ($\text{I}\kappa\text{B}\alpha$) (Figure S6B). We also detected a number of peaks that were significantly higher in stimulated Treg compared to Tconv. This final set of p65 peaks specific to stimulated Treg cells was defined by identifying stimulated Treg binding sites differentially enriched over both unstimulated Treg and stimulated Tconv cells. Of the 432 genes putatively associated with these 859 peaks, several fall within the well-described set of Treg signature genes such as *Foxp3*, *Ilkzf2*, and *Lrrc32* (Figure 7F). GSEA of this “Treg-stim enriched” population was enriched in genes highly expressed in Treg versus Tconv (Figure S6C). Only a few peaks were specifically detected in stimu-

lated Tconv but not Treg cells, suggesting that p65 binding could be influenced by a Treg-specific environment that does not exist in a Tconv-specific landscape. The distribution of peak distances from the nearest transcription start site (TSS) and the distribution of genomic annotations associated with those peaks showed significant variation between different samples (Figure S6D). In contrast with constitutive peaks, Treg stim-enriched peaks showed marked increase in the fraction of peaks located in distal intergenic regions, suggesting that they might be binding to putative enhancers. These peaks contributed to the overall increase in Treg stim peaks (Figure 7A). Our data demonstrated that TCR+CD28-induced NF- κ B binding is different in Treg and Tconv, suggesting that the Treg-lineage environment influences NF- κ B binding and function.

We next looked for p65 binding motif enrichment in each gene subset using SeqGL (Setty and Leslie, 2015), as described in STAR Methods. We found an enrichment for a Rel Homology domain-like motif nearby both Treg stim-enriched and Treg-Tcon shared summits (Figure 7C). The peaks shared by Tconv and Treg were enriched with motifs for various transcription factors such as CREB, ETS, and RUNX1 in addition to NF- κ B REL-binding motifs (Figure 7C). On the other hand, Treg-enriched peaks were predominantly enriched only with REL-binding motifs. This suggests that preferential binding of p65 to those sites in Treg over Tconv might not be due to unique unconventional binding motifs.

We then analyzed whether Treg-specific p65-binding could simply rely on a differential chromatin accessibility in Treg and Tconv cells. DNase hypersensitivity (HS) experiments have previously demonstrated a discrete chromatin accessibility in Treg and Tconv (Samstein et al., 2012). To assess a possible role for accessibility in p65-binding, we aligned their DNase HS sequencing data in Treg and Tconv cells to our p65 ChIP-seq dataset. Interestingly, in Treg-enriched peaks, chromatin accessibility was significantly higher in Treg versus Tconv, whereas the opposite trend was observed in Treg-Tconv shared peaks (Figure 7D). This was illustrated by higher DNase HS signals in Treg at numerous Treg-associated genes, such as *Foxp3*, *Ilkzf2*, or *Lrrc32* (Figure 7G). Similar results were obtained when using a recently reported (Kitagawa et al., 2017) ATAC-Seq dataset (Figure 7E). This correlation suggests that chromatin accessibility yields increased p65 binding at Treg-enriched loci, resulting NF- κ B-dependent regulation of the Treg specific transcriptional program.

To further elucidate the direct contribution of p65 binding to the differential gene expression of Tconv and Treg cells, we next focused our analysis on Treg and Tconv specific genes. We first defined a set of 587 “Treg signature” genes whose

Figure 4. Complete Ablation of the Canonical NF- κ B Pathway Drives a Scurfy-like Autoimmune Syndrome

(A) Survival curves of WT (*Foxp3*^{YFP-cre}*Rela*^{+/+}*Rel*^{+/+}) and *Foxp3*^{YFP-cre}*Rela*^{fl/fl}*Rel*^{fl/fl} littermates. (B) H/E staining of lung and liver sections from 2- to 3-week-old mice. Scale bars represent 100 μ m; original magnification: 100X. (C–I) Lymphoid tissues of 2- to 3-week-old mice were analyzed by flow cytometry. (C) Total cell count in spleen and LN. (D) Representative CD44 and CD62L expression in spleen T cells. (E and F) Percentage and numbers of naive (CD44^{low}CD62L^{hi}), TCM (CD44^{hi}CD62L^{hi}), and TEM (CD44^{hi}CD62L^{low}) in spleen T cells. (G) Representative IL-17A and IFN- γ expression in spleen CD4⁺ T cells. Numbers indicate the percentage in quadrants. (H) Percentage and numbers of IFN- γ ⁺CD4⁺ T cells in spleen. (I) Representative FoxP3 expression in spleen CD4⁺ T cells. Numbers indicate the percentage in the gate. (J) Cumulative percentage and number of Treg cells in spleen. (K and L) *in vivo* colitis suppression assay. (K) Weight curves, shown as percentage of original weight. (L) Representative colon histology 6 weeks after transfer. Scale bars represent 100 μ m; original magnification: 100X. In (C) and (F)–(J), mean \pm SEM is shown. Data is representative or cumulative of five mice per group from two experiments. **p* < 0.05, ***p* < 0.005. See also Figure S4.

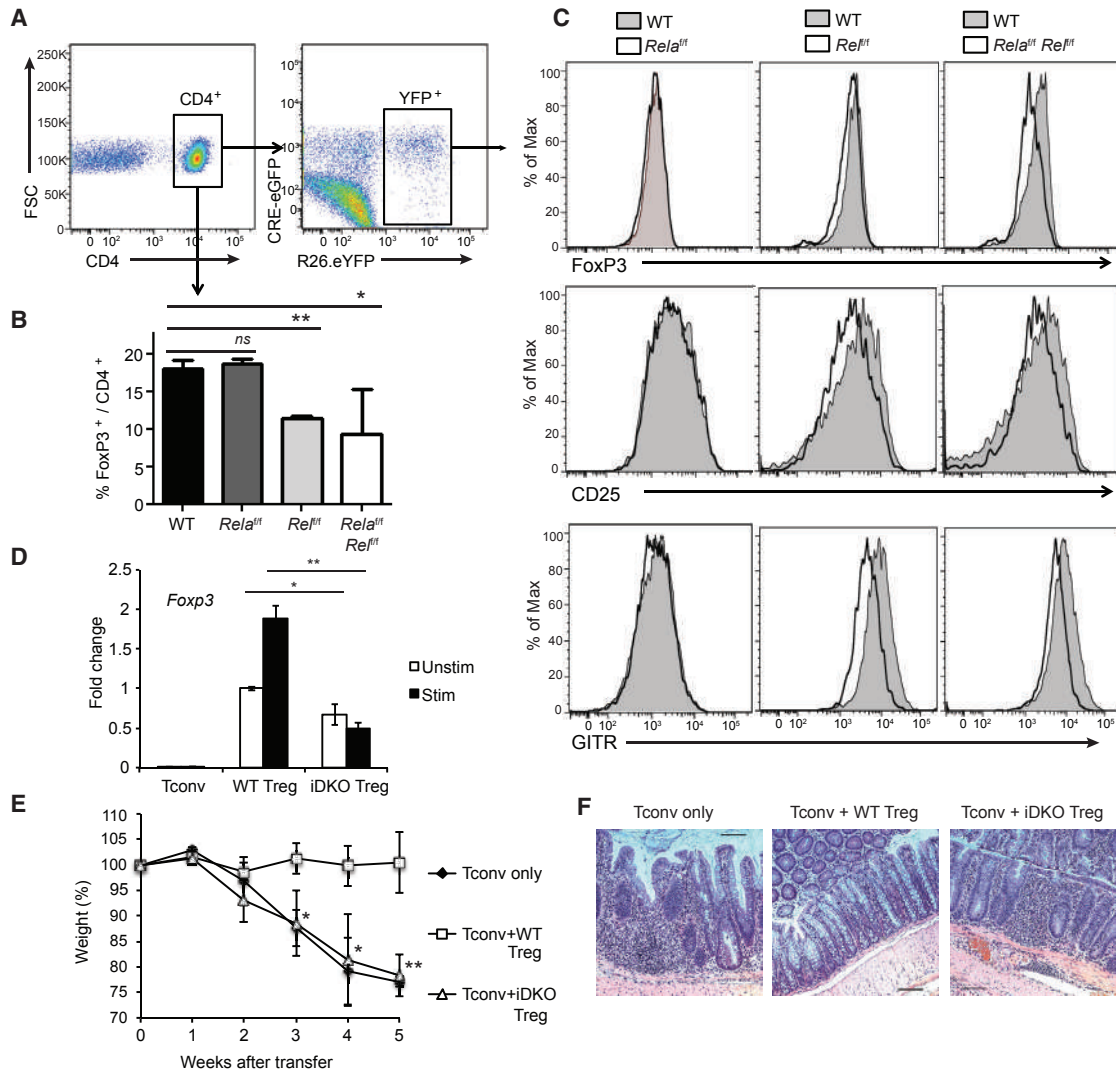


Figure 5. Tonic Canonical NF- κ B Signaling Maintains Treg Homeostasis and Function in Peripheral Mature Treg Cells

(A) Inducible deletion of *Rela*, *Rel*, or both *Rela* and *Rel* in Treg cells was achieved by tamoxifen treatment of *Foxp3*^{eGFP-Cre-ERT2}*xRosa26*^{stop-eYFP} *Rela*^{fl/fl}, *Rel*^{fl/fl}, and *Rela*^{fl/fl} *Rel*^{fl/fl} mice. YFP⁺ cells were gated from CD4⁺ cells and used for flow cytometry analysis.

(B) Cumulative percentage of FoxP3⁺ Treg cells in total spleen CD4⁺ T cells.

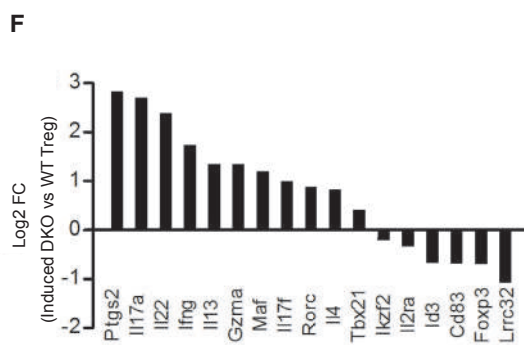
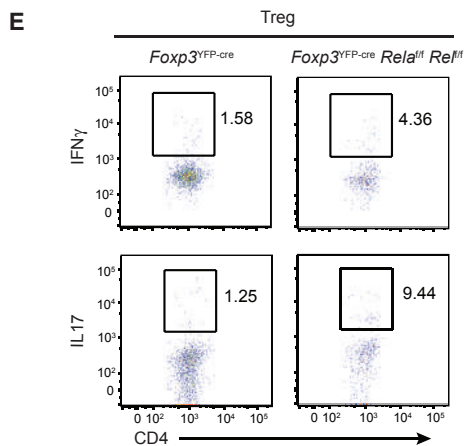
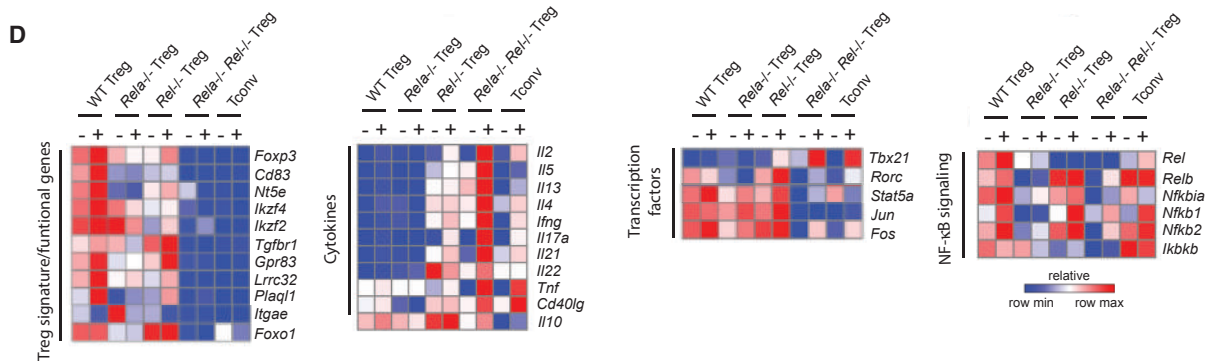
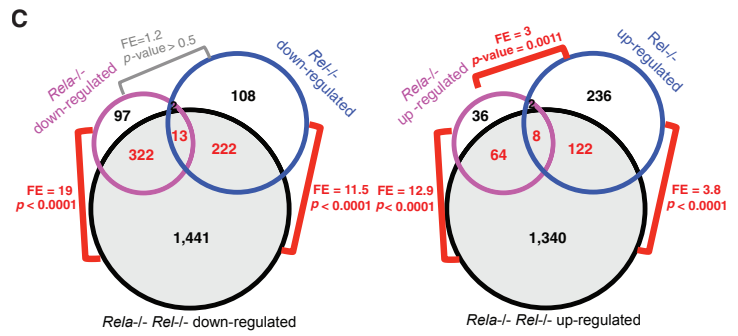
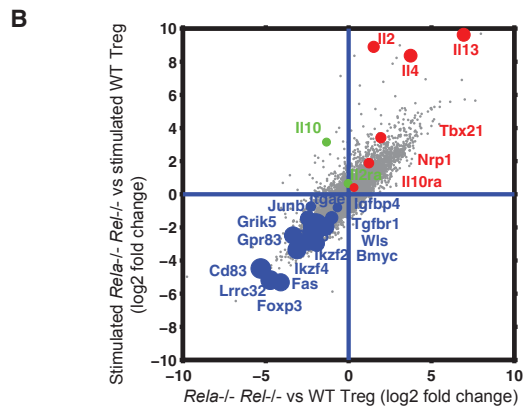
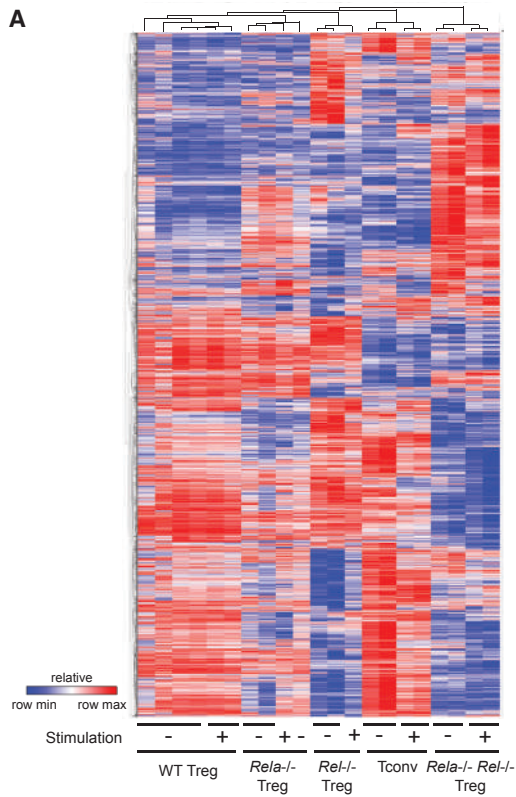
(C) YFP⁺ “recombined” Treg cells were sorted and stained for FoxP3, CD25, and GITR. A representative histogram is shown for each genotype.

(D) Treg cells with inducible deletion of *Rela* and *Rel* after tamoxifen treatment were stimulated or not with anti-CD3 and anti-CD28 antibodies for 3 hr. RNA was prepared from those Treg cells and the expression level of *Foxp3* mRNA was examined by qPCR. The mean \pm SD is shown. Data is representative of six mice per group from three experiments.

(E and F) *in vivo* colitis suppression assay using sorted YFP⁺ recombined Treg cells. (E) Weight curves, shown as percentage of original weight. (F) Representative colon histology 5 weeks after transfer. Scale bars represent 100 μ m; original magnification: 100X. In (C) and (F), mean \pm SD is shown. Data is representative or cumulative of five mice per group from three experiments. **p* < 0.05, ***p* < 0.005.

expression was significantly higher in WT Treg than WT Tconv, based on public datasets (Ye et al., 2014) (Figure 7F). As a counterpart, we used a “Tconv signature” set composed of 211 genes, as well as the total coding genome. We assessed the potential association between p65 binding at Treg-stim enriched, Treg-Tconv shared and constitutive peaks, FoxP3 binding peaks (Samstein et al., 2012), Tconv-Treg and Treg *Rela*^{-/-} *Rel*^{-/-}—WT differentially expressed genes, and Tconv-Treg signature genes. We found a strong enrichment of Treg-specific p65 binding in Treg-signature genes. Expression of many of these genes

was dependent on NF- κ B, as their expression was downregulated in *Rela*^{-/-} *Rel*^{-/-} Tregs (Figure 7F). Interestingly, the fraction of genes bound by both FoxP3 and p65 was higher in Treg-signature genes than in the rest of the genome (Figure 7F and S6E), further underlining the importance of these transcription factors in Treg-specific gene expression. This was illustrated by FoxP3 binding in the vicinity of p65 binding at *Foxp3*, *Ilkzf2*, and *Lrrc32* loci (Figure 7G). Taken together, our results showed that NF- κ B directly directed the expression of Treg-essential genes by accessing specific, open loci in Treg cells.



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DISCUSSION

Even though Tconv and Treg cells develop from the same pool of thymocyte progenitor cells, they have diametrically opposed functions in the immune system. Consistent with their common developmental lineage, Treg and Tconv cells share many of the same cell surface receptors and intracellular signaling pathways. This common nature of T cell signaling events in both Treg and Tconv cell represents a significant barrier to the development of immunomodulatory therapies. Thus, targeting of a common signaling mechanism in both cell types might result in a single agent simultaneously promoting and inhibiting Tconv responses. Given the importance of Tregs in both beneficial peripheral tolerance and detrimental tumor tolerance, it is therefore essential that we understand how the same TCR signaling leads to such different biological functions in Treg and Tconv cells.

It has been proposed that activation of the canonical NF- κ B pathway upon TCR and CD28 triggering is important for normal Treg differentiation. For instance, mice lacking any member of the CARMA-1-Bcl-10-Malt1 complex exhibit a profound lack of FoxP3⁺ thymocytes (Molinero et al., 2009), whereas constitutive activation of NF- κ B through an IKKEE transgene in T cells increases thymic Treg cells (Long et al., 2009). Furthermore, germline deletion of c-Rel leads to reduced transition into the Treg progenitor step and subsequent maturation into FoxP3⁺ Treg cells (Grigoriadis et al., 2011; Isomura et al., 2009). However, deletion of *Rel* does not fully eliminate Treg cells, leaving unclear the role of the canonical NF- κ B pathway. The study of *Rela*^{-/-} mice was complicated by the embryonic lethality of these mice although indirect evidence pointed toward a role for p65 in FoxP3 expression (Deenick et al., 2010; Isomura et al., 2009; Soligo et al., 2011). In the current study, we demonstrated a crucial requirement for both canonical NF- κ B subunits in the generation of FoxP3⁺ Treg precursors. Canonical NF- κ B signaling, along with IL-2 to STAT-5 and I κ B-NS signals (Lio and Hsieh, 2008; Schuster et al., 2012), is crucial for the subsequent expression of FoxP3 and for mature Treg homeostasis in secondary lymphoid organs. We also showed a partial redundancy as absence of both p65 and c-Rel led to nearly complete abolishment of Treg cells in the thymus and the periphery.

To date, only a moderate role for NF- κ B was previously described in the in vitro iTreg polarization (Jana et al., 2009; Visekruna et al., 2010). Here we showed that naive T cells fully lacking canonical NF- κ B were unable to give rise to iTreg, even

in the presence of exogenous IL-2, whereas deletion of only *Rela* or *Rel* had nearly no effect. Thus, our studies demonstrated a redundant but crucial role for canonical NF- κ B activity in iTreg development and highlighted a specific role for each NF- κ B subunit in Treg development in vivo and in vitro.

To examine the role of NF- κ B in Tregs more precisely, we performed Treg-specific conditional deletion of *Rela* and/or *Rel*. The profound autoimmunity observed upon deletion of *Rela* in Tregs was consistent with recently reported results (Messina et al., 2016). However, these mouse models showed that both p65 and c-Rel played essential, although partially redundant, roles in the function of Treg cells. In contrast to its essential role in Treg development, we observed little effect of *Rel* deletion on the ability of Tregs to suppress systemic lymphoproliferative disease. In contrast, mice in which both subunits are deleted in Treg cells display a *Scurfy*-like phenotype with severe lethal lymphoproliferative and autoimmune disease despite only modest reductions in Treg numbers. Therefore, canonical NF- κ B is essential for the function of both Treg and Tconv cells.

The graded phenotype observed in mice lacking c-Rel, p65, or both NF- κ B subunits in Treg offers some hints as to the relative importance of specific canonical NF- κ B regulated genes in Treg function. That is, while *Foxp3*^{YFP-cre}*Rela*^{f/f}*Rel*^{f/f} mice exhibit a *Scurfy*-like phenotype, mice lacking *Rela* in Tregs display a relatively delayed, though still severe phenotype, whereas mice lacking only *Rel* in Tregs show a substantially delayed and mild phenotype. Thus, it might be expected that an analysis of gene expression between genotypes would be instructive in understanding the basis of this graded phenotype. Although expression of specific candidate genes known to be important for Treg function were clearly altered, it seemed that graded changes in expression of several target genes, likely due to partial compensation between canonical NF- κ B subunits, accounted for the difference in Treg suppressive phenotypes observed. When the canonical NF- κ B pathway was completely ablated in Treg cells by deleting both *Rela* and *Rel*, Tregs not only lost suppressive function and their expression of Treg-signature genes but also upregulated the expression of certain Tconv genes, especially inflammatory cytokines. This was due to a specific function of c-Rel, as *Rel*-deficient Tregs, but not *Rela*-deficient Tregs, displayed increased expression of *Ifng* among the cytokines. These data suggested that in Tregs, c-Rel, specifically, was essential for the suppression of effector cytokine expression. Further analysis of the functions of c-Rel

Figure 6. Canonical NF- κ B Shapes the Molecular Identity of Treg Cells

Treg and Tconv cells of the indicated genotypes were FACS-sorted, in vitro-activated or not for 3 hr and submitted to RNA-seq.

(A) Heatmap created by unsupervised hierarchical gene clustering shows normalized expression of transcripts whose expression was changed in at least 1 condition (FC > 1, ANOVA p < 0.05).

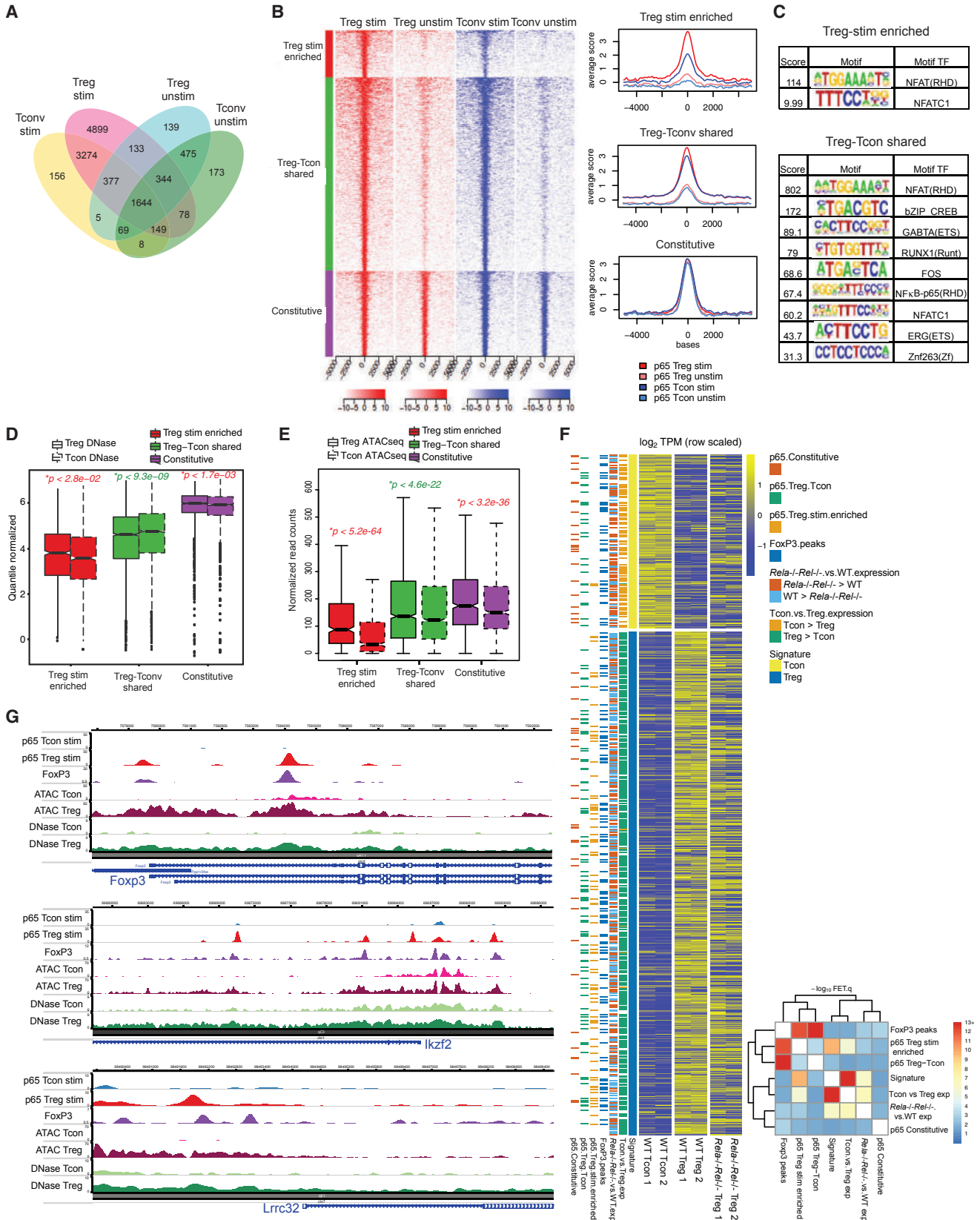
(B) Log₂ fold changes of *Rela*^{-/-}*Rel*^{-/-} Treg versus WT Treg and stimulated *Rela*^{-/-}*Rel*^{-/-} Treg versus stimulated WT Treg. Representative genes from downregulated genes were indicated as blue and representative genes from upregulated genes were indicated as red. The size of the selected genes indicates p values by ANOVA.

(C) 5-way ANOVA analysis was performed to assess the association between genes and different factors (factor 1: Treg or Tconv; factor 2: stimulated or not; factor 3: *Rela*^{-/-}*Rel*^{-/-} or not; factor 4: *Rela*^{-/-} or not; factor 5: *Rel*^{-/-} or not). The Venn diagrams depict downregulated (top) and upregulated (bottom).

(D) Representative genes from the genes with significantly changed expression in *Rela*^{-/-}*Rel*^{-/-} Treg cells were categorized by their function and the expression change was presented as heatmaps.

(E) Representative flow cytometry plots from intracellular staining for IFN- γ and IL-17 in CD4⁺ FoxP3⁺ Treg cells from *Foxp3*^{YFP-cre} and *Foxp3*^{YFP-cre} *Rela*^{f/f}*Rel*^{f/f}.

(F) Treg cells were isolated from *Foxp3*^{eGFP-Cre-ERT2}*xRosa26*^{stop-eYFP} WT and *Foxp3*^{eGFP-Cre-ERT2}*xRosa26*^{stop-eYFP} *Rela*^{f/f}*Rel*^{f/f} after tamoxifen treatment and expression of various genes were examined by qPCR. Log₂ fold changes (Log₂ FC) of gene expression in inducible DKO versus WT were shown. See also Figure S5.



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and p65 in Tregs demonstrated a unique role for c-Rel in activated Treg function and maintenance of tumor tolerance (Grinber-Bleyer et al., 2017). Overall, canonical NF- κ B regulated a lineage specific transcriptome comprising multiple genes with a broad role in the maintenance of Treg identity and function, which suppressed a conventional CD4⁺ T cell phenotype in Tregs.

While canonical NF- κ B is well-known to be critical for effector cell differentiation and expression of inflammatory cytokines in Tconv cells, our findings indicate that the same signaling pathway regulated a dissimilar lineage defining transcriptional program in Treg cells. Thus, the canonical NF- κ B pathway, in both cases activated through ligation of the same TCR complex, activated a qualitatively and quantitatively distinct transcriptional program with opposing biological functions. Two potential mechanisms help explain the divergent role of canonical NF- κ B in Treg and Tconv cells: the extent of NF- κ B activation and access to κ B DNA binding sites. Tregs depend on constitutive TCR signaling for maintenance of suppressive function (Levine et al., 2014). Thus, we, like others (Messina et al., 2016), saw higher levels of nuclear canonical NF- κ B subunits in circulating Tregs compared to Tconv cells, consistent with an ongoing role for NF- κ B in the regulation of the Treg specific transcriptome (data not shown). However, increased canonical NF- κ B activation in Tregs compared with Tconv cells did not account for the divergent transcriptional programs regulated by NF- κ B in the two cell types. TCR ligation induced equivalently robust canonical pathway activation in both cell types, yet NF- κ B regulated distinct sets of genes in Treg and Tconv cells. This suggested that changes in the chromatin landscape in Treg cells might be a major determinant of Treg identity. Consistent with this idea, NF- κ B p65 ChIP-seq identified substantial differences in chromatin binding between Treg and Tconv cells, following TCR stimulation. In particular, the number and intensity of many peaks were higher in Treg cells compared to Tconv cells. These Treg-enriched peaks were often found in Treg-associated genes, such as *Foxp3* or *Ikzf2*, further confirming a direct Treg-lineage specific role for NF- κ B. This specific binding was not due to an altered binding motif. However, DNase-seq and ATAC-seq analyses revealed greater chromatin accessibility in Treg than Tconv cells at these loci. This suggested that lineage-associated p65-binding relied on a Treg-specific chromatin structure rather than on a specific DNA sequence.

The molecular mechanism driving distinct p65 binding in Treg cells remains to be elucidated. A possibility is that other

Treg-specific transcription factors might drive NF- κ B activity, or cooperate with p65, to regulate Treg gene expression. Given that FoxP3 is the lineage specific transcription factor that differentiates Treg and Tconv cells, it is possible that FoxP3 itself coordinates Treg-specific p65-binding and activity. It was previously suggested that FoxP3 and p65 might influence NF- κ B activity, for instance on the *Tnfrsf18* (GITR) locus (Tone et al., 2014). Analysis of our ChIP-seq and public data showed binding for both p65 and Foxp3 on multiple Treg genes, including *Foxp3* itself, *Ikzf2*, *Lrrc32*, or *Ctla4*. Other transcription factors, such as Foxo1/3 or ETS-1, were also suggested to influence the expression of NF- κ B-dependent Treg signature genes (Kerdiles et al., 2010; Ouyang et al., 2012; Samstein et al., 2012). This suggests that other lineage specific factors likely also contributed to the lineage-specific transcriptional program regulated by canonical NF- κ B. Understanding how different chromatin modifying and transcription factors orchestrate the establishment of a Treg-specific transcriptome would be of great interest.

In summary, our studies demonstrated an absolute requirement for canonical NF- κ B for regulatory T cell function in vivo. Given the known roles of canonical NF- κ B in conventional T cell responses, these data suggested that considerable caution is need for the development of immunomodulatory therapeutics that target the NF- κ B pathway. In this regard, the differential roles of p65 and c-Rel in Tregs, identified here, as well as in B and T cell immune responses, previously described, suggest that approaches that selectively target specific components of the NF- κ B signaling pathway might have greater utility compared to inhibitors that shut down the entire pathway, e.g., inhibitors that target IKK. Finally, these studies demonstrated an intriguing functional plasticity for the NF- κ B pathway where even within closely related cellular lineages, the same NF- κ B subunits could regulate highly divergent transcriptional programs with profound biological ramifications.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODELS
 - Mice

Figure 7. NF- κ B Regulates the Treg-Associated Transcriptional Program

(A–F) WT Tconv and Treg cells were isolated and stimulated or not for 3 hr. p65 ChIP was performed and subjected to library preparation and sequencing as described in the Methods section. (A) The Venn diagram represents the number of p65-binding peaks and overlapping and non-overlapping peaks between four groups. (B) Heatmap showing the distribution of mean log₂ p65-ChIP/input signal for Treg stim/unstim (red) and Tconv stim/unstim (blue) around peaks summits, +/- 5 kb flanks from TSS, from each group: Treg-specific (red bar), Treg-Tconv shared (green bar), and constitutive (purple bar). The log₂ p65-ChIP/input signal is also presented as histograms on the right. (C) Enrichment of motifs in Treg-specific peaks and Treg-Tconv shared peaks as determined by the SeqGL algorithm. (D) Comparison of quantile normalized, library-size normalized, DNase accessibility signals between Treg and Tconv around summits (+/- 150bp) of three different peak sets. Mann-Whitney U test p values comparing Treg versus Tconv signal are also provided. (E) Comparison of ATAC-seq signals between Treg and Tconv of three different peak sets. (F) The log₂ TPM counts for two sets of RNA-seq data for Tconv, WT Treg and *Rela*^{-/-}*Rel*^{-/-} Treg (Right side columns of the heatmap) corresponding to Tconv signature and Treg signature genes were displayed as a heatmap. Genes with significant expression changes in *Rela*^{-/-}*Rel*^{-/-} versus WT Treg and Tconv versus Treg were indicated. Genes, within its -10 kb to +5 kb coordinates, associated with p65 constitutive peaks, Tconv-Treg shared peaks, Treg stim-specific peaks and FoxP3 peaks are also indicated along the leftside of the heatmap. The cluster heatmap shows the pairwise -log₁₀ p values for fisher exacts comparing the indicated annotation groups. (G) Example genes from the set of genes including p65 Treg stim-enriched peaks. Tracks were created in WashU epigenome browser and display normalized p65, FoxP3, ATAC-seq, and DNase HS sequencing signal. See also Figure S6.

● METHOD DETAILS

- Cell Isolation
- Flow Cytometry and Sorting
- In Vitro Treg Differentiation
- Western Blotting
- In Vivo Suppression Assay
- In Vitro Suppression Assay
- Quantitative RT-PCR (qRT-PCR)
- Inducible Deletion of Genes by tat-cre Treatment
- Inducible Deletion of Genes by Tamoxifen Treatment
- Histology
- RNA-Seq
- Chromatin Immunoprecipitation (ChIP) Assay for Sequencing
- ChIP Sequencing (Illumina)
- Preliminary Processing of Sequencing Data
- Identification of Regions of Enrichment (peak-calling)
- Peak Annotation
- Differential Detection
- RNA-Seq Analysis
- Transposase-Assessible Chromatin (ATAC)-Seq Analysis

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2017.08.010>.

AUTHOR CONTRIBUTIONS

H.O. and Y.G.-B. carried out the majority of the experiments and helped write the paper. W.L., D.M., P.W., Z.W., D.B., J.W., R.R., carried out and analyzed certain experiments, N.H., U.K., and R.M.S. provided mice strains, M.S.H. analyzed the data and helped write the paper, and S.G. conceived of the study, analyzed the data, wrote the paper, and secured funding.

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The Alternative NF- κ B Pathway in Regulatory T Cell Homeostasis and Suppressive Function

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CD4⁺Foxp3⁺ regulatory T cells (Tregs) are essential regulators of immune responses. Perturbation of Treg homeostasis or function can lead to uncontrolled inflammation and autoimmunity. Therefore, understanding the molecular mechanisms involved in Treg biology remains an active area of investigation. It has been shown previously that the NF- κ B family of transcription factors, in particular, the canonical pathway subunits, c-Rel and p65, are crucial for the development, maintenance, and function of Tregs. However, the role of the alternative NF- κ B pathway components, p100 and RelB, in Treg biology remains unclear. In this article, we show that conditional deletion of the p100 gene, *nfkb2*, in Tregs, resulted in massive inflammation because of impaired suppressive function of *nfkb2*-deficient Tregs. Surprisingly, mice lacking RelB in Tregs did not exhibit the same phenotype. Instead, deletion of both *relb* and *nfkb2* rescued the inflammatory phenotype, demonstrating an essential role for p100 as an inhibitor of RelB in Tregs. Our data therefore illustrate a new role for the alternative NF- κ B signaling pathway in Tregs that has implications for the understanding of molecular pathways driving tolerance and immunity. *The Journal of Immunology*, 2018, 200: 2362–2371.

Maintenance of immune homeostasis and tolerance is achieved through multiple feedback mechanisms. CD4⁺Foxp3⁺ regulatory T cells (Tregs) represent 5–15% of the CD4⁺ T cell population and develop both in the thymus (natural Treg [nTreg]) and the periphery (peripheral Treg [pTreg]). They play a pivotal role in the control of innate and adaptive immune responses (1), and are generally characterized by

the constitutive expression of CD25 and the forkhead-box transcription factor Foxp3, which is required for their suppressive activity (2). Perturbations in the homeostasis and/or function of these cells are associated with the development of autoimmune diseases such as type 1 diabetes and rheumatoid arthritis (3). Depletion of Tregs in mice leads to a lethal multifocal inflammation; moreover, mutations in the *foxp3* locus are responsible for the *Scurfy* phenotype in mice and the IPEX (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked) syndrome in humans (4). Thus, Treg development, maintenance, and function must be tightly regulated through various molecular mechanisms. In recent years, several studies have demonstrated a crucial role for the NF- κ B transcription factor in the development of Tregs and the expression of Foxp3 (5–7).

NF- κ B signaling can be separated in two main pathways. The canonical pathway leads to the phosphorylation and degradation of I κ B α/β and consequent nuclear translocation of the NF- κ B1 p50 protein bound to either c-Rel or p65, whereas the alternative pathway leads to activation of NF- κ B-inducing kinase (NIK), which promotes processing of NF- κ B2 from the full-length precursor protein p100 to the p52 form, which results in formation of transcriptionally active p52:RelB complexes (8, 9). The biological processes regulated by these two NF- κ B pathways are distinct. The canonical p65 and c-Rel subunits are well-defined inducers of inflammation and have clear roles in the activation of B and T cells, whereas the alternative NF- κ B RelB:p52 heterodimer has primarily been implicated in lymphoid tissue organogenesis and cell migration (9, 10). NF- κ B2 and RelB are important regulators of immune tolerance, because *NIK*^{-/-}, *relb*^{-/-}, and *nfkb2*^{-/-} mice develop spontaneous autoimmunity (reviewed in Ref. 11). Importantly, the two NF- κ B pathways are triggered by different receptors and signaling pathways. Upon engagement of the TCR/CD28 Ag receptor, the canonical pathway is activated and NF- κ B complexes containing c-Rel can regulate de novo expression of Foxp3 in developing thymocytes, and therefore c-Rel-deficient animals exhibit fewer nTregs (5–7, 12–15). Recently, we and others have demonstrated a central role for the NF- κ B p65 subunit

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Abbreviations used in this article: BM, bone marrow; GSEA, Gene Set Enrichment Analysis; KO, knockout; LN, lymph node; NIK, NF- κ B-inducing kinase; nTreg, natural Treg; pLN, peripheral LN; pTreg, peripheral Treg; Tconv, conventional CD4⁺ T; Tg, transgenic; Treg, regulatory T cell; WT, wild-type.

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in the maintenance of mature Treg identity and in the prevention of autoimmunity (16, 17). In contrast, c-Rel, but not p65, is required for the homeostasis of Tregs during antitumor responses (18). These observations highlight canonical NF- κ B signaling as a master regulator of Treg development and function, and demonstrate the discrete functions of individual NF- κ B subunits in Treg-dependent immune tolerance.

The alternative NF- κ B signaling pathway, by contrast, is largely inactive during normal T cell homeostasis. In lymphocytes, NF- κ B2 resides predominantly in the cytoplasm in its unprocessed p100 form, where it is believed to function as an inhibitor of other NF- κ B proteins (9). However, activation of a subset of TNFR family members expressed in lymphocytes, including OX40, CD40, or LT- β R, leads to activation of the alternative pathway. Specifically, these receptors direct the recruitment and inhibition of the TRAF2/3/cIAP1/2 ubiquitin ligase complex, which allows the stabilization and activation of NIK. The alternative pathway also appears to be indirectly involved in Treg development. For example, *NIK*^{-/-} mice and NIK mutant *aly/aly* mice have reduced numbers of Tregs (19, 20). The same observation was made more recently in mice when IKK α was conditionally deleted in T cells (21). Finally, constitutive expression of NIK in T cells induced the peripheral expansion of poorly functional Tregs (22). However, enforced expression of NIK can activate both alternative and canonical pathways (23), and therefore the specific role for the alternative NF- κ B pathway remained unclear. Likewise, germline deletions of *relb* or *nfkb2* were associated with developmental and autoimmune defects associated with changes with lymphoid organogenesis, obscuring analysis of their functional role in Tregs (9). To overcome these difficulties, we have now used conditional deletion of *nfkb2* and/or *relb* in both total T cells and Tregs, and demonstrated a critical function for NF- κ B2 in maintaining Treg homeostasis. Surprisingly, rather than functioning as a crucial component of alternative pathway-dependent transcriptional complexes, we found that the key function of p100 in Tregs was as a negative regulator of p52-independent, RelB-containing complexes. Hence our studies suggest that p100 and RelB have critical and unexpected functions in Treg homeostasis and suppressive function.

Materials and Methods

Animals and cell lines

Nfkb2-floxed and *relb*-floxed mice were generated by U. Klein (Columbia University, New York, NY) (24). CD4^{cre} [Tg(CD4-cre)^{1Cw1}], Foxp3^{CRE-YFP} [Foxp3^{tm4(YFP/cre)Ayr}], CD45.1 (Ptprc^a Pepc^b/BoyJ), and RAG1^{-/-}, all on a C57BL/6J background, were originally purchased by the Jackson Laboratory and maintained in our animal facility. C57BL/6J mice were purchased from the Jackson Laboratory. All mice were kept in specific pathogen-free conditions in the animal care facility at Columbia University (New York, NY). All mouse experiments were approved by the Institutional Animal Care and Use Committee of Columbia University.

Flow cytometry

Cells were isolated from thymus, spleen, and lymph nodes (LNs) by mechanical desegregation in PBS+FBS 3%. Colon lamina propria lymphocytes were isolated upon digestion and Percoll for intracellular cytokines analyses, and cell suspensions were incubated 3 h with PMA (50 ng/ml; Sigma) and ionomycin (1 μ g/ml; Sigma) in the presence of Golgi Plug (BD Biosciences). Cells were then stained with mAbs purchased from eBioscience or Tonbo Biosciences. Foxp3 and cytokine staining were performed using the eBioscience kit and protocol. Cells were acquired on an LSR II (BD Biosciences) and analyzed with FlowJo (Tree Star) software. Cell sorting was achieved on a FACSria II (BD Biosciences).

In vitro Treg differentiation

CD4⁺CD44^{low}CD25⁻ naive T cells were FACS-sorted from splenocyte suspensions. A total of 10⁵ T cells were cultured in complete RPMI 1640

(Life Technologies) with 10⁵ T cell-depleted, mitomycin C-treated wild-type (WT) splenocytes and 2.5 μ g/ml anti-mCD3 (Bio X Cell), in the presence of 10 ng/ml mIL-2 (PeproTech) and grading doses of human TGF- β 1 (PeproTech), for 4 d at 37°C. Cells were then stained for flow cytometry analysis.

Suppression assays

For in vitro assays, LN-derived CD45.1⁺ naive conventional CD4⁺ T (Tconv) cells were magnetically isolated (Miltenyi) and labeled with CellTrace Violet Proliferation Tracker (CTV; Life Technologies). They were cultured with T cell-depleted, mitomycin C-treated WT splenocytes and 2.5 μ g/ml anti-mCD3, in the presence or not of FACS-sorted WT or *nfkb2*^{-/-} CD4⁺YFP⁺ Tregs. Proliferation of CD4⁺CD45.1⁺ T cells was assessed by FACS at day 4. The percentage of suppression was calculated as described. For in vivo assays, 4 \times 10⁵ naive CD45.1⁺ Tconv cells were isolated as described earlier and transferred with or without 1 \times 10⁵ Tregs to the retro-orbital sinus of 6- to 9-wk-old RAG1^{-/-} mice. Recipients were then weighed every week and euthanized when weight loss was >30%.

Western blotting

Total lysates were extracted using radioimmunoprecipitation assay buffer and protease inhibitors with SDS. Cytosolic and nuclear fractions were fractionated using the classical hypo- and hypertonic lysis buffers protocol. Twenty micrograms of protein extracts was run in polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with polyclonal anti-NF- κ B2 (K-27), RelB (C-19), p65 (C-20), c-Rel (C, sc-71), HDAC-1 (H51) (Santa Cruz), and monoclonal anti-GAPDH (10R-G109a; Fitzgerald) Abs, followed by HRP-coupled secondary Abs from Jackson ImmunoResearch.

Quantitative RT-PCR and RNA sequencing

Total RNA was extracted using a Qiagen RNeasy Mini Kit with DNase treatment. For quantitative PCR, RNA was reverse transcribed by Superscript III (Invitrogen). cDNAs were used for PCR with SYBR Green reagents (Quanta Biosciences, Gaithersburg, MD) on a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA). The data were normalized to GAPDH expression. Primers sequences can be sent under request. For RNA sequencing, RNA was extracted from Tregs isolated from three to four pooled mice per genotype. Libraries were prepared using an Illumina TruSeq Library Kit and sequenced by an Illumina 2500 instrument. Upon sequencing, raw FASTQ files were aligned on the mm10 genome using STAR aligner with default parameters (25). Aligned fragments were then counted and annotated using Rsamtools v3.2 and the TxDb.Mmusculus.UCSC.mm10.knownGene version 3.1.2 transcript database, respectively. Normalized fragments per kilobase per million mapped reads were obtained using the robust fragments per kilobase per million mapped reads estimate function of DESeq2 v1.10.1. Differentially expressed genes were obtained using the DESeqResults function of the same package. For gene set enrichment analysis, we acknowledge our use of the Gene Set Enrichment Analysis (GSEA) software and Molecular Signature Database (MSigDB) including the ImmuneSigDB (C7 collection) (26, 27). We also used the Panther Database for further function analysis (28). The Gene Expression Omnibus accession number is GSE108532 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108532>).

Histology

Colons were washed and fixed in 4% formalin acetate. Five-micrometer sections were then prepared from the middle section of the colon; three to four sections, each separated by 300 μ m, were prepared for each sample. Tissues were then stained with H&E. Quantification of abscesses was performed in a double-blind fashion. The mean number of abscesses among the sections is shown.

Statistical analyses

Experimental groups were compared statistically using the nonparametric Mann-Whitney *U* test or the unpaired, two-tailed Student *t* test.

Results

Nfkb2 restrains the Treg pool size

To investigate the T cell-intrinsic role of NF- κ B2 p100/p52 in the differentiation and function of CD4⁺ T cell subsets, we first ablated *nfkb2* in total T cells using the *CD4*^{cre} deleter strain. This led to the efficient depletion of p100 in splenic CD4⁺ T cells. As

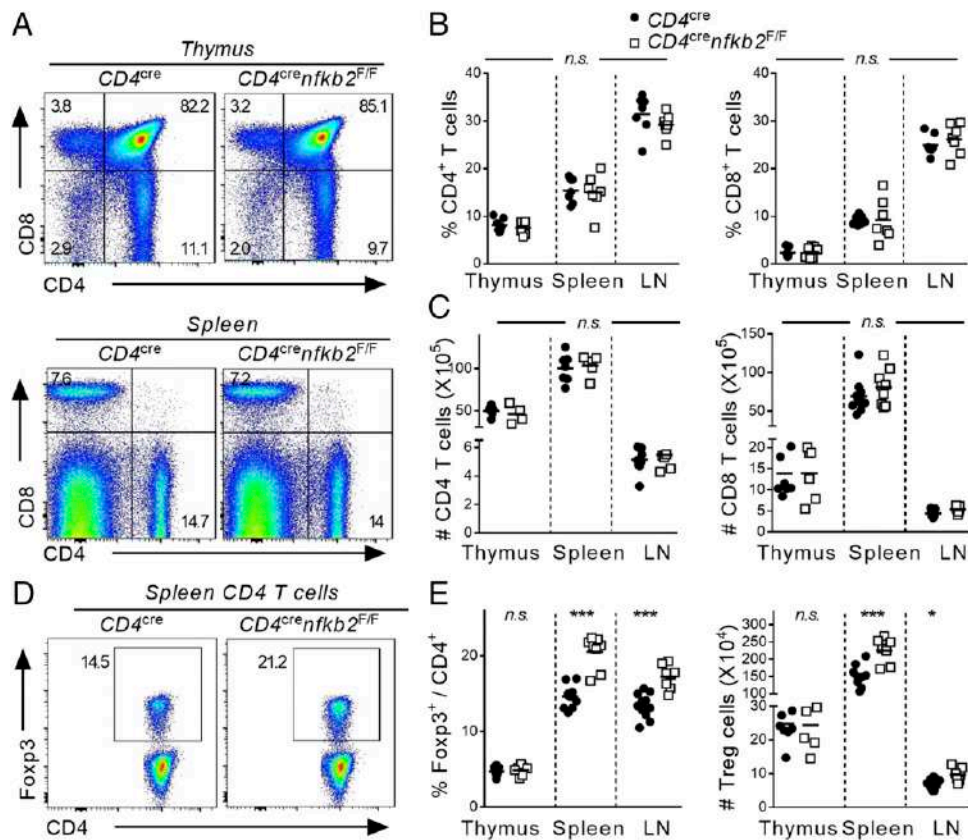


FIGURE 1. T cell-specific ablation of *nfkb2* drives peripheral Treg expansion. Thymus, spleen, and pLNs of 6- to 8-wk-old *CD4^{cre}* and *CD4^{cre}nfkb2^{F/F}* mice were stained for FACS. **(A)** Dot plot showing CD4 and CD8 expression in gated total thymus (top) and spleen (bottom) live cells. Numbers indicate the percentage in each quadrant. **(B and C)** Proportion (B) and absolute numbers (C) of live TCR- β^+ CD4 $^+$ and CD8 $^+$ in the indicated tissues. **(D)** Expression of Fosp3 in gated spleen TCR- β^+ CD4 $^+$ live cells. Numbers indicate the percentage in gate. **(E)** Percentage in TCR- β^+ CD4 $^+$ and absolute numbers of Tregs. Data are representative (A and D) or the pool (B, C, and E) of three experiments. (B–D) Each dot represents a mouse. * $p < 0.05$, *** $p < 0.001$. n.s., nonsignificant.

expected, p52 expression was not observed at steady-state in WT cells (Supplemental Fig. 1A and data not shown). Deletion of *nfkb2* was not associated with significant changes in the proportion and numbers of CD4 and CD8 T cells in both primary and secondary lymphoid organs (Fig. 1A–C). Next, we examined the differentiation of Fosp3 $^+$ Tregs. The proportion and number of immediate Fosp3 $^-$ Treg precursors and mature Fosp3 $^+$ Tregs were similar in the thymus of WT and knockout (KO) animals (Fig. 1E, Supplemental Fig. 1B). However, we observed a significant increase in the percentage of Tregs in the spleen and peripheral LNs (pLNs) upon *nfkb2* deletion (Fig. 1D, 1E). This increase in the fraction of Tregs corresponded to an increase in the absolute number of Tregs in these tissues (Fig. 1E), consistent with there being an expansion of Treg numbers rather than a decrease in the Tconv populations. Moreover, the activation state of *nfkb2*-deficient Tregs was altered, as demonstrated by significantly increased expression of CD44 and Ki67 relative to WT Tregs (Supplemental Fig. 1C). Interestingly, we also observed a trend toward decreased expression of Fosp3 in *nfkb2* $^{-/-}$ Tregs (Supplemental Fig. 1D). Thus, ablation of *nfkb2* in T cells led to a selective expansion of the peripheral Treg population without apparent effects on the thymic differentiation of Tregs.

Although we did not observe any changes in CD4 or CD8 T cell phenotype, we wished to confirm that these changes in Treg homeostasis were due to intrinsic changes in p100/p52 function in Tregs. We therefore selectively deleted *nfkb2* in Tregs using the *Fosp3^{CRE}* deleter mice. In 6- to 8-wk-old animals, there were no obvious signs of systemic inflammation or autoimmunity. Consistent with the lack

of gross phenotypic changes in these mice, there were no changes in thymic Treg differentiation or in the distribution or effector function of conventional CD4 (Tconv) and CD8 T cells in the mutant mice (data not shown). However, consistent with the results obtained with *Cd4^{CRE}* mice, the Treg population was significantly expanded in the spleen and LN of *Fosp3^{CRE}nfkb2^{F/F}* animals when compared with littermate controls (Fig. 2A, 2B). Again, *nfkb2*-deficient Tregs displayed significantly impaired expression of Fosp3, whereas Treg activation markers were upregulated in *nfkb2*-deleted Tregs (Fig. 2C and data not shown). Taken together, these results demonstrate an important role for p100, and/or p52, in the regulation of Treg homeostasis.

Although these results demonstrate a role for p100/p52 in Tregs, we wondered whether *nfkb2* controlled Treg homeostasis in a cell-intrinsic manner or indirectly, for example, by regulating production of a factor that was driving Treg expansion. Therefore, to formally demonstrate a Treg-autonomous role for *nfkb2*, we performed mixed bone marrow (BM) chimera experiments. In such an experimental setting, any cell-extrinsic defect in the KO BM would either be compensated for by the presence of WT BM or be revealed by an effect on WT Tregs. Lethally irradiated WT recipients were transplanted with a 1:1 mixture of congenic WT BM and either *Fosp3^{CRE}* or *Fosp3^{CRE}nfkb2^{F/F}* BM (Fig. 2D). Eight weeks after BM reconstitution, the proportion of Tregs in KO spleen and LN compartments was dramatically increased when compared with their WT counterparts (Fig. 2E). Consistent with the increase in deleted Tregs, upregulation of the Ki67 proliferation marker was also selectively observed in Tregs in which *nfkb2*

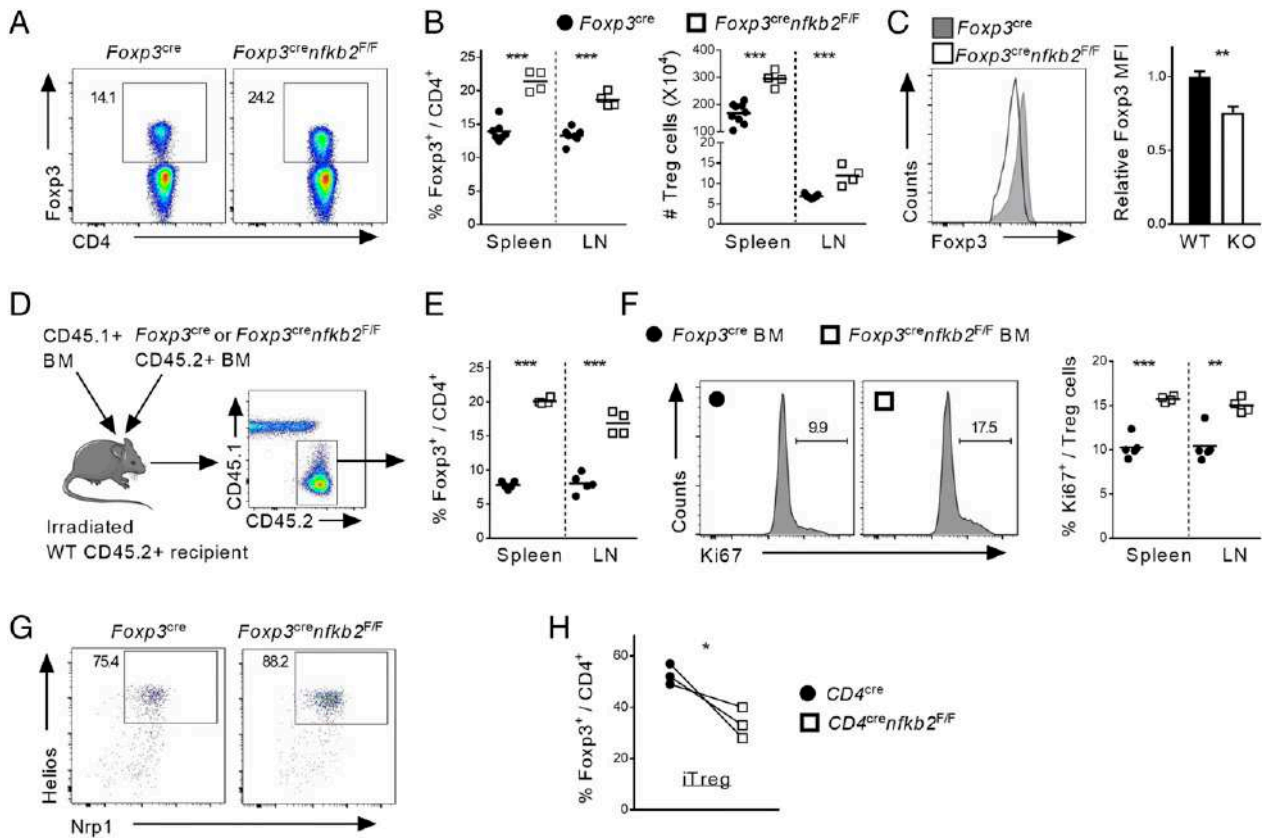


FIGURE 2. Intrinsic NF- κ B2 p100/p52 signaling restricts the peripheral Treg pool. (A–C) Spleen and pLNs of 6- to 8-wk-old *Foxp3^{Cre}* and *Foxp3^{Cre}nfkb2^{F/F}* mice were stained for FACS. (A) Expression of Foxp3 in gated spleen TCR- β^+ CD4⁺ live cells. Numbers indicate the percentage in gate. (B) Percentage in TCR- β^+ CD4⁺ and absolute numbers of Tregs. (C) Expression of Foxp3 and pooled MFI in gated spleen TCR- β^+ CD4⁺Foxp3⁺ Tregs. (D–F) The cell-intrinsic role of *nfkb2* in Tregs was evaluated in mixed BM chimeras. (D) Experimental design. (E) Percentage of Tregs in TCR- β^+ CD4⁺CD45.2⁺ cells. (F) Representative Ki67 expression in gated spleen in TCR- β^+ CD4⁺CD45.2⁺Foxp3⁺ Tregs, and pooled percentage of Ki67⁺ in Tregs. Numbers indicate the percentage in gate. (G) Expression of Nrp-1 and Helios in gated spleen in TCR- β^+ CD4⁺Foxp3⁺ Tregs from *Foxp3^{Cre}* and *Foxp3^{Cre}nfkb2^{F/F}* animals. (H) Naive T cells from *CD4^{Cre}* and *CD4^{Cre}nfkb2^{F/F}* LNs were cultured under in vitro–differentiated Treg (iTreg) polarization conditions for 4 d. The percentage of Treg in each genotype in three experiments is shown. Data are representative (A and C–G) or the pool (B and H) of two to three experiments. (B, E, and F) Each dot represents a mouse. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

was deleted. Thus, *nfkb2* has a cell-intrinsic function in restricting Treg proliferation in the periphery. Next, we assessed whether these expanded Tregs were of thymic origin or derived from a peripheral conversion of Tconv cells. The percent expression of two genes associated with nTregs, the membrane receptor Nrp-1 and the transcription factor Helios, were not affected by loss of *nfkb2*. This suggested that the enhanced *nfkb2*^{-/-} Treg expansion was occurring in nTregs of thymic origin (Fig. 2G). We also measured the effect of ablating *nfkb2* on the in vitro conversion of Tconv into Foxp3⁺ Tregs, which mimic pTregs. After 4 d of culture, the proportion of Foxp3⁺ cells was slightly reduced in the absence of *nfkb2* (Fig. 2H). Therefore, NF- κ B2 does not have effects on either nTreg or pTreg development that explain the expansion of the peripheral Treg population. Instead, the data suggest that *nfkb2* is a key, cell-autonomous regulator of nTreg homeostasis.

Mild autoimmune syndrome in aged *Foxp3^{CRE}nfkb2^{F/F}* mice

As described earlier, young *Foxp3^{CRE}nfkb2^{F/F}* mice did not display abnormal inflammation or autoimmunity. However, beginning around 12 mo of age, we began to observe variable weight loss and hunched posturing in the *Foxp3^{CRE}nfkb2^{F/F}* animals. Histological examination revealed increased immune infiltrate in the colons of *Foxp3^{CRE}nfkb2^{F/F}* mice, whereas other tissues (lungs, liver, and kidney) were unaffected (Fig. 3A and data not shown).

Of note, these localized colonic infiltrates were not associated with significant histological changes at the mucosa (Fig. 3A). FACS analysis revealed increased numbers of IFN- γ -producing CD4 and CD8 T cells in the colon lamina propria of *nfkb2*-deficient mice compared with WT controls, whereas the IL-17A⁺ cell population was unchanged (Supplemental Fig. 2A, 2B). Next, the lymphoid tissue composition was analyzed. The cellularity of both spleen and pLNs was significantly increased in *Foxp3^{CRE}nfkb2^{F/F}* animals (Fig. 3B). Interestingly, the CD4⁺ T cell compartment was significantly expanded, leading to an increased CD4/CD8 ratio (Supplemental Fig. 2C). Moreover, we observed enhanced activation of both CD4⁺ Tconv and CD8⁺ T cells, as shown by the increased expression of CD44 and Ki67 (Fig. 3C–E). Strikingly, we observed a nearly 2-fold increase in the proportion of CD4 and CD8 T cells expressing IFN- γ (Fig. 3F–G). However, the expression of IL-17A, TNF, and IL-2 was not significantly different between WT and KO mice (Fig. 3H, Supplemental Fig. 2D). Finally, we could not detect any change in the levels of serum TNF and total IgG (data not shown). Thus, the expression of NF- κ B2 by Tregs prevents the late development of a mild autoimmune syndrome, likely mediated by Th1 CD4 and Tc1 CD8 T cells.

Given the expansion of Tregs in young *Foxp3^{CRE}nfkb2^{F/F}* animals, we hypothesized that the observed autoimmune phenotype in aged animals was likely the consequence of an impaired Treg suppressive function. Indeed, FACS analysis revealed that the

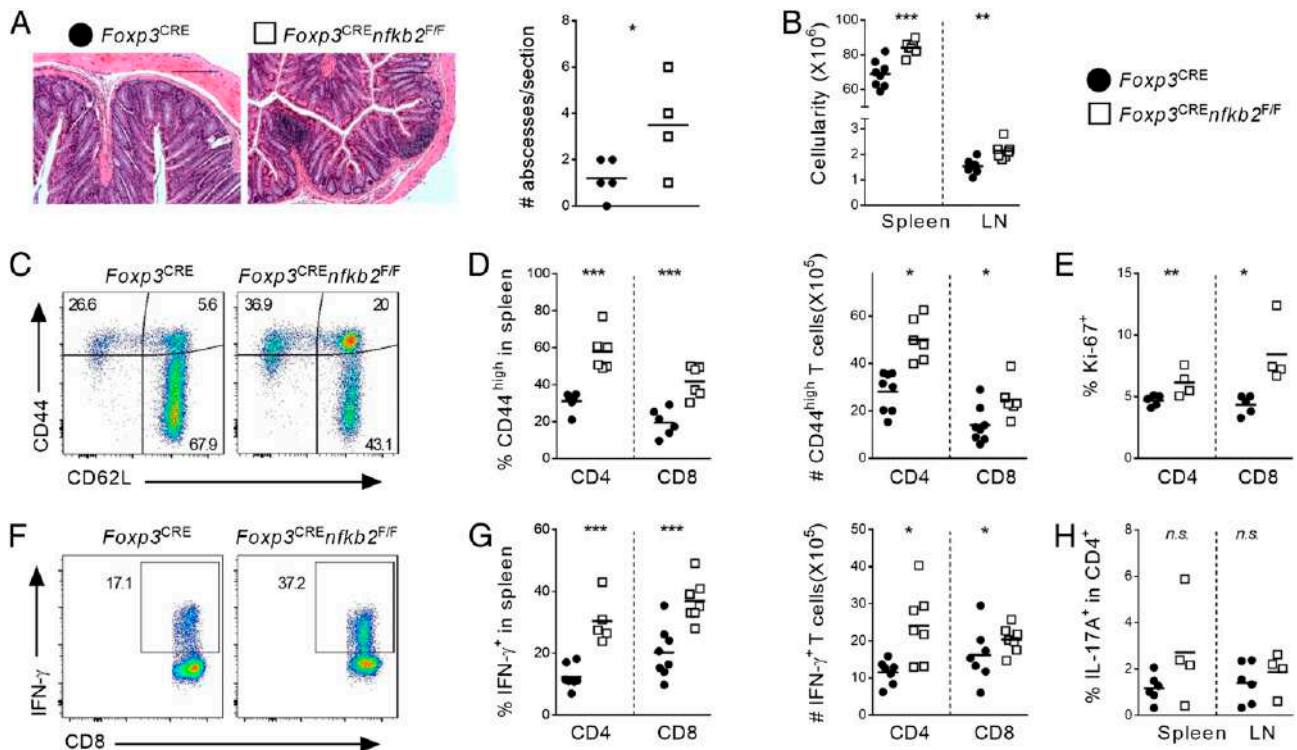


FIGURE 3. Uncontrolled inflammation in aged *Foxp3^{cre}nfkb2^{F/F}* mice. Tissues of 12-mo-old *Foxp3^{cre}* and *Foxp3^{cre}nfkb2^{F/F}* mice were analyzed by histology and FACS. **(A)** Left, Representative section of colon stained with H&E. Scale bars, 200 μ m. Original magnification $\times 10$. Right, Quantification of abscesses (immune infiltrates) in each colon section. **(B)** Gross live cell count in spleen and LN. **(C)** CD44 and CD62L expression in gated spleen TCR- β^+ CD8 $^+$ cells. Numbers indicate the percentage in each quadrant. **(D)** Percentage [in TCR- β^+ CD4 $^+$ Foxp3 $^-$ (CD4) and TCR- β^+ CD8 $^+$ (CD8)] and absolute numbers of CD44 $^{\text{high}}$ T cells. **(E)** Percentage of Ki67 $^+$ cells in spleen CD4 and CD8 cells. **(F)** Expression of IFN- γ upon PMA-ionomycin restimulation in gated spleen TCR- β^+ CD8 $^+$ cells. Numbers indicate the percentage in gate. **(G)** Percentage (in CD4 and CD8) and absolute numbers of IFN- γ^+ T cells. **(H)** Percentage of IL-17A $^+$ cells in CD4 cells. Data are representative (A, C, and F) or the pool (A, B, D, E, G, and H) of three experiments. (A, B, D, E, G, and H) Each dot represents a mouse. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. n.s., nonsignificant.

inflammatory syndrome observed in aged *Foxp3^{cre}nfkb2^{F/F}* mice was associated with continued expansion of Tregs (Fig. 4A). Therefore, we next examined the suppressive capacity of *nfkb2*-deficient Tregs in vitro and in vivo. For the in vitro assay, the proliferative capacity of responder CD4 T cells was measured in the absence or presence of varying ratios of WT or KO Tregs. *Nfkb2*-deficient Tregs exhibited reduced inhibitory activity at each ratio relative to WT Tregs (Fig. 4B). We next assessed the activity of WT and *nfkb2^{-/-}* Tregs in vivo using a T cell transfer colitis model (29). Although WT Tregs effectively prevented weight loss and reduced IFN- γ expression by splenic Tconv cells transferred into *RAG1^{-/-}* mice, *nfkb2^{-/-}* Tregs were considerably less effective (Fig. 4C, 4D). This was not due to a loss of the Treg population per se, because the proportion of *nfkb2^{-/-}* Tregs 5 wk after transfer was three times higher than that of WT Tregs (Fig. 4E). To better understand this loss of Treg suppressive function, we measured the mRNA expression of several well-characterized genes involved in Treg function. Whereas the expression of *Tgfb1*, *Lag3*, and *CD73* was unaffected in Tregs lacking NF- κ B2, *Ctla4* mRNA was strongly downregulated (Supplemental Fig. 2E). This was associated with a decreased expression of intracellular CTLA-4 in *nfkb2^{-/-}* Tregs, both in unmanipulated *Foxp3^{cre}nfkb2^{F/F}* mice and in the T cell transfer colitis model (Supplemental Fig. 2F). Taken together, these results demonstrate that the expression of NF- κ B2 is required to restrict Treg proliferation and also to maintain optimal suppressive capacity.

NF- κ B RelB hyperactivity in *nfkb2*-deficient cells alters Treg homeostasis

We next explored the molecular mechanism driving dysregulated Treg homeostasis in the absence of NF- κ B2. NF- κ B2 can act as

both a negative regulator of other Rel-proteins, by sequestering them through interaction via its ankyrin domains, and as a positive regulator by generating the p52 subunit, which can associate with other NF- κ B subunits to form transcriptionally active NF- κ B heterodimers. Therefore, we first sought to examine the expression and activation state of the transcriptionally active NF- κ B subunits, RelB, p65, and c-Rel. Both the expression and stimulation of induced nuclear localization of the canonical NF- κ B subunits RelA and c-Rel were unaffected in *nfkb2^{-/-}* Tregs (Fig. 5A). In contrast, there was a dramatic increase in the nuclear accumulation of RelB in TCR/CD28-stimulated *nfkb2^{-/-}* Tregs (Fig. 5A). Therefore, the data suggested that NF- κ B2 predominantly functions as an inhibitor of RelB activation in Tregs. To test the hypothesis that the primary function of NF- κ B2 in Tregs is to regulate RelB activation, we deleted *relb* in both total T cells (using *CD4^{cre}*) and Tregs (using *Foxp3^{cre}*). RelB protein was undetectable in splenic T cells of *CD4^{cre}relb^{F/F}* mice (Supplemental Fig. 3A). Surprisingly, *Relb* deletion did not have a significant effect on either the overall distribution of CD4 and CD8 T cells in lymphoid organs or the corresponding number of Tregs (Supplemental Fig. 3B, 3C). Strikingly, ablation of *relb* in the *CD4^{cre}nfkb2^{F/F}* background restored a normal proportion and number of peripheral Tregs (Fig. 5B). Hence loss of NF- κ B2 resulted in increased RelB activation, and the Treg expansion observed in *nfkb2*-deficient animals was dependent on RelB.

We next sought to determine whether the altered in vivo suppressive function observed in *Foxp3^{cre}nfkb2^{F/F}* mice was also dependent on RelB. Therefore, we next measured inflammation/autoimmunity symptoms in Treg-specific, *Foxp3^{cre}nfkb2^{F/F}relb^{F/F}*,

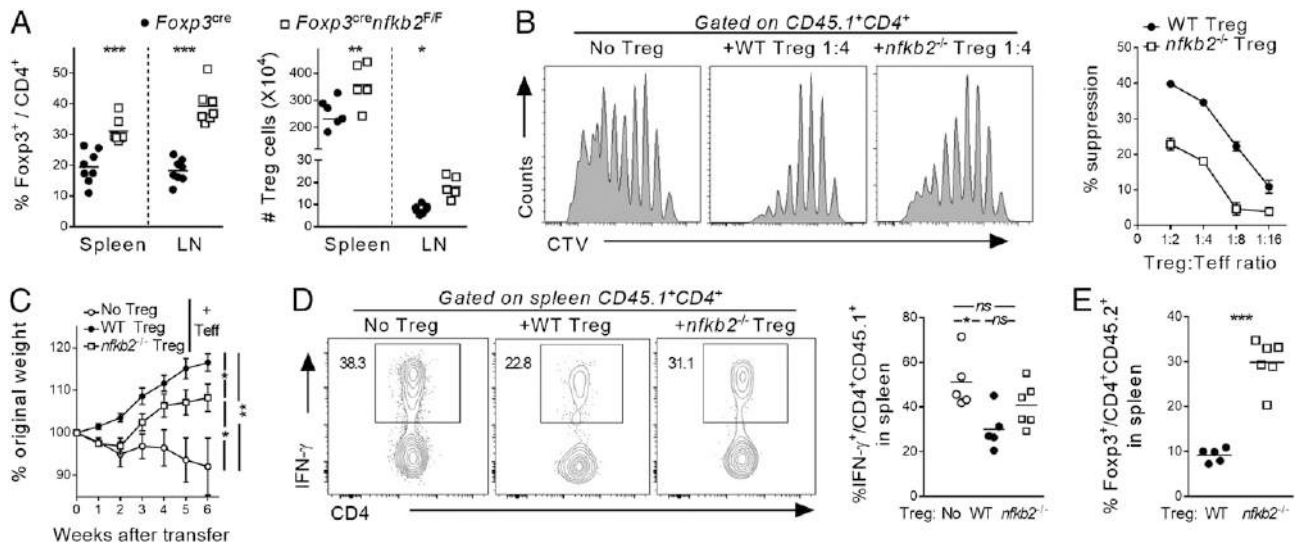


FIGURE 4. Impaired suppressive function of *nfkb2*-deficient Tregs. **(A)** Spleen and pLNs of 12-mo-old *Foxp3^{cre}* and *Foxp3^{cre}nfkb2^{F/F}* mice were analyzed by FACS. The percentage of Tregs among CD4 T cells and their absolute numbers are shown. **(B)** In vitro suppressive function of Tregs sorted from *Foxp3^{cre}* (WT) and *Foxp3^{cre}nfkb2^{F/F}* (*nfkb2^{-/-}*) animals was assessed as described in *Materials and Methods*. Left, Representative proliferation of responder T cells at day 4. Right, Mean \pm SEM of suppression of responder T cells proliferation by Tregs at different ratios. **(C–E)** In vivo suppression assay, as described in *Materials and Methods*. **(C)** Mean \pm SEM percentage of original weight. **(D)** Representative (left) and pooled (right) expression of IFN- γ in gated CD45.1⁺CD4⁺ T cells at day 50. **(E)** Percentage of Foxp3⁺ Tregs among live CD45.2⁺CD4⁺ cells. **(A, C, and E)** Data are the pool of three pooled experiments. **(B)** Data are from one out of three experiments. **p* < 0.05, ***p* < 0.005, ****p* < 0.001. n.s., nonsignificant.

double-KO mice. Consistent with the normalization of Treg homeostasis upon RelB loss, no increase in colonic abscesses was evident in 10- to 12-mo-old *Foxp3^{CRE}nfkb2^{F/F}relb^{F/F}* mice (Fig. 5C, 5D). Moreover, the global cellularity of spleen and LN was also normal (Fig. 5E). As in the *CD4^{cre}* deleter strain, *Foxp3^{CRE}nfkb2^{F/F}relb^{F/F}* mice also displayed normal numbers of peripheral Tregs (Fig. 5F). Finally, we examined the activation state of conventional CD4 and CD8 T cells. Again, the level of CD44 and IFN- γ expression remained unchanged in KO mice when compared with WT littermates (Fig. 5G–I, Supplemental Fig. 3D–F). Taken together, these data indicate that p100 is an essential negative regulator of RelB in Tregs and that the uncontrolled expansion of Tregs, and the subsequent autoimmunity that develops after deletion of *nfkb2*, is secondary to enhanced RelB activation.

NF- κ B2 regulates the Treg transcriptome

To further explore how NF- κ B2 controls Treg homeostasis, we compared the transcriptomes of WT, *nfkb2^{-/-}*, *relb^{-/-}*, and *nfkb2^{-/-}relb^{-/-}* Tregs by RNA sequencing. To ensure an optimal activation of the alternative NF- κ B pathway, we stimulated Tregs with anti-CD3, anti-CD28, anti-OX40, and IL-2. We initially analyzed global changes in gene expression between the genotypes (Fig. 6A, 6B). Surprisingly, we found that loss of NF- κ B2 resulted in a far broader effect on the Treg transcriptome than did loss of RelB. Specifically, the analysis of the *nfkb2^{-/-}* Treg transcriptome was notable for the large number of upregulated transcripts (175 upregulated versus 84 downregulated), reinforcing the idea the NF- κ B2 predominantly affects Treg function through its I κ B-like repressor activity. Interestingly, the dysregulated gene set in *nfkb2^{-/-}* Tregs showed only minor overlap with other genotypes. Furthermore, *relb* deletion did not impair gene expression, which was at odds with its role in B cells (24) but was consistent with the lack of a detectable phenotype in *Foxp3^{CRE}relb^{F/F}* mice. Remarkably, however, dysregulated expression of the vast majority of genes in *nfkb2*-deficient Tregs was rescued upon *relb* ablation (Fig. 6B). Therefore, these data further support the idea that NF- κ B2 primarily acts as an inhibitor of RelB-mediated

transcription. Interestingly, neither partial nor complete ablation of the alternative NF- κ B pathway drove a change in the expression of other NF- κ B subunits genes (Supplemental Fig. 4A). GSEA and other gene ontology analyses revealed significant changes in the expression of genes related to proliferation, survival, and migration, as well as cytokine expression (Fig. 6B, Supplemental Fig. 4B). We observed a dramatic increase in the expression of genes involved in cell proliferation and antiapoptotic functions in *nfkb2*-only deleted cells (Fig. 6C). The expression of the proapoptotic gene *Bcl2l11*, which encodes Bim, was downregulated in *nfkb2^{-/-}* Tregs, whereas the kinase *Kit* was upregulated. These genes were restored to the WT expression levels upon additional ablation of *relb*. This perturbed expression profile may explain the specific accumulation of Tregs in the tissues of *nfkb2*-deficient mice.

It has previously been suggested that the alternative pathway may control the expression of migration and homing molecules in stromal cells and innate immune cells. In Tregs, the expression of numerous chemokines and chemokine receptors were modified by the absence of NF- κ B2, but not RelB (Fig. 6C). However, most of these genes were upregulated and not downregulated, revealing a previously unknown facet of the alternative NF- κ B pathway as an inhibitor of expression of homing molecules in Tregs.

Finally, we observed changes in the expression of many genes related to inflammation and Th cell differentiation. *Nfkb2^{-/-}* Tregs displayed a significant overexpression of inflammatory molecules, in particular cytokines and transcription factors that are hallmarks of Th17 effector cells, such as *Il-17a*, *Il-21*, *Ccl20*, and *Rorc* (Fig. 6C, Supplemental Fig. 4B). This was further confirmed by quantitative RT-PCR and flow cytometry analyses (Fig. 6D, 6E). Moreover, the aberrant expression of most of these genes was “rescued” by knocking out *relb*, underscoring a deleterious role for uncontrolled RelB activation on the Treg-associated molecular profile. These results are consistent with a predominantly inhibitory function of NF- κ B2 in the expression of cell cycle and proinflammatory genes. As we recently demonstrated, the canonical NF- κ B subunits p65 and c-Rel are critical for maintaining

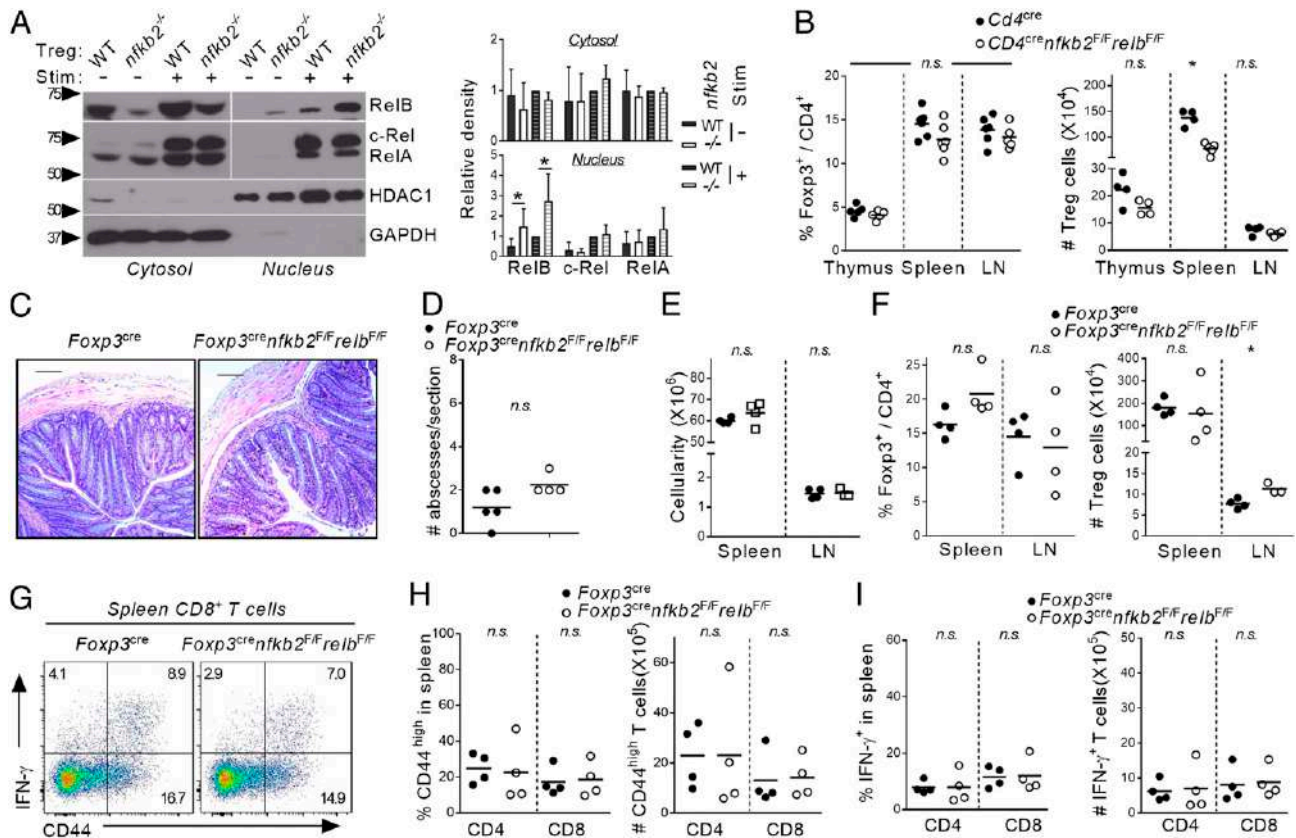


FIGURE 5. Overactivation of RelB in the absence of NF- κ B2 drives uncontrolled Treg expansion and inflammation. **(A)** Tregs were sorted from *Foxp3^{cre}* and *Foxp3^{cre}nfk2^{F/F}* spleen and LN, and activated overnight with anti-CD3/CD28 Abs and IL-2. Cytosolic and nuclear lysates were separated by SDS-PAGE, and membranes were blotted with the indicated Ab. **(B)** Thymus, spleen, and pLNs of 6- to 8-wk-old *CD4^{cre}* and *CD4^{cre}nfk2^{F/F}relb^{F/F}* mice were stained for FACS. The percentage in TCR- β^+ CD4⁺ and absolute numbers of Tregs are shown. **(C-F)** Tissues of 12-mo-old *Foxp3^{cre}* and *Foxp3^{cre}nfk2^{F/F}relb^{F/F}* mice were analyzed by histology and FACS. **(C)** Representative section of colon stained with H&E. Scale bars, 200 μ m. Original magnification \times 10. **(D)** Quantification of abscesses (immune infiltrates) in each colon section. **(E)** Gross live cell count in spleen and LN. **(F)** Percentage in TCR- β^+ CD4⁺ and absolute numbers of Tregs. **(G-I)** Cells were restimulated with PMA-ionomycin before FACS analysis. **(G)** CD44 and IFN- γ expression in gated spleen TCR- β^+ CD8⁺ cells. Numbers indicate the percentage in each quadrant. **(H and I)** Percentage (in CD4 and CD8 cells) and absolute numbers of CD44^{high} and IFN- γ^+ T cells, respectively. Each dot represents a mouse. Data are representative (A, C, and G) or pooled (A, B, D-F, H, and I) from two to four experiments. * $p < 0.05$. n.s., nonsignificant.

the expression of Treg hallmark genes (16). However, there was not a significant positive or negative correlation in gene expression between *Foxp3^{CRE}Rela^{F/F}Rel^{F/F}* and *Foxp3^{CRE}nfk2^{F/F}* Tregs (Supplemental Fig. 4C), suggesting that perturbations in gene expression in these two genotypes were distinct, and further supporting the idea that the inhibitory effect of NF- κ B2 is on RelB-containing complexes. Therefore, taken together, our observations demonstrate a novel and unique function of NF- κ B2 in maintaining Treg homeostasis and function.

Discussion

Recently, significant progress has been made in understanding the intrinsic role of the canonical NF- κ B pathway in the biology of Tregs (16–18). Indeed, we have shown that conditional deletion of p65 in Tregs drove a systemic autoimmune syndrome, which was further aggravated by deletion of c-Rel. c-Rel itself exhibited a critical function in maintaining Treg homeostasis specifically during antitumor immunity (18). We now demonstrate that the alternative NF- κ B pathway, acting through its NF- κ B2 subunit, is also crucial for the maintenance of Treg homeostasis and function. Forced activation of the alternative pathway by deleting TRAF3 (*Traf3^{-/-}* mice), or overexpressing NIK (NIK-transgenic [Tg] mice), leads to increased numbers of Tregs (22, 30). Based on these findings it would have been predicted that loss of *nfk2*

would result in impaired Treg expansion. Instead, surprisingly, we find that deletion of *nfk2* drives a cell-autonomous increase in Treg numbers in secondary lymphoid organs. This is likely due to the expansion of nTregs in the periphery because in vitro-differentiated Treg generation was only slightly impaired.

Interestingly, Treg numbers returned to baseline upon additional deletion of RelB (i.e., *nfk2^{-/-}relb^{-/-}* double KO). Of note, in both *Foxp3^{CRE}relb^{F/F}* and *Foxp3^{CRE}nfk2^{F/F}relb^{F/F}* mice, the relative proportion of Tregs was not decreased compared with WT mice. This suggested that NF- κ B2 functions to prevent aberrant RelB activation, which otherwise has no role in Treg expansion at steady-state. Previous studies had indirectly addressed the role of the alternative NF- κ B pathway in Treg expansion. For instance, several reports showed a cell-intrinsic requirement for NIK in the peripheral homeostasis of Tregs (20, 31). Our data suggest that this result is likely due to the effect of NIK on the function of canonical NF- κ B. A similar decrease of Tregs was observed in conditional IKK α -KO mice (21); but once again, because IKK α can also activate the canonical NF- κ B pathway (32), this suggests that the effects of NIK or IKK α relied more on impaired p65 or c-Rel activation, rather than on a diminished activation of the alternative NF- κ B pathway.

Nfk2 encodes for both p100 and p52. In this study, we show that deletion of *nfk2* can drive uncontrolled Treg activation;

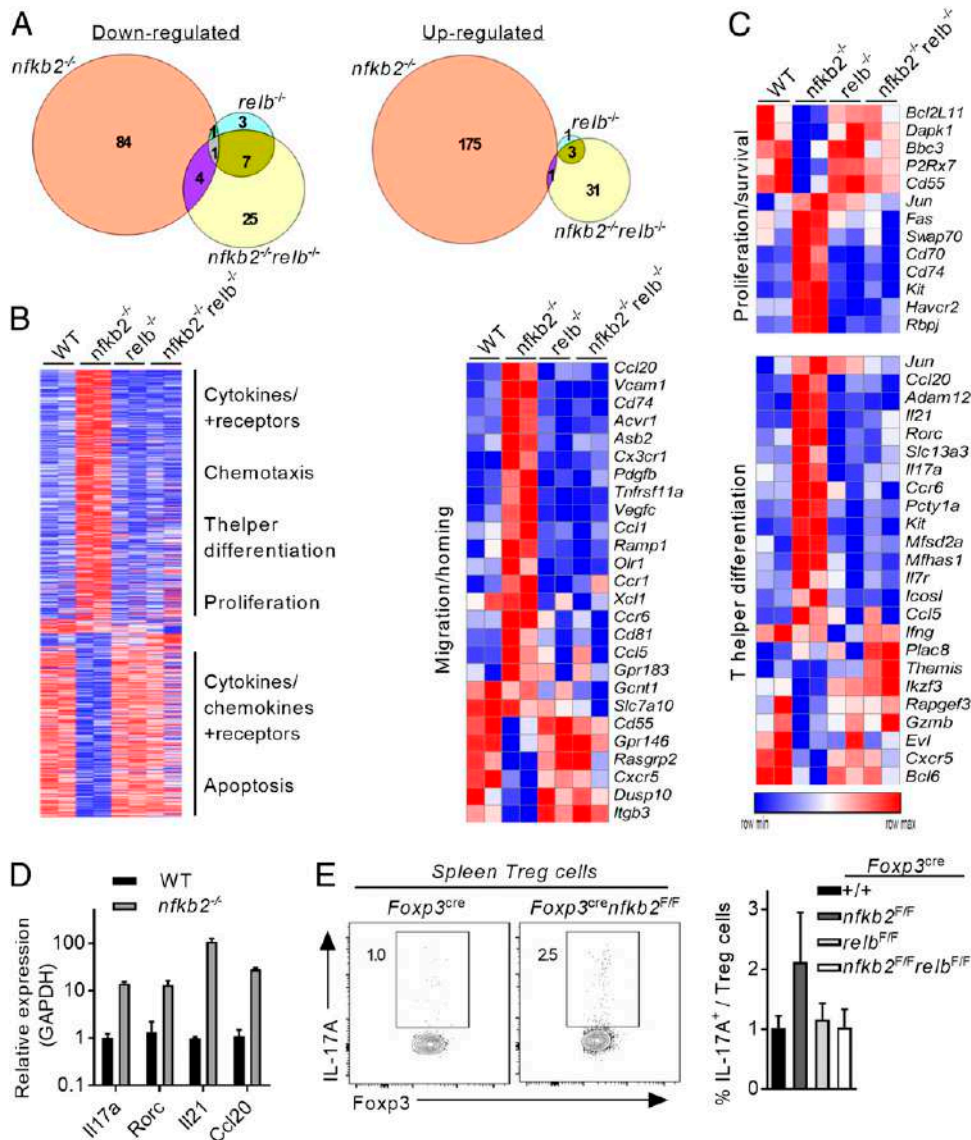


FIGURE 6. NF-κB2 represses aberrant expression of inflammatory and homing genes in Tregs. Tregs were sorted from *Foxp3^{cre}*, *Foxp3^{cre}nfk2^{fl/fl}*, *Foxp3^{cre}relb^{fl/fl}* mice and *Foxp3^{cre}nfk2^{fl/fl}relb^{fl/fl}* mice (three mice/genotype), activated overnight, and processed for RNA sequencing. **(A)** Venn diagrams showing genes with a fold-change <0.6 and >1.5 and a *p* value <0.05 compared with WT Tregs. **(B)** Unsupervised hierarchical row clustering. Only genes with changed expression in at least one genotype is shown. The biological functions indicate selected enriched pathways upon GSEA and Metacore analysis. **(C)** Heat maps showing the relative expression of selected genes enriched in different biological processes. **(D)** Quantitative RT-PCR quantification of selected genes. Mean ± SEM of expression relative to GAPDH is shown. **(E)** Spleens of 12-mo-old mice were analyzed by FACS. Left, Representative expression of IL-17A in gated Tregs; numbers indicate the percentage in the gate. Right, Mean ± SEM of IL-17A expression in spleen Tregs. Data are from two (A–C) or three (D and E) experiments.

however, it is not clear whether this effect results from the ablation of p100, p52, or both. Previous studies have suggested that p100 can act as a repressor of TCR-triggered signaling events; for instance, overexpression of p100 in cell lines decreased initial NF-κB activation and IL-2 transcription upon TCR triggering (33). Similarly, constitutive activation of NIK in c-IAP^{H570A} mutant mice, or in NIK-Tg animals, led to overactivation of CD4⁺ T cells (22, 34). Moreover, T cells isolated from mice carrying a germline deletion of *nfk2* were also more proliferative in vitro (34). Finally, *NIK*-deficient T cells were slightly hyporesponsive in vitro and in vivo (19, 31). In summary, these studies suggest that Treg expansion in *nfk2*-conditional KO animals was likely due to the absence of the inhibitor p100, rather than the absence of transcriptionally active p52-containing complexes. Mechanistically, several studies have demonstrated the role of p100 as a potent inhibitor of both canonical and alternative NF-κB signaling. For

example, the p100 C-terminal ankyrin domains have been shown to physically interact and inhibit the translocation of RelB, p65, and c-Rel (19, 33, 35). Most compelling, however, are studies highlighting RelB as the critical target of p100 in the cytosol of mouse embryonic fibroblasts, Hela cells, dendritic cells, and T cells (36–39). Consistent with this, our studies in Tregs revealed that the nuclear translocation of RelB, but not p65 or c-Rel, was significantly increased in the absence of *nfk2*. This suggested that an aberrant RelB activation was responsible for the accumulation of Tregs in vivo. This hypothesis was confirmed by abolishment of *nfk2*-deficient Treg expansion upon the additional ablation of *relb*.

Analysis of the transcriptional landscape associated with our KO lines further confirmed the specific role of NF-κB2 in restraining the activity of the other NF-κB subunits. Indeed, many genes whose expression was increased in *nfk2*^{-/-} Tregs were

normalized in the *nfk2^{-/-}relb^{-/-}* cells. Because we did not observe a correlation of the *nfk2^{-/-}* Treg gene signature with the *Rela^{-/-}Rel^{-/-}* Treg gene signature, it supported our hypothesis that *nfk2* deletion did not affect the function of the canonical NF- κ B transcription factors. To our surprise, only a small number of genes were affected by the loss of RelB, or both NF- κ B2 and RelB, suggesting that transcription by alternative NF- κ B subunits was mainly expendable for the maintenance of Treg identity in the steady-state. However, it remains possible that p52 and/or RelB could display a more prominent function in Treg biology during acute or chronic inflammation, which is known to drive the engagement of a number of TNFR family members and, therefore, leads to alternative NF- κ B activation and p100 processing (11, 40).

Previous studies have shown a role for the alternative pathway in the expression of chemokines and adhesion molecules by stromal cells upon LT- β R triggering (9). This pattern was only partially confirmed in BAFF and CD40-stimulated *nfk2^{-/-}* B cells (24). In our experiments, we actually observed an increase in the expression of migration/homing genes, upon CD3/CD28/OX40 stimulation of *nfk2^{-/-}* Tregs, but not in double-KO (*nfk2^{-/-}relb^{-/-}*) Tregs. Another striking difference between *nfk2^{-/-}* and double-KO Tregs is the significant enrichment of a Th17 cell signature in the absence of p100 alone. The increased expression of *Rorc* or *Il17a* might explain why these p100-deficient Tregs partially lose their inhibitory function, even when the expression of suppressive cytokines or costimulation markers remain intact. IL-17-producing Foxp3⁺ Tregs have been implicated in the development of certain autoimmune and chronic inflammatory conditions including psoriasis (41, 42). Interestingly, high numbers of IL-17⁺Foxp3⁺ cells were found in the lamina propria and blood of patients with inflammatory bowel disease and mouse models of colitis (43, 44). Therefore, it is possible that development of colonic inflammation in aging *Foxp3^{CRE}nfk2^{F/F}* animals relies on the aberrant expression of inflammatory cytokines by Tregs.

Even though aging *Foxp3^{CRE}nfk2^{F/F}* mice had an expanded Treg population in their pLNs, the conventional CD4 and CD8 T cells exhibited increased activation and secretion of inflammatory cytokines, leading to undesired infiltration in nonlymphoid tissues. This suggested that despite a large increase in numbers, these Tregs had impaired suppressive activity. This functional deficit was confirmed both in vitro and in vivo. Mice with conditional deletion of *Ctla4* in Tregs also develop a severe autoimmune syndrome despite a significant increase in their number of Tregs (45), and we observed reduced CTLA-4 expression by *nfk2^{-/-}* Tregs. This may partially explain both Treg expansion and their lack of suppressive function in *Foxp3^{CRE}nfk2^{F/F}* mice. Moreover, uncontrolled activation of RelB might be deleterious for Tregs. This possibility has been previously addressed indirectly. For instance, the triggering of the GITR receptor, which induces activation of the alternative pathway (46), led to increased T cell survival and proliferation (47). This drove a significant Treg accumulation in GITRL-Tg animals (48). Meanwhile, Treg suppressive function was impaired by GITR signaling (49). These data also fit our model. Therefore, all of these observations may help unmask a specific role for the alternative pathway in Treg homeostasis.

Based on the previous analyses of TNFR superfamily members in Tregs and the data presented in this article, we propose a model in which p100 is a crucial regulator of RelB and Treg homeostasis and function. Following TCR stimulation, induced synthesis of p100 results in the inhibition of RelB-containing complexes. However, upon activation of the alternative NF- κ B pathway, for

example, through OX40 or GITR, increased processing of p100 results in the activation of RelB-containing complexes leading to Treg expansion coupled with loss of Treg suppressive function. This model is consistent with recent reports analyzing OX40 and GITR function and provides a molecular explanation for the ability of these alternative NF- κ B pathway stimuli to inhibit Treg function and tumor tolerance. Likewise, these data provide further insight into how constitutive activation of the canonical and alternative NF- κ B pathways might drive pathology in patients with chronic inflammation and autoimmune disease (11, 40, 50). Therefore, taken together, our data demonstrate that the adequate balance of NF- κ B activity, regulated by NF- κ B2 p100, is essential for maintaining optimal Treg function and immune tolerance.

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Disclosures

The authors have no financial conflicts of interest.

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Review

NF- κ B in Cancer Immunity: Friend or Foe?

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Abstract: The emergence of immunotherapies has definitely proven the tight relationship between malignant and immune cells, its impact on cancer outcome and its therapeutic potential. In this context, it is undoubtedly critical to decipher the transcriptional regulation of these complex interactions. Following early observations demonstrating the roles of NF- κ B in cancer initiation and progression, a series of studies converge to establish NF- κ B as a master regulator of immune responses to cancer. Importantly, NF- κ B is a family of transcriptional activators and repressors that can act at different stages of cancer immunity. In this review, we provide an overview of the selective cell-intrinsic contributions of NF- κ B to the distinct cell types that compose the tumor immune environment. We also propose a new view of NF- κ B targeting drugs as a new class of immunotherapies for cancer.

Keywords: onco-immunology; immunotherapy; NF-kappaB

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1. Introduction: The Growing Role of Immunity in Cancer

The past decades have been marked by a remarkable interest in research on onco-immunology, i.e., the interactions between cancer and the immune system. This has culminated with the approval of anti-Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and Programmed Death (PD)-1/PD-Ligand 1 monoclonal antibodies (mAbs) and CD19-targeted Chimeric Antigen Receptor (CAR)-T-cell infusions as frontline therapies for a variety of cancers. Despite the fact that these treatments are not effective in all patients and all types of diseases, they established the revolutionary concept that the immune system can be manipulated to treat cancer. Many different immune cell types, surface receptors and secreted factors are involved in the different steps of cancer progression. In order to highlight novel efficient and safe immunotherapies, it is necessary to apprehend this complexity of immune responses to cancer. Recent technological developments such as single-cell sequencing, high-dimensional flow and mass cytometry, and multiparameter immunofluorescence techniques, have enabled the fine dissection of immune cell subsets, their phenotype and function, revealing a landscape even more complicated than originally thought. However, little is known about the intracellular pathways that dictate the development and fate of different cell subsets in the tumor environment. It is becoming evident that understanding these intracellular cascades and transcription factors may highlight potential therapeutic targets. Among those, the Nuclear Factor kappa light chain enhancer of activated B cells (NF- κ B) transcription factors exhibit various and sometimes confusing effects on most immune subsets involved in immune responses to cancer.

2. A Simplified View of the Signaling to NF- κ B

NF- κ B is a family of 5 transcription factors that all share a Rel Homology Domain (RHD): NF- κ B1 (p105/p50, encoded by *Nfkb1*), NF- κ B2 (p100/p52, *Nfkb2*), RelA (*Rela*), RelB

(*Relb*) and c-Rel (*Rel*) (reviewed in [1]). In the resting state, NF- κ B subunits are retained in the cytosol by I κ B proteins and by unprocessed p105 and p100 that act as inhibitors through the ankyrin repeats in their C-terminal portion. The engagement of diverse receptors leads to the nuclear translocation of homo- or heterodimers of NF- κ B subunits, which can then activate or repress gene transcription.

The signaling cascade leading to NF- κ B activation was historically split in two pathways. In innate immune cells, activation of the canonical pathway is classically driven by the engagement of Pattern Recognition Receptors (PRRs) such as Toll-Like Receptors (TLRs). This leads to the recruitment of adaptors such as MyD88 or Toll/IL-1R domain-containing adaptor-inducing IFN- β (TRIF) and the activation of Tumor necrosis factor Receptor-associated Factor (TRAF)6. Similarly, cytokine receptors or members of the Tumor Necrosis Factor Receptor superfamily (TNFRSFs) can engage TRAF2/5. Additionally, in adaptive immune cells, engagement of the antigen receptor (BCR and TCR for B and T cells, respectively) recruits Protein Kinase C (PKC) β /PKC θ to enable the formation of the Caspase Recruitment domain-containing Membrane-Associated guanylate kinase protein-1 (Carma1)/ B-cell lymphoma/leukemia 10 (Bcl10) and Mucosa-Associated Lymphoid Tissue lymphoma translocation protein 1 (Malt1), or CBM complex [2]. All these signals lead to the activation of the I κ B kinase (IKK) complex (composed of the α and β catalytic subunits and γ scaffolding protein), which by phosphorylating Inhibitor of NF- κ B (I κ B α) and p105 triggers their proteasomal degradation and processing to the active p50 protein, respectively. This culminates in the rapid and transient translocation of NF- κ B subunits to the nucleus. The alternative NF- κ B pathway is activated in response to the engagement of a number of TNFRSFs, such as CD40 on dendritic cells (DCs) and B cells, B cell-Activating Factor Receptor (BAFF-R) on B cells or TNFR2 on T cells. Through the recruitment of TRAF2/3, this allows the stabilization of NF- κ B-inducing kinase (NIK) and the activation of IKK α / α homodimers that phosphorylate p100 and lead to its processing into active p52. p52/RelB dimers are then translocated to the nucleus. Importantly, converging evidence now indicates that the canonical and alternative pathways, as well as subunits, are highly intricate. Hence, it may be more relevant to report the composition and roles of NF- κ B subunits and dimers, rather than oversimplifying NF- κ B to two distinct pathways.

As one of the most studied family of transcription factors, NF- κ B has been highlighted for its diverse roles in most biological processes, with a particular focus on inflammation, adaptive immunity and oncogenesis. Indeed, extensive research has identified both cell-autonomous and cell-extrinsic roles for NF- κ B in cancer. For instance, canonical NF- κ B controls tumor cell proliferation, tumor-mediated angiogenesis as well as metastasis. This is nicely demonstrated by the reduced tumor burden detected in mouse models of K-Ras induced lung cancer carrying conditional ablation of *Ikkkb* or *Rela*, or transgenic expression of an I κ B α -superrepressor; similar observations were made in melanoma among other cancers [3–6]. However, opposite results were described in murine liver cancer or squamous cell carcinoma, where canonical NF- κ B inhibition increased tumor growth, thus highlighting the cell type-specific functions of this pathway and a possible role for NF- κ B as a tumor suppressor in certain settings [7,8]. In addition to these cell-intrinsic functions, NF- κ B activation in tumors appears to impact their immune environment. It is well established that tumor cells produce a number of NF- κ B-dependent cytokines and chemokines that affect the recruitment and phenotype of immune cells in the tumor bed, and the outcome of cancer [9–12]. Moreover, NF- κ B controls the mRNA expression and protein stability of PD-L1 in tumor cells, thereby promoting the inhibition of cytotoxic CD8⁺ T cells [13,14]. Finally, NF- κ B plays many cell-autonomous roles in the function of immune cells in the tumor environment, clearly impacting cancer outcome. This will be the focus of the present review.

3. NF- κ B in Innate Immunity and Inflammation

The development and progression of some tumors, such as colon or liver cancers, have been tightly associated with inflammatory cells and mediators. Because NF- κ B, through its function in innate immune cells, is a critical orchestrator of the inflammatory process, it is often viewed as the main molecular link between inflammation and carcinogenesis. However, its roles may be multifaceted depending on the cell subset, the type of cancer and the signaling pathways that are engaged (Figure 1).

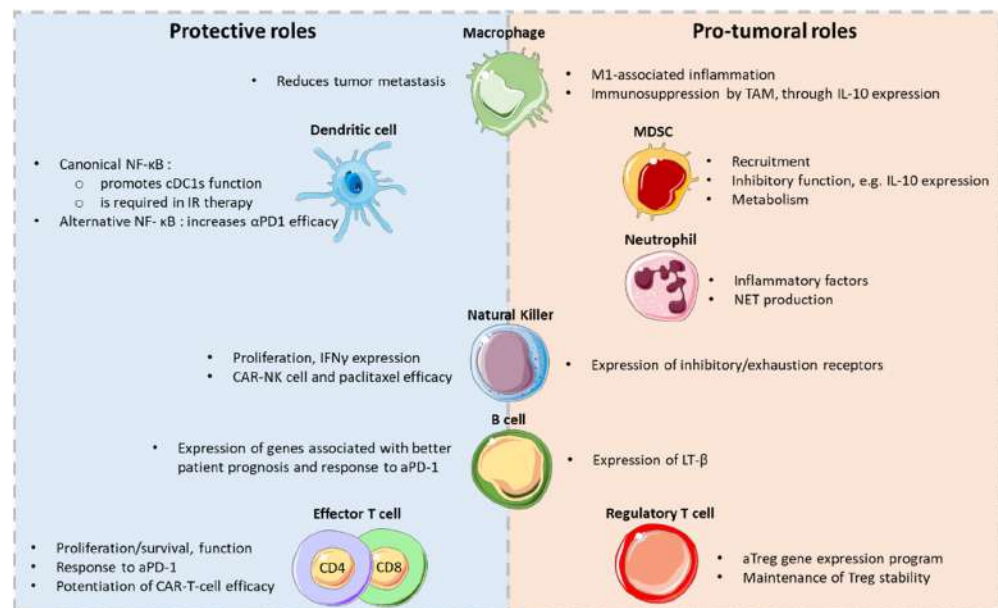


Figure 1. Dual roles of NF- κ B in tumor immunity. The figure depicts some of the known functions of NF- κ B in immune cell subsets in the tumor environment. Some figure elements were obtained from Servier Medical Art (<https://smart.servier.com/>).

3.1. Pro-Tumoral Roles of NF- κ B in Innate Immune Cells: Macrophages and MDSCs

3.1.1. Macrophages

Tumor-Associated Macrophages (TAMs) are a major component of the immune infiltrate of solid tumors [15]. Different flavors of macrophages can be found and often co-exist in the tumor microenvironment (TME): ‘M1’ macrophages are potent producers of pro-inflammatory factors, whereas ‘M2’ macrophages display anti-inflammatory, immunosuppressive and pro-angiogenic properties [16]. Ordinarily, high TAM infiltration is correlated with bad prognosis in many cancers irrespective of their polarization status [17].

In vitro assays have unequivocally demonstrated the role of canonical NF- κ B in the expression of inflammatory cytokines by macrophages- in other words, polarization towards an M1 phenotype [16,18]. Engagement of the TLR-Myd88 axis, as well as TNFR1, enables transcription of *Tnf*, *Il6* or *Il12b* among others, through canonical NF- κ B. In contrast, p50 homodimers have regulatory activity, which is essential for an M2 “tolerant” phenotype in vitro and in vivo [19,20]. Alternative NF- κ B subunits seem to be expendable for the expression of inflammatory cytokines [21]; instead overexpression of RelB inhibits *Tnf* expression in M1-polarizing conditions [22].

Seminal studies from Michael Karin’s group have established the pro-tumoral function of the canonical NF- κ B pathway in macrophages, in the context of inflammation-driven cancer. Ablation of *Ikkkb* or *Rela* in macrophages using the Lysozyme (LysM)-CRE strain, leads to decreased tumor growth in mouse models of cigarette-smoke induced lung cancer and colitis-associated colon cancer [23–25]. Conversely, in models of breast,

ovarian cancer and glioblastoma, canonical NF- κ B activation in macrophages has an anti-inflammatory effect, preventing antitumor immunity and allowing tumor escape, in particular through the secretion of interleukin (IL)-10 [26–29]. Thus, depending on the tumoral setting, NF- κ B exerts its deleterious functions by promoting either inflammation or immunosuppression; these separate functions have been discussed in the past [30]. Of note, this pathogenic role of canonical NF- κ B was confirmed in patients with lung cancer, in which RelA activation in lung myeloid cells is associated with poor survival [25]. At odds with these reports, it was proposed that IKK β may exert a protective function against tumor cell dissemination. Indeed, metastasis of breast cancer and melanoma cells was drastically increased in mice with myeloid-restricted deletion of *Ikkbb*, due to impaired innate and adaptive immunity to tumors [31–33]. Nevertheless, opposite results were found using a similar model of breast cancer metastasis [29]. These discrepancies may be attributable to the generalized use of the LysM-CRE strain in macrophage biology, which drives expression of the recombinase not only in macrophages but also in highly immunosuppressive MDSCs that are described below [34]. These complex functions of NF- κ B could also be harnessed in a therapeutic perspective. Cell therapy using *Ikkbb* or *Nfkb1*-deficient macrophages favors strong anti-tumor immunity and tumor rejection [27,35]. Conversely, as discussed in later sections, in vivo stimulation of TLRs using agonistic molecules, has a strong therapeutic potential in cancer, perhaps through its effect on macrophages [36]. Finally, the natural flavonoid baicalin, promotes the repolarization of TAM to M1 macrophages by increasing Relb and p52 expression, leading to decreased growth of hepatocellular carcinoma in mice [37].

Taken together, further exploration is needed to fully establish the role of NF- κ B in the different tumor types and steps of cancer development. Deciphering these precise molecular mechanisms, in particular the selective roles of NF- κ B subunits, will help provide a rationale for the targeting of this pathway in cancer. In the future, the development of more specific tools (conditional gene ablation, cell depletion, etc.) will undoubtedly clarify these dual functions.

3.1.2. MDSCs

Myeloid-Derived Suppressor Cells (MDSCs) have emerged in the past years as an important player in cancer immunity. They compose a heterogeneous subset of innate immune cells defined by their immunosuppressive properties, in particular their expression of IL-10, Transforming Growth Factor (TGF)- β , or Indoleamine 2,3 DiOxygenase (IDO) among other factors, rather than by their phenotype that can overlap with other myeloid subsets [38]. Consequently, high MDSC infiltration is usually correlated with a poor prognostic value in cancer [39]. In mice, depletion of MDSCs (using poorly specific anti-Gr1 or anti-Ly6C mAbs) leads to enhanced antitumor immunity and reduced tumor burden [40,41]. The biology of MDSCs and the therapeutic potential of their targeting is currently under scrutiny, NF- κ B appearing to largely support their deleterious function.

It was proposed that NF- κ B could support the activation and expansion of MDSCs [42,43]. In pre-clinical models of cancer, both the canonical and the alternative pathway were reported to be implicated. It was first shown that TLR2/TLR4- MyD88-mediated signaling was required for the optimal function of MDSCs and suppression of anti-tumor immunity in models of colon cancer and fibrosarcoma, suggesting an involvement of canonical NF- κ B activation [44,45]. Similarly, TNFR/TNFR2 signaling promotes MDSC function, especially IL-10 production, in an NF- κ B-dependent manner [46]. More directly, the inhibitory function of MDSCs was strongly reduced in *Nfkb1*^{-/-} animals- though their number was increased in the tumor bed- resulting in the reduced growth of transplanted murine fibrosarcoma [47]. In addition, LysM-CRExRela-Flox mice transplanted with glioblastoma cells showed reduced levels of MDSCs and pro-tumoral TAMs, suggesting a role for RelA in MDSC recruitment [28]. Finally, a recent study elegantly described the role of c-Rel in regulating the development, function and metabolism of MDSCs during

tumorigenesis [48]. Indeed, LysM-CRExRel-Flox mice display reduced growth of melanoma and thymoma cells, and the administration of the specific c-Rel inhibitor R96A delayed tumor growth and potentiated anti-PD1 therapy. This fully established the critical function of all three canonical NF- κ B subunits in the deleterious functions of MDSCs. Moreover, activation of the NIK/RelB-p52 axis induces *IDO* expression in human MDSC-like cells cultured with breast cancer cells, suggesting that alternative NF- κ B subunits may also promote immunosuppression in tumors through its role in MDSCs [49].

Of note, some literature also supports a pro-tumoral role for NF- κ B in neutrophils during cancer. The high activation of the canonical pathway in Tumor-Associated Neutrophils (TAN) suggests its pro-tumoral role by inhibiting apoptosis of TANs, promoting autophagy and inducing a pro-inflammatory state [50–53]. Finally, indirect evidence highlights a role for NF- κ B in Neutrophil extracellular traps (NET) production [51], a known propagator of metastasis [54]. Altogether, this large body of evidence demonstrates the deleterious facet of NF- κ B activation in innate immune cells through its effects on macrophages and MDSCs. However, the picture is way more complex than it seems and NF- κ B can also suppress tumor growth through its pivotal functions in other innate immune subsets: DCs and NK cells.

3.2. NF- κ B in the Promotion of Anti-Tumor Immunity: DCs and NK Cells

3.2.1. DCs

DCs are the main bridge between innate and adaptive immune responses. Through their critical function as Antigen Presenting Cells (APCs) and their cytokine and chemokine expression, they can shift the balance between tumor immunity and tolerance [55]. DCs can come in different flavors defined by their phenotype and function in the TME. Type 1 Conventional DCs (cDC1s) have an optimal ability to cross-present tumor antigens to CD8⁺ T cells and produce high amounts of IL-12, making them potent anti-tumor cells [56], and the accumulation of cDC1s in the TME is of good prognosis in many cancers [57]. Furthermore, cDC1s are required for the response to anti PD-1 therapies in mice [58]. Type 2 cDCs (cDC2s) are classically associated with the priming of CD4⁺ T cells. Interestingly, intra-tumoral cDC2 density was also shown to be correlated with abundant CD4⁺ T cells and anti-PD-1 therapy response in melanoma patients [59]. Finally, plasmacytoid DCs (pDCs), characterized by their high expression of type I interferons, have complex roles in tumor immunity that largely rely on the type of stimulation and their activation status [60,61].

Extensive research has highlighted the various roles of NF- κ B in the biology of DCs. This stems from the fact that most of the stimuli that induce DC maturation (PRR, cytokines, etc.) are known activators of the NF- κ B pathways (reviewed in [62]). Briefly, canonical NF- κ B subunits were historically described as important regulators of DC maturation and survival following TLR and CD40 stimulation [63–65], though this is still controversial and may in fact depend on the type of stimuli and DC subset [66]. In contrast, it is established that c-Rel is absolutely critical for the expression of *Il12p35* (which, together with *Il12p40*, encodes IL-12), which emphasizes its role in the function of cDC1s [63,66,67]. The NIK/RelB-NF- κ B2 axis is also involved in DC biology, particularly for the stability of the cDC2 lineage, and is required for optimal antigen cross-presentation [68–71]. To add another layer of complexity, it was shown that canonical NF- κ B signaling induced by TLR engagement, drives the translocation of p50/RelB dimers, which enables optimal DC maturation [72]. Collectively, this highlights the overlaps between canonical and alternative subunits [73]. In contrast with these immune-stimulatory properties, NF- κ B activation in DCs is also critical to maintain immune tolerance. This is clearly illustrated by the spontaneous autoimmune syndrome observed in mice with conditional ablation of *Ikkb* in CD11c⁺ DCs, which is due to impaired homeostasis of migratory DCs and a subsequent defect of Foxp3⁺ regulatory T cells (Treg cells) in lymph nodes [74]. Moreover, the alternative pathway, through IKK α activation following CD40 stimulation,

was proposed to promote the expression of the immunosuppressive molecule IDO- in particular in pDC-, thereby contributing to immune tolerance, for instance through the induction of Treg cells [75,76].

These diverse functions have obvious implications in cancer immunity. Indeed, NF- κ B activation is often actively dampened in DCs of the TME, which leads to reduced DC maturation and function, through mechanisms that may involve soluble factors such as Fibrinogen-like protein 2 (FGL2) and Vascular Endothelial Growth Factor (VEGF) or immune checkpoints such as PD-1 [77]. The cell-autonomous functions of NF- κ B in DCs are illustrated in different settings of cancer therapy. For instance, DC-restricted ablation of *Rela* abolishes the therapeutic effect of ionizing radiation (IR) in several models of transplanted tumors, while ablation of *RelB* improves this therapeutic efficacy, highlighting opposing functions of canonical and alternative NF- κ B subunits in this IR-induced signaling cascade [78]. In line with this, adenovirus-mediated overexpression of the *RelA* RHD in bone-marrow-derived DCs (BMDCs) increases their APC function and cytokine expression. Indeed, transfer of such transgenic cells to tumor-bearing mice leads to reduced tumor growth and enhanced survival [79]. In vivo administration of CD40 agonists as well as Second Mitochondria-derived Activator of Caspases (SMAC) mimetics, drives the activation of the alternative NF- κ B pathway and increases the secretion of IL-12 [80]. When combined to anti-PD-1 therapy, this regimen leads to tumor rejection in a NIK-dependent fashion [81]. Finally, it is likely that many TLR agonists with therapeutic activity in cancer, act in part by enhancing NF- κ B activation in DCs, though this has so far not been directly demonstrated [82,83]. Thus, NF- κ B in DCs appears as an interesting target in cancer therapy. However, the roles of each subunit may vary depending on the context and deciphering their DC-intrinsic selective contributions to different cancer types and therapies may provide valuable insight and rationale into the use of DC-based immunotherapies.

3.2.2. NK Cells

Through their potent cytokine production and cytotoxic functions, Natural Killers (NK) cells are powerful antitumor agents. Their enrichment in the tumor bed is associated with good prognosis in many cancers. In mouse models, NK cells are able to delay tumor growth or prevent metastasis, depending on the model (reviewed in [84]). NK cell activation and cytotoxicity largely relies on the engagement of several activating receptors. Interestingly, many of these, such as CD244, NKG2D, CD226, NK1.1, Ly49D, or Nkp30, trigger the activation of canonical NF- κ B subunits in a CBM complex-dependent manner [85–88]. Similarly, IL-18, one of the master cytokines enabling NK cells function in cancer, activates NF- κ B through MyD88 [89–91].

Consequently, NF- κ B was shown to play a role in different aspects of NK cell biology. Inhibition of NF- κ B with a non-degradable I κ B α in NK cells reduced their proliferation [92]. Conversely, constitutive IKK β activation leads to hyper-activated NK cells [93]. Interestingly, *Nfkb1*^{-/-} NK cells display a strong increase in proliferation, while *Rel* ablation has no effect, highlighting NF- κ B1 as a negative regulator of NK proliferation [94]. In patients with *IKBKB* or *IKBKG* mutations, impaired IFN γ expression by NK cells was detected [95,96]. Similar observations were made upon chemical or genetic inhibition of canonical NF- κ B in murine NK cells [86,87,92]. However, the exact contribution of each subunit remains unknown to date, as NF- κ B1 and c-Rel appear to be expendable for NK cell function in vivo [94]. Finally, several reports using mouse NK cells suggested a role for canonical NF- κ B subunits in the expression of *Prf1* (encoding perforin) and *Gzmb* (granzyme B) [97,98].

Despite this body of evidence, only few studies have directly investigated the function of NF- κ B in NK cells in the context of cancer. It was proposed that HIF-1 α expression, induced by hypoxia in the TME, inhibited IL-18-mediated NF- κ B activation in tumor-infiltrating NK cells, contributing to their defective function and uncontrolled tumor progression in the RMA/S lymphoma model that exhibit impaired expression of

Major Histocompatibility Complex (MHC)-I [99]. In a model of pancreatic cancer, stimulation of NK cells with the polysaccharide SEP enhanced their cytotoxicity through a TLR4/ Mitogen-Activated Protein Kinase (MAPK)/MAPKs/NF- κ B pathway, which was associated with delayed tumor growth [100]. Moreover, it was proposed that the therapeutic effect of paclitaxel in breast cancer, may rely on enhanced NK cell function in an NF- κ B-dependent manner [101]. Finally, CAR-NK cells engineered to express an artificial Myd88/CD40 signaling node display enhanced anti-tumor activity, likely through increased NF- κ B activation [102]. Importantly, NF- κ B can also exhibit a deleterious facet, as it was suggested to promote the expression of Tim-3 and NK cell dysfunction in human esophageal cancer [103]. Hence, although NF- κ B mainly displays anti-tumor properties through its function in NK cells, further studies are warranted to fully apprehend their therapeutic potential and discriminate the dual roles of NF- κ B in NK cell function and exhaustion.

4. NF- κ B in Adaptive Immunity to Cancer

4.1. B Cells

B cells are often found in large quantities in the TME [104] but seem to have divergent functions in cancer immunity depending on the tumor context. B cells were originally shown to promote cancer growth in mouse models, for instance through their expression of soluble factors such as IL-8 or lymphotoxin (LT)- β [105–107], or more directly through antibody secretion [108]. In addition, a subset of “regulatory” B cells (Breg cells) can express inhibitory cytokines such as IL-10, TGF- β or IL-35 and favor tumor progression [104,109–111]. In line with these pre-clinical observations, the depletion of B cells using anti-CD20 mAbs, had beneficial effects in patients with colon cancer, though results were disparate in melanoma patients [112,113]. Conversely, B cells were also shown to exert anti-tumor functions through their Ab secretion (driving ADCC, complement-dependent lysis or opsonization of tumor antigens by APCs) as well as their APC capacity [114–116]. B-cell infiltration is associated with good prognosis in the vast majority of cancers [117]. The organization of B cells and T cells in Tertiary Lymphoid Structures (TLS) is a powerful predictor of the efficacy of PD-1-blockade therapy in human melanoma, renal cell carcinoma and sarcoma [118–120].

NF- κ B plays important functions in the maturation and thus the function of B cells. NF- κ B activation can be driven by the engagement of the antigen receptor (BCR) as well as many members of the TNFRSF family (in particular BAFF-R and CD40 for B cells). Conditional ablation of both *Rel* and *Rela* in mature B cells decreases their numbers in the spleen [121]. Similar observations were made with conditional *Ikk β* KO animals [122]. Upon immunization with T cell-dependent antigen sheep red blood cells, c-Rel is essential for germinal center B cell maintenance, whereas RelA is required for plasma cell development [123]. Interestingly, the alternative pathway is also involved in germinal center B cell development, as shown in mice harboring conditional ablation of *Relb* and *Nf κ b2* in mature B cells [124]. Interestingly, c-Rel and RelB may have complementary functions in B-cell homeostasis [125].

Despite this large body of evidence characterizing NF- κ B in non-pathological states, only few studies have directly assessed the functions of NF- κ B in B cells in cancer. In a mouse model of prostate cancer, it was demonstrated that IKK β drives the expression of LT- β by B cells, which in turn augments tumor growth [107], highlighting the deleterious function of B cells in this setting. Conversely, it was recently shown that human melanoma secretomes drive the expression of many NF- κ B-dependent genes in B cells, including activation markers and co-stimulation molecules such as CD69, CD30 or CD137, as well as chemokines such as CCL3 or CCL4 [112]. This enrichment was correlated with improved survival of patients and better response to anti-PD-1 checkpoint blockade therapy. However, it remains unclear whether NF- κ B actually plays an active role in the orchestration of anti-tumor immune responses. More mechanistic studies, in particular in

conditional KO mouse models, are therefore required to elucidate the implication of NF- κ B subunits in B cells during cancer.

4.2. T Cells

T cells are probably the most scrutinized cells in cancer immunity. Whereas CD8⁺ T cells and IFN γ -producing Thelper (Th) 1 cells exhibit potent anti-tumor functions and are generally of good prognosis in solid tumors [126,127], their Foxp3⁺ regulatory T cell (Treg cells) counterparts are master inhibitors of cancer immunity (reviewed in [128]). Their enrichment in the TME is associated with poor prognosis in a large number of cancers [129]. Strikingly, NF- κ B plays many roles in these distinct T-cell subsets.

4.2.1. Effector CD4⁺ and CD8⁺ T-Cell Subsets

NF- κ B was originally described as an important regulator of T-cell homeostasis, i.e., proliferation, survival and expression of specific cytokines [130]. T cells isolated from *Nfkb1*^{-/-} or *Rel*^{-/-} animals, exhibit a reduced proliferative capacity in vitro when compared to control cells [131,132]. Similarly, Shifrut et al. identified RelA as a critical regulator of human CD8⁺ T-cell proliferation using a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 screening approach [133]. This observation was further validated in mice [134]. This phenotype seems to rely on both cell-intrinsic defects (e.g., impaired expression of the cell cycle factor c-Myc and the anti-apoptotic protein Bcl-xL [135,136]) and extrinsic factors, in particular the reduced production of IL-2, which when added to cultures rescues the proliferative defects of *Rel*-deficient T cells [135]. In addition, NF- κ B has cell-autonomous roles in the polarization towards different flavors of Th cells. RelA and c-Rel are crucial for the expression of RAR-related orphan receptor (ROR) γ t in differentiating Th17 cells and have been involved in the expression of IFN γ by Th1 cells [137–140]. A recent study pinpointed possible functions of RelA and NF- κ B2 in the expression of *Gata3* and *Il4*, respectively, by murine Th2 cells [141]. Finally, Th9 cells, which can exhibit potent anti-tumor properties, may rely on either canonical or alternative NF- κ B activation, depending on the stimulus [142,143]. NF- κ B is also involved in the maturation and function of CD8⁺ T cells. For instance, RelA activation downstream of TCR engagement, is required for optimal expression of *Eomes* [144]. The expression of IFN γ by CD8⁺ T cells is also NF- κ B dependent, as shown in mice with conditional ablation of *Rela* in T cells [139], and in patients carrying loss-of-function mutations in *IKBKB* and *REL* [96,145]. Finally, PKC- θ and Bcl10 are required for the cytotoxic function of CD8⁺ T cells in vitro, implying of the need for canonical NF- κ B activation in this mechanism [146].

Altogether, this suggests that NF- κ B likely plays a role in controlling T-cell activation, survival and/or function in the context of cancer. In line with this, NF- κ B activation is dysregulated in T cells from patients and mice with renal cancer [147,148]. Moreover, peripheral anergic T cells, which resemble tumor-infiltrating exhausted T cells, exhibit reduced RelA nuclear translocation [149]. More directly, the function of NF- κ B in T-cell immunity to cancer was explored in a seminal study by Barnes and al, where the authors used mice carrying conditional ablation of *Ikkbb* in total T cells. Inhibition of canonical NF- κ B activation leads to increased tumor growth in an immunogenic model of fibrosarcoma [150]. This was associated with reduced effector function and cytotoxic activity of CD8⁺ T cells. This phenotype was also observed in *Carma1*-deficient mice. In a reverse set of experiments, the same group found that constitutive activation of IKK β (Ikk β -CA) in T cells, strongly decreases tumor growth in a CD8⁺ T cell-dependent manner [151]. Engineering WT or TCR-transgenic CD8⁺ T cells with an active form of IKK β (Ikk β -CA), enables potent tumor rejection. Similarly, ablation of *Tnfrsf3* in TCR-Tg T cells, which increases canonical NF- κ B activation, augments their therapeutic potential in transplanted mouse melanoma [152]. Overall, this highlights an interest for genetically-augmented NF- κ B activation in T-cell-based cancer therapies. It would now be interesting to decipher which cell types and which NF- κ B subunit(s) are required for this control of tumor growth. A recent report suggests that p50/p50 homodimers are able to directly

repress *Gzmb* expression in CD8⁺ T cells, consequently, *Nfkb1*^{-/-} mice exhibit a partial resistance to colon tumorigenesis [153]. In addition, as described above, NF- κ B subunits orient the polarization toward Th cell subsets. Importantly, both protective and deleterious functions were attributed to discrete Th cell subsets in cancer immunity [154]. It is thus likely that this function will impact tumor growth, though the link has not yet been determined. Finally, NF- κ B seems to be important for the reinvigoration of T cells following checkpoint-blockade therapies, and this point will be largely discussed below in Section 5.1.

4.2.2. Foxp3⁺ Treg Cells

Unlike its function in promoting immunity by controlling effector T cell function, NF- κ B is also a master regulator of Treg cell biology and thus regulates immune tolerance. Activation of RelA and c-Rel, through a PKC θ /CBM/IKK axis, is critical for the initiation of Foxp3 expression in immature Treg cells in the thymus [155–158]. Consequently, germline or conditional ablation of one of the aforementioned molecules leads to a significant decrease in the number of Foxp3⁺ Treg cells in the thymus and secondary lymphoid tissues. In addition, NF- κ B activation is required to maintain the homeostasis and identity of mature peripheral Treg cells. Treg-restricted ablation of *Carma1*, *Bcl10*, *Malt1*, *Ikkkb* or *Rela* using the Foxp3-CRE strain, leads to a multifocal and lethal autoimmune syndrome, characterized by the loss of Treg signature genes and the expression of effector genes such as inflammatory cytokines [158–162]. On the other hand, activation of the alternative pathway seems to have a rather deleterious effect, as transgenic expression of *Nik* [163], as well as ablation of *Nfkb2* [164,165], also drive autoimmune symptoms. The latter can be rescued by additional ablation of *Relb*, which by itself is expendable for Treg cell homeostasis [166].

This master function of NF- κ B in Treg cells has strong implications in cancer immunity. Indeed, the growth of transplanted tumors is strongly reduced in mice with Treg-specific deletion of either *Carma1*, *Bcl10* or *Malt1* [159–161]. This is consistent with our original observation that melanoma burden is significantly impaired in mice with conditional *Rel* ablation in Treg cells [167]. Interestingly, germline ablation of *Rel* gives rise to the same phenotype [48]. Mechanistically, ablation of the CBM/c-Rel axis leads to the generation of ‘unstable’ Treg cells that produce important amounts of IFN γ and TNF. Thus, Treg cells become potent anti-tumor cells, as the transfer of *Carma1*^{-/-} Treg cells is sufficient to reduce cancer growth in an IFN γ -dependent fashion [159]. In line with all these observations, administration of Malt1 inhibitors (mepazine, MI-2), IKK β inhibitor (KINK-1) or c-Rel inhibitors (pentoxifylline, IT-603 or R96A) reduces Treg cell ‘fitness’ in the tumor, i.e., their accumulation and/or their function [48,159,162,167,168]. Consequently, these drugs have a significant therapeutic effect in different pre-clinical models of cancer, when administered alone or combined with anti-PD-1/PD-L1 mAbs, tumor vaccines or chemotherapy.

Collectively, this demonstrates that NF- κ B is at the interface between T-cell immunity and tolerance to tumors. This highlights a strong rationale for targeting this pathway in cancer immunotherapy.

5. NF- κ B Modulation at the Era of Immunotherapy: Activation or Inhibition?

5.1. NF- κ B Activation to Enhance Effector T-Cell Function in Cancer

5.1.1. Putative Functions of NF- κ B in T-Cell-Targeting Immunotherapies

Cancer immunotherapies developed in the past decades have revolutionized patient care. In particular, immune checkpoint-blockade therapies such as anti-CTLA-4 and anti-PD-1/PD-L1 antibodies, and CAR-T-cells, appear as game changers for the treatment of numerous solid cancers and hematological malignancies, respectively. However, these agents are not effective in all patients, and it is therefore critical to understand the molecular mechanisms underlying the response and resistance to such therapies.

Interestingly, a number of studies converge to consider the NF- κ B pathway as a possible mediator of the anti-tumor properties of current immunotherapies.

Bulk and single-cell transcriptomic analyses of tumor specimens obtained from patients before and after anti-CTLA4 and/or anti-PD1 administration, have unequivocally demonstrated that the up-regulation of NF- κ B-dependent genes in both tumor and immune cells, underlies the clinical response to checkpoint-blockade therapies [169,170]. In particular, a number of supposedly NF- κ B-regulated cytokines and chemokines are up- and down-regulated in patients who respond to immunotherapy [171]. However, it remains unclear whether NF- κ B activation is a proxy of T-cell activation or is actually required for improving anti-tumor responses. Mechanistically, it was proposed that the engagement of either CTLA-4 or PD-1 on T cells, may reduce NF- κ B activation through the recruitment of Src homology region 2 domain-containing phosphatase-2 (SHP-2) and Protein Phosphatase (PP)2A, leading to reduced signaling downstream of TCR and CD28 [172–175]. Subsequently, CTLA-4 or PD-1-blockade would lead to increased NF- κ B activity in T cells. In line with this hypothesis, it was shown that CD28 engagement, which drives NF- κ B activation among other pathways, is critical for the efficacy of anti-PD-1 therapy in mice [176]. Moreover, the expression of IFN γ and CD127, two canonical NF- κ B-dependent genes, are required for tumor clearance upon CTLA-4/PD-1 dual blockade [177].

Improvement of NF- κ B activity in cellular therapies to cancer is also a promising approach. As stated above, ablation of *Tnfrsf3* (encoding the NF- κ B inhibitor A20) or expression of Ikk β -CA augments the anti-tumor activity of transferred T cells [151,152]. Recently, a genome-wide CRISPR approach revealed that ablation of the RNA helicase *Dhx37* in murine T cells improved tumor clearance by increasing RelA activity [178]. In addition, possible implication of NF- κ B was reported in the expansion of CD19- CAR-T-cell carrying a 4-1BB signaling domain. Indeed, engagement of the CAR in these cells drives sustained NF- κ B activation in a TRAF1-dependent mechanism [179]. Both the canonical and alternative NF- κ B pathway are activated following CD19 recognition, and expression of a dominant-negative NIK peptide reduces CAR-T cell expansion and survival ex vivo [180]. Further studies are now required to elucidate the direct function of discrete NF- κ B subunits in the therapeutic effect of CAR-T cell administration in vivo.

These findings strongly advocate for a beneficial role of NF- κ B activation in the response to checkpoint-blockade and CAR-T-cell-based therapies, though direct evidence of its requirement is lacking.

5.1.2. Stimulation of NF- κ B by TLRs and TNFRSFs Agonistic Reagents as Novel Immunotherapies

The therapeutic potential of TLR agonists in mouse models of cancer has been largely documented (reviewed in [83]). This has led to numerous clinical trials in which patients are treated with synthetic TLR3, 4, 7 and 9 agonists in combination with other immunotherapies or conventional treatments [181]. Because TLR signaling through NF- κ B is a master inducer of innate immune cell activation, maturation and inflammatory cytokine expression, it is likely that the anti-tumor effect of these drugs relies on NF- κ B activation. Similar hypotheses can be made for Stimulator of Interferon Genes (STING) and CD40 agonists, which drive NF- κ B activation in DCs (and B cells for CD40) [78,182,183]. In addition, a number of initiatives currently aim at exploring the therapeutic potential of antibodies triggering activation of “stimulatory” checkpoints on T cells, i.e., co-stimulation receptors of the TNFRSF family such as 4-1BB or Glucocorticoid-Induced TNFR (GITR). Engagement of these receptors has been proposed to induce or sustain NF- κ B activation in combination with TCR stimulation [184]. Engagement of all these receptors improves immunity to cancer and reduces tumor growth in pre-clinical models [185]. These observations are contributing to pushing these agents toward clinical applications [185]. However mechanistically, it is still unclear whether the therapeutic efficacy of these molecules relies on NF- κ B activation. Furthermore, the specific

delineation of NF- κ B subunits in this process is unknown. Novel tools, such as mice carrying lineage-specific NF- κ B ablation, will help unravel these issues and may provide a mechanistic rationale for the use of these new therapies.

5.2. NF- κ B Inhibitors as Immunotherapeutic Agents?

5.2.1. Modulation of Tumor Immunity by NF- κ B Inhibitors

At odds with this possible protective function in T cells, it is clear that NF- κ B can have pathogenic functions in cancer, in particular through its well-established roles in malignant cells. Consequently, a myriad of inhibitors of the NF- κ B pathway have been tested for their anti-cancer properties, with encouraging results in pre-clinical models. These compounds were shown to directly impact tumor cell survival, proliferation, or invasiveness in in vitro assays and in vivo. In addition, it is becoming evident that NF- κ B inhibitors largely modulate the tumor immune response. Indeed, therapeutic intervention at each step of the NF- κ B pathway (i.e., Cellular Inhibitor of Apoptosis Protein (cIAPs), CBM and IKK complexes, I κ Bs, and c-Rel) was shown to enhance cancer immunity to some extent. A large panel of modifications in the immune composition of the tumor infiltrate and the function of immune cells was described, and some relevant examples are highlighted in Table 1. The precise reasons for this immune stimulation are not clearly defined, though the following mechanisms can be proposed (Figure 2):

Table 1. Effect of NF- κ B inhibitors on cancer immunity. This table highlights some of the studies aiming at evaluating monotherapy-induced immune responses in the context of cancer in vivo. In vitro assays and other diseases are not discussed.

Agent/Mechanism.	Tumor Model	Impact on Immune Cells	References
Mepazine/MI-2, MALT-1 inhibitor	Mouse melanoma	↑ CD8 ⁺ T cells, ↑ IFN γ by Treg cells in TILs; efficacy lost in RAG ^{-/-} mice	[159]
Curcumin, prevents I κ B α degradation, NF- κ B translocation	Mouse breast, colon, lung cancer	↑ T cells and IFN γ expression, ↓ Treg cell proportion in TILs	[186–188]
	Mouse breast, bladder lung cancer	↓ MDSCs in TILs, ↑ M1 macrophages	[189–191]
	Mouse glioblastoma	↑ NK cells; NK-cell-dependent therapeutic effect	[192]
	Patients with lung cancer	↑ IFN γ expression by CD4 ⁺ T cells, ↓ Treg cell proportions in PBMCs	[193]
KINK-1, IKK β inhibitor	Mouse melanoma	↑ CD8 ⁺ T-cell infiltration and function in tumors	[162]
SMAC mimetics (CIAP antagonists), inhibit canonical NF- κ B, enhance alternative NF- κ B	Mouse Multiple myeloma	↑ macrophage phagocytosis; macrophage-dependent therapeutic effect	[194]
	Mouse glioblastoma, breast cancer	↑ T-cell in TILs; CD8 ⁺ T-cell-dependent therapeutic effect	[195]
IT-603, c-Rel inhibitor	Mouse thymoma, melanoma	↓ circulating T cells; ↑ IFN γ expression by Teff/Treg cells in TILs	[196,167]
PTXF, c-Rel inhibitor	Mouse breast cancer, melanoma	↑ CD8 ⁺ T cells, ↓ Treg cells in TILs; efficacy lost in RAG ^{-/-} mice	[168,167]
R96A, c-Rel inhibitor	Mouse melanoma	↓ inhibitory function of MDSCs	[48]

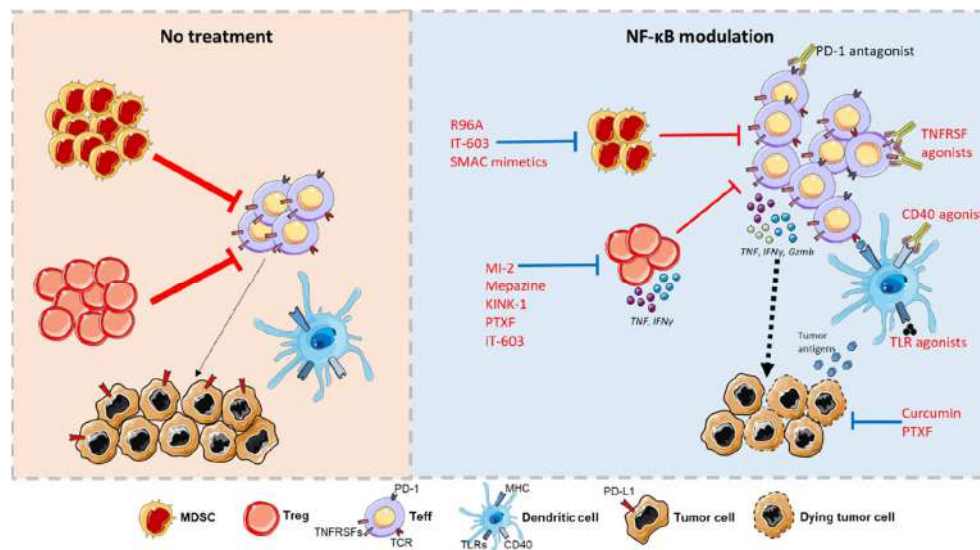


Figure 2. Proposed mechanism for the immunotherapeutic effects of NF- κ B modulating agents. In the absence of treatment, MDSCs and Treg cells actively inhibit CD4⁺ and CD8⁺ Teff cells; tumor-derived Ag presentation by DCs is low. NF- κ B inhibitors increase the numbers and activity of Teff cells by decreasing the recruitment and fitness of MDSCs and impairing Treg cell identity, relieving the suppression of Teff cells. Moreover, these treatments reduce PD-L1 expression and induce tumor cell death, leading to enhanced Ag-presentation by DCs. Conversely, stimulation of TNFRSFs and TLRs on DCs and Teff cells activates NF- κ B to increase their maturation numbers and function. All these agents potentiate the therapeutic effect of anti-PD-1 mAbs. Some figure elements were obtained from Servier Medical Art (<https://smart.servier.com/>).

(i) One possible mechanism relies on tumor cell-targeting effects of NF- κ B inhibitors, which would in turn enhance immune responses. Tumor cell death induced by Bortezomib was proposed to enhance DC maturation and stimulation of potent anti-tumor effector T cells and NK cells [197]. Moreover, the chemokine expression profile of tumor cells may be affected by NF- κ B inhibition, leading to enhanced tumor infiltration, as shown with the IKK inhibitor BMS-345541 [198] or curcumin [199]. NF- κ B is also well-known to favor the expression and stability of PD-L1 at the surface of tumor cells, with the administration of curcumin to breast-tumor bearing mice decreasing PD-L1 expression and subsequently enhancing CD8⁺ T-cell infiltration [14].

(ii) Aside from these tumor-targeting effects, NF- κ B inhibitors likely function by relieving the suppression exerted by MDSCs and Treg cells. The latter, in particular, are impaired in the tumor tissue following administration of mepazine, KINK-1 and pentoxifylline (PTXF) (reviewed in [200]). Similarly to what was highlighted in Section 4.2.2 using genetically-engineered mice, agents targeting the NF- κ B pathway are able to specifically decrease Treg cell numbers and impair their stability, leading to the expression of effector cytokines such as IFN γ or TNF, in the TME but not in lymphoid tissue. This suggests that these therapies should cause limited adverse effects in normal tissues. Thus, the effects of NF- κ B inhibitors on cancer immunity vary depending on the drug and cancer model. Generally, all of these reagents lead to an increase in the number and/or the function of cytotoxic CD8⁺ T cells in the tumor microenvironment. Importantly, these changes in immune cells do not seem to be solely a side effect of tumor cell targeting, but rather play an active role in cancer elimination. For instance, administration of mepazine or PTXF fails to reduce tumor burden in *RAG1*^{-/-} animals that are devoid of B and T cells [159,167].; In addition, the therapeutic effect is lost when CD8⁺ T cells are depleted by a monoclonal antibody, highlighting the importance of T cells in the therapeutic potential of NF- κ B inhibitors.

5.2.2. Combination of NF- κ B Inhibitors and Other Immunotherapies

The main challenge in cancer immunotherapy is now to identify drugs that are able to overcome innate or acquired resistance to anti-PD-1 or anti-CTLA-4 checkpoint-blockade therapies. Combination treatments associating NF- κ B inhibitors to other immunotherapies are emerging as a promising step forward in cancer care (Table 2). In the context of anti-PD-1-therapy-resistant tumors, the co-administration of either curcumin, bortezomib, SMAC mimetics, mepazine, PTXF or R96A enhances anti-tumor immunity and delays cancer growth. The combination of anti-PD-1 and bortezomib is currently investigated in clinical trials in multiple myeloma patients. NF- κ B inhibitors can also be associated with cytokines (IL-12, IL-18, IFN α), oncolytic viruses or other antibody-based therapies (anti-CTLA4, anti-CD38). The relative lack of efficacy of NF- κ B inhibitors observed in clinical trials of the past decades may therefore be overcome by their association with immunotherapies.

Table 2. Examples of combination of NF- κ B inhibitors with other immunotherapies.

Organism.	Combination Therapy	Cancer Type(s)	References
Mouse Studies	RELA shRNA + α PD-1	Hepatocellular carcinoma	[201]
	curcumin + α CTLA-4	Breast cancer	[14]
	curcumin + α PD-1/PDL-1	Diverse	[189,199,202]
	Bortezomib + IL12	Multiple myeloma	[203]
	Bortezomib + reovirus	Multiple myeloma	[204]
	Mepazine + α PD-1/PD-L1	Melanoma	[159,160]
	BAY11-7082 + rmIL-18	PDAC	[205]
	SMAC mimetics + oncolytic VSV	Breast cancer	[206]
	SMAC mimetics + α PD-1	Diverse	[81,207]
	PTXF/IT-603/R96A + α PD-1/PD-L1	Diverse	[48,167]
Human Studies	PTXF + α PD-1 + chemotherapy	Breast cancer	[168]
	Bortezomib + α CD38 + dexamethasone	Multiple myeloma	[208]
	Bortezomib + α PD-1	Multiple myeloma	[209]
	Bortezomib + α SLAMF7	Multiple myeloma	[210]
	Bortezomib + Interferon α 2B	Melanoma	[211]
	SMAC mimetics + α PD-1	Diverse	[212]

5.3. Limitations of NF- κ B-Targeting Therapies and Future Directions

Despite great expectations following the early development of NF- κ B inhibitors for the treatment of inflammatory diseases and cancer, very few of these molecules have reached advanced phases of clinical trials. This is due to either modest efficacy or severe adverse effects. In light of recent progress in understanding NF- κ B mediated regulation of immune responses, one could now envision a revival of this class of drugs. However, several aspects of the immense power of NF- κ B must be taken into account before we can safely move towards large-scale clinical applications:

The type of cancer: It is very likely that the putative efficacy of NF- κ B inhibitors will depend on the type of malignant tissue and its immune environment. It was proposed that NF- κ B inhibition would be especially relevant for the treatment of inflammation-driven cancers and NF- κ B-“addicted” tumors [213]. Hypothetically, NF- κ B activation may in some settings have deleterious roles in tumor-cell autonomous biology, e.g., apoptosis or chemokine/cytokine expression, though there is to our knowledge, no direct evidence of this. Moreover, the composition and quality of the immune response must be carefully evaluated when it comes to NF- κ B inhibition. In the case of cold tumors, or conversely in cancers undergoing a partial anti-tumor response, it is possible that NF- κ B inhibition may have no effect or even reduce the activity of effector cells.

The cell-specific targeting: In pre-clinical models of cancer, it is likely that NF- κ B inhibitors are partially efficient because they primarily target suppressive cells that are largely enriched in the tumor environment. However, it is important to note that NF- κ B is also a master regulator of potent anti-tumor effector cells as described above. Therefore, the duration, dosage and specificity of these treatments must be tightly controlled to avoid deleterious effects. For instance, Heuser et al. showed that administration of high doses of KINK-1 impaired CD8 T-cell function, whereas lower doses displayed therapeutic effects in melanoma [162]. Recent advances in drug engineering may also help in the development of more specific, thus more potent and safer, drugs. Antibody-drug conjugates, allowing the delivery of the payload to defined cell types, are currently under development [214]. This system could be used to deliver NF- κ B inhibitors to either tumors cells or immunosuppressive cells. Consistently, curcumin conjugated to anticancer Abs, displays promising tumoricidal activity in pre-clinical models of melanoma and glioblastoma [215,216].

The precise delineation of the roles of NF- κ B subunits: It is increasingly evident that NF- κ B subunits exhibit distinct functions in immune cell subsets and cancer cells. These selective contributions may underlie the lack of efficacy of certain NF- κ B-targeting agents and explain their toxicity. The use of conditional knock-out and knock-in animal models, as well as large-scale CRISPR screens and modern sequencing techniques, should provide, both at the whole-organism and single cell levels, the gene expression patterns controlled by each member of the NF- κ B family. In addition, the modeling of interactions between tumor cells and their microenvironment using organoid systems, will allow the development of screening approaches and the identification of selective modulators of the NF- κ B pathway. This will undoubtedly help the discovery of unique therapeutic targets for the treatment of cancer.

6. Conclusions

Immune responses to cancer are complex and vary depending on the type of malignancy. However, the growing knowledge on the dozens of innate and adaptive immune cell subsets has enabled the identification of novel therapeutic targets. In this review we have highlighted the multifaceted roles of 5 members of the NF- κ B family of transcription factors, in orchestrating tumor immunity. Targeting NF- κ B in cancer may not be a revolutionary concept as it finds its roots in the early 90s, but there is now a strong rationale for the revival of NF- κ B-targeting therapies. Indeed, in addition to their direct effect on tumor cell survival, proliferation or metastatic potential, modulators of the NF- κ B pathways are also likely to act as immune-stimulatory agents. New efforts must now be directed at identifying agents able to selectively activate or repress specific NF- κ B subunits, and to ensure their efficacy, delivery mode and safety.

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A T-cell-intrinsic function for NF- κ B RelB in experimental autoimmune encephalomyelitis

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Abstract

NF- κ B (NF- κ B) is a family of transcription factors with pleiotropic functions in immune responses. The alternative NF- κ B pathway that leads to the activation of RelB and NF- κ B2, was previously associated with the activation and function of T cells, though the exact contribution of these NF- κ B subunits remains unclear. Here, using mice carrying conditional ablation of RelB in T cells, we evaluated its role in the development of conventional CD4⁺ T (Tconv) cells and their function in autoimmune diseases. RelB was largely dispensable for Tconv cell homeostasis, activation and proliferation, and for their polarization toward different flavors of Thelper cells *in vitro*. Moreover, ablation of RelB had no impact on the capacity of Tconv cells to induce autoimmune colitis. Conversely, clinical severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS) was significantly reduced in mice with RelB-deficient T cells. This was associated with impaired expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) specifically in the central nervous system (CNS). Our data reveal a discrete role for RelB in the pathogenic function of Tconv cells during EAE, and highlight this NF- κ B subunit as a putative therapeutic target in MS.

Introduction

Classically split into two separate activation pathways, the NF- κ B family is composed of 5 subunits that all share a Rel homology domain and associate as homo- or heterodimers to modulate gene expression¹. In T cells, stimulation of the canonical pathway is classically triggered by the engagement of T-cell receptor (TCR)/CD28 molecules, which drives the activation of a Carma1-Bcl-10-Malt-1/inhibitor of κ B kinase (IKK) $\alpha/\beta/\gamma$ cascade. This, in turn, leads to the degradation of I κ B α and the nuclear translocation of NF- κ B1 (p105/p50, encoded by *Nfkb1*), RelA (or p65, encoded by *Rela*) and c-Rel (encoded by *Rel*) proteins². The alternative NF- κ B pathway is generally triggered by the engagement of different members of the tumor necrosis factor receptor (TNFRSF) superfamily, and consists in the activation of NIK, which phosphorylates IKK α , driving the processing of NF- κ B2 (p100/p52, encoded by *Nfkb2*) and culminating in the nuclear translocation of p52 and RelB (*Relb*) subunits. Importantly, it is

now clearly established that canonical and alternative subunits can dimerize with each other under certain conditions, conferring novel functions to NF- κ B and adding another layer of complexity to its signaling³⁻⁵. A number of studies have reported the multiple functions of NF- κ B in inflammation and immunity. In particular, it was suggested to exert critical roles in the development and homeostasis of CD4⁺ T lymphocytes^{6,7}.

CD4⁺ T cells are pivotal actors of adaptive immune responses. Following stimulation, naïve conventional CD4⁺ T (Tconv) cells can polarize into different flavors of T helper (TH) cells depending on the cytokine milieu to mount protective responses against specific classes of pathogens⁸. In addition to these protective roles, TH cells are involved in the pathogenesis of different immune disorders, such as allergy or autoimmunity. For instance, TH1 and TH17 cells, characterized by their secretion of interferon gamma (IFN γ) and interleukin (IL)-17 (IL-17A/F), respectively, have deleterious activities in tissue-specific autoimmune diseases, including inflammatory bowel disease and MS, as demonstrated in mouse models and suggested in human pathology^{9,10}. More specifically, so-called pathogenic TH17 cells (also reported as TH1.17 or TH17* in the literature) produce appreciable amounts of GM-CSF, driving clinical symptoms in murine models of MS such as EAE^{11,12}. At odds with these pro-inflammatory cells, another subset of CD4⁺ T cells, called regulatory T cells (Treg cells) and characterized by its high expression of the transcription factor Foxp3, is crucial for the suppression of autoimmunity¹³. Hence, depletion of Treg cells exacerbates EAE severity¹⁴.

Canonical NF- κ B subunits are largely implicated in T-cell activation, survival, as well as in the polarization toward the TH17 lineage and the development of autoimmunity¹⁵⁻¹⁷. Moreover, these proteins are critical for Treg cell development and function^{18,19}. In contrast, the T-cell-intrinsic functions of the alternative pathway remain unclear. Mice and patients with germline mutations in *Map3k14* (encoding NIK) or *Relb* exhibit spontaneous T-cell activation and profound immunodeficiency, though this may rely on T-cell-extrinsic mechanisms²⁰⁻²³. Tconv cells isolated from mice harboring either germline or T-cell-restricted ablation of *Map3k14*, displayed a normal TH1 and TH2 polarization capacity, but impaired TH17 differentiation *in vitro*^{24,25}. Consequently, *Map3k14* ablation conferred full resistance to EAE. In contrast, experiments using animals with a non-processible form of p100 or with constitutive activation of RelB, suggested that RelB could directly inhibit TH17 differentiation^{26,27}. In line with this, *Relb*^{-/-} T cells, though they may have an impaired TH1 differentiation ability *in vitro*²⁸, induced severe EAE upon transfer to immunodeficient recipients, associated with enhanced TH17 cytokines in the inflamed CNS²⁹. However, conflicting results were also reported²⁷, and the specific contribution of RelB to TH polarization and T-cell mediated autoimmunity is thus still debated. This is largely due to the fact that the study of cell-autonomous functions was so far prevented by the early and lethal autoimmunity arising in mice with germline deletion of *Relb*²³. To overcome this issue, we used mice carrying specific *Relb* ablation in T cells. We found that T-cell development *in vivo*, TH polarization *in vitro* and T-cell-transfer-induced colitis did not require RelB activity. Conversely, the severity of EAE was significantly reduced in mice with *Relb*-deficient T cells, associated with impaired accumulation of GM-CSF⁺ T cells in the inflamed CNS. Our results reveal a discrete contribution of RelB in T-cell biology and highlight this NF- κ B subunit as a putative therapeutic target for the treatment of MS.

Results

RelB is dispensable for T-cell homeostasis at steady-state.

To investigate the T-cell-autonomous functions of RelB, we crossed mice carrying *Relb*-floxed alleles to the CD4^{cre} strain, allowing the conditional ablation of *RelB* in T-cells (*Relb*-cKO)³⁰.

Relb-cKO offspring were viable and mutant mice displayed no signs of autoimmunity up to 12 months after birth (data not shown). Importantly, flow cytometry analyses in adult animals showed that T-cell distribution in the thymus remained unchanged compared to CD4^{cre} control mice (Figure 1A and data not shown). The proportion and quantity of CD4⁺ and CD8⁺ mature T cells in the spleen and peripheral lymph nodes were not affected by the absence of RelB (Figure 1A). Furthermore, *in vivo* activation and proliferation levels, measured through the expression of Ki67, CD44 and CD62L, were normal (Figure 1B-C). Since NF- κ B signaling has been associated with the expression of inflammatory cytokines by T cells, we measured IFN- γ , IL-2 and TNF α expression by Tconv cells following *ex vivo* polyclonal restimulation of lymphoid tissues. Once again, the proportion of cytokine-producing T cells did not differ between control and *Relb*-cKO mice (Figure 1D). Together, these data showed that *RelB* ablation did not impact steady-state homeostasis and function of Tconv cells.

In vitro properties and TH polarization are maintained in the absence of RelB.

Next, we evaluated the impact of *Relb* ablation on T-cell behavior *in vitro*. Naive Tconv cells isolated from the spleen and LN of control and *Relb*-cKO mice, displayed similar levels of proliferation following CD3/CD28 stimulation, in the presence or absence of IL-2 (Figure 2A). In the same setting, the proportion of IFN γ ⁺, IL-2⁺, TNF α ⁺ and GM-CSF⁺ cells was unaltered, as well as the amount of secreted cytokines (Figure 2B-C). Following stimulation in TH1-, TH2- or iTreg-polarizing conditions, we detected normal expression levels of IFN γ /T-bet, GATA-3, and Foxp3, respectively, suggesting that RelB was expendable for these TH subsets (Figure 3A-C). Over the last few years, several reports have pondered over the implication of the alternative NF- κ B pathway in the biology of TH17 cells. We thus carefully examined the TH17 polarization potential of *Relb*-deficient Tconv cells. Surprisingly, the ability of Tconv cells to convert into IL-17A⁺ and ROR γ t⁺ TH17 cells when cultured with anti-CD3, feeders, TGF- β and IL-6 was not impacted by the absence of *Relb* (Figure 3D). Similar results were obtained following the addition of IL-1 β and IL-23, and using plate-coated anti-CD3 and -CD28. Thus, though it was described that RelB activation may inhibit IL-17A production by TH17 cells, its ablation did not seem to modify the TH17 polarization capacity of naive Tconv cells.

RelB expression is not required for T-cell mediated colitis.

To test the TH polarization capacity and pathogenic function of Relb-deficient T cells *in vivo*, we next used a T-cell-transfer model of colitis, in which naïve Tconv cells are transferred to immunodeficient RAG2^{-/-} recipients. Transfer of control Tconv induced pronounced colon inflammation, leading to detectable weight loss (Figure 4A). We observed a similar phenotype when *Relb*-deficient Tconv were used, which was also confirmed by histological analyses (Figure 4B-C). FACS analyses 6 weeks after cell transfer revealed equivalent lymphoid tissue reconstitution and similar colon infiltration by Tconv, suggesting that RelB was dispensable for lymphopenia-induced proliferation and survival in immunocompromised hosts (Figure 4D). Moreover, the proportion of Foxp3⁺ iTreg cells was unaltered between the two strains, thereby recapitulating our *in vitro* data (Figure 4E). The expression of “TH1” markers such as T-Bet, IFN γ or TNF α was also stable (Figure 4F). Interestingly, whereas the overall proportion of ROR γ t⁺ T cells was not impacted by the absence of RelB, we detected a significant increase in the percentage of IL-17A⁺ cells specifically in the mesenteric lymph nodes (Figure 4G). Thus, in this setting, RelB may act as an inhibitor of IL-17A production, as previously reported in other systems. However, the clinical outcome did not differ, presumably because the expression of IL-17A and GM-CSF in the colon was independent of RelB (Figure 4G). Collectively, this showed that RelB expression was not involved in the pathogenic function of TH cells upon transfer to lymphopenic hosts.

RelB promotes T-cell pathogenicity in EAE

To directly assess the role of RelB in a murine model of T-cell mediated autoimmunity, we immunized control and mutant mice with MOG₃₃₋₅₅ peptide to induce EAE. As expected, immunization of control mice led to detectable and severe clinical symptoms in most animals, characterized by a peak in the disease 2 weeks after induction, and followed by a spontaneous partial remission (Figure 5A-C). Interestingly, even though the onset of disease was not delayed in *Relb*-cKO animals, maximal scores were significantly lower than that in the control group, suggesting that RelB may limit the pathogenic function of T cells without affecting their initial priming. To understand this mechanism, we assessed the proportion and phenotype of T cells in the inflamed CNS and draining lymph nodes 20 days after immunization. Although we observed similar numbers of total and CD4⁺ T cells in draining lymph nodes (dLN), T-cell accumulation was drastically decreased in the brain and spinal cord of *Relb*-cKO animals (Figure 5D-E). The proportion of Foxp3⁺ Treg cells remained intact, suggesting a Tconv cell-intrinsic functional impairment (Figure 5F). The proportion of activated (CD44^{hi}) and proliferating (Ki67⁺) cells was equivalent between strains (Figure 5F), indicative of an intact T-cell priming but an impaired migration toward the CNS, and/or a defective survival *in situ*. We thus assessed the TH profile of each strain. The expression of TNF α as well as TH1 (T-bet and IFN γ) and classical TH17 (ROR γ t and IL-17A) markers was unaffected in *Relb*-cKO animals (Figure 5G, H). We detected a specific and significant decrease in the proportion and numbers of GM-CSF⁺ Tconv cells in the brain of *Relb*-cKO mice (Figure 5I). A similar trend was observed in the inflamed spinal cord, though the values did not reach statistical significance (Figure 5I). Overall, the absolute numbers of GM-CSF⁺ Tconv cells, which directly impact the severity of the disease, were dramatically decreased in the brain and spinal cord of mutant mice. Altogether, these data highlight a pathogenic T-cell-intrinsic function of RelB in CNS autoimmunity, through its role in GM-CSF expression.

Discussion

The major roles of the canonical NF- κ B pathway in TH17 cell polarization and pathogenic function in autoimmunity are well established. However, despite growing literature, the roles of the alternative pathway and subunits remain poorly defined. This lack of knowledge is partly due to the absence of adequate mouse models to study the cell-autonomous functions of RelB. In the present study, although *Relb* expression was largely dispensable for Tconv biology at steady-state and was not required for TH polarization *in vitro*, the absence of *Relb* significantly improved clinical symptoms of EAE by limiting the pathogenic function of T cells in the inflamed CNS. These findings further our knowledge on the role of the alternative NF- κ B pathway in immune responses.

Our *Relb*-cKO model was viable, unlike mice carrying a germline deletion of *Relb*, and no autoimmune symptoms or inflammation could be detected in our animals, thus confirming previous literature establishing that the lethal autoimmunity and spontaneous T-cell activation observed in *Relb*^{-/-} mice is independent of the absence of RelB in T cells^{31,32}. As such, the phenotype and activation levels of *Relb*-deficient T cells was largely comparable to that of control cells. We next used our *Relb*-cKO strain to fully decipher the cell-autonomous function of RelB in T-cell features *in vitro*, including cell proliferation, cytokine expression and polarization toward different Thelper lineages. To date, conflicting data have illustrated the putative contribution of alternative NF- κ B subunits to TH polarization. For instance, total T cells from *Relb*^{-/-} animals, but not *Nfkb2*^{-/-} or *Map3k14*^{-/-} mice, displayed defects in TH1 polarization^{25,27,28}. Because we detected intact IFN γ and T-bet expression in TH1 cells from *Relb*-cKO mice, this early observation was likely due to a cell-extrinsic phenomenon. The role of this pathway in *in vitro*-differentiated TH17 cells is even more debated. Ablation of NIK or p100 reduced IL-17A expression in TH17 cells^{25,27}. At odds with these reports, it was shown that forced activation and overexpression of RelB in T cells significantly impaired TH17

polarization. Mechanistically, following OX-40 engagement, RelB could directly bind to the *Il17* locus and recruit histone modifiers to inhibit *Il17a* expression²⁹. Conversely, we showed here that T-cell-restricted ablation of *Relb* did not modify the ability of Tconv to polarize into ROR γ t⁺ IL17-A⁺ cells *in vitro*. This confirmed the conclusions of a previous report using *Lck^{cre}Relb^{flox}* animals³². Thus, whereas strong activation of RelB may be deleterious for IL-17A expression, its absence did not impact polarization toward TH17 cells *in vitro*.

In addition, RelB was dispensable for lymphopenia-induced proliferation and colon inflammation following transfer of Tconv to immunodeficient recipients. This represents an important insight into the pathophysiology of IBD. Indeed, it was shown that NIK activity was integral, both in intestinal epithelial cells and dendritic cells, for the development of IBD in mouse models^{33,34}. In addition, *Chuk^{-/-}* T cells (deficient for IKK α), that have reduced activation of the canonical and alternative pathways, fail to induce IBD when transferred to *Rag1^{-/-}* recipients³⁵. Our experiments demonstrate that RelB activation *per se* was not involved in the pathogenic function of T cells in IBD, suggesting a cell type-specific contribution of the alternative NF- κ B pathway in intestinal autoimmunity.

Interestingly, T-cell-intrinsic *RelB* expression was critical to induce severe symptoms of EAE. This observation is in stark contrast with previous reports showing either no effect²⁷ or a deleterious impact²⁹ of *Relb* ablation on the function of T cells during EAE. However, these two studies used cell transfer experiments to address the question. Our results are to our knowledge the first to directly investigate the function of RelB in T cells using an active EAE model in unmanipulated animals. Thus, similarly to what was observed in oligodendrocytes or dendritic cells, RelB appears to exert a pathogenic effect in T cells during CNS autoimmunity^{36,37}.

From our results, RelB seems to be implicated in discrete steps of the pathogenic function of T cells during EAE, rather than being involved in the differentiation of the whole TH17 lineage *in vivo*. First, we observed a major decrease in the number of T cells, both in the brain and spinal cord of *Relb*-cKO animals, 20 days after EAE induction. Because T-cell numbers and phenotype were intact in the dLN, this suggested an impaired *in situ* accumulation of T cells in the inflamed tissues. As the proportion of proliferating cells was untouched, this phenomenon was likely due to a defective survival of *Relb*-deficient T cells. This was in accordance with observations made in NIK-deficient animals²⁵. In addition to this reduced accumulation, we detected a specific impairment of GM-CSF⁺ Tconv cells in the brain of mutant mice, whereas the proportion of ROR γ t⁺ and IL-17A⁺ cells was not significantly altered. The precise molecular mechanism underlying this defective GM-CSF expression remains unclear. In contrast to c-Rel and p52, it was proposed that RelB could not directly bind and activate the putative κ B-binding site in the *Csf2* promoter in mouse T cells²⁷. However, divergent conclusions were reached using human cell lines³⁸. It would be of interest to measure the binding of RelB to the *Csf2* locus in CNS-infiltrating Tconv cells during EAE, for instance using ChIP-Seq methods. Nevertheless, the low number of T cells that can be isolated from such non-lymphoid tissues poses a major technical challenge.

It would also be important to elucidate how RelB modulates GM-CSF expression in T cells. RelB was suggested to exert its pleiotropic functions in neuroinflammation through different molecular mechanisms³⁹: (i) activation of the alternative NF- κ B pathway leading to translocation of p52-RelB dimers, (ii) 'canonical-like' association of Relb-p50 and RelB-RelA dimers, and (iii) epigenetic modifications driven by RelB monomers or homodimers. Again, ChIP experiments as well as the use of mouse models with conditional ablation of other NF- κ B subunits would be relevant tools to address this specific question.

Collectively, our data offer new insights in the regulation of T-cell immunity by the different members of the NF- κ B family. While c-Rel seems critical to kickstart the expression of ROR γ t and the TH17 lineage¹⁶, we herein propose that RelB, exerts selective functions in the pathogenic programming of TH cells during ongoing CNS inflammation, rather than in the priming phase of the disease. Thus, we highlight this understudied NF- κ B subunit as a putative therapeutic target in EAE and MS.

Methods

Mice

Relb-Floxed mice were previously described⁴⁰. CD4^{cre} (Tg(CD4-cre)^{1Cw1}) on a C57Bl/6J background were purchased from the Jackson Laboratory. *Rag2*^{-/-} and C57Bl/6 CD45.1 (*Ptprc^a Pepc^b/BoyJ*) mice were purchased from Charles River France. Mice were bred and used in specific-pathogen-free (SPF) conditions at the CRCL animal facility (Anican). Animals were housed in individually ventilated cages with temperature-controlled conditions under a 12-h-light/dark cycle with free access to drinking water and food. Adult (6-28-week-old) male or female mice were used for all experiments. Studies were conducted in accordance with the animal care guidelines of the European Union and French laws. Protocols were validated by the local Animal Ethic Evaluation Committee (committee C2A15) (protocol CLB-2019061914498113).

Adoptive cell transfer-induced colitis

Donor mice were euthanized by cervical dislocation and spleens and peripheral lymph nodes were harvest in ice cold NaCl. Sterile single cell suspensions were obtained by mechanical dilaceration through a 70 μ m cell strainer. Red blood cells were lysed in RBC lysis buffer (NH₄Cl 0.154M+ NaHCO₃ 0.14M + EDTA 0.1mM) for 10 minutes at room temperature. Naïve CD4⁺ T cells were negatively isolated using the CD4⁺ T Cell Isolation Kit (Miltenyi), with a purity >90%. Cells were resuspended at 20x10⁶ cells/mL in ice cold PBS 1X and injected intravenously in the retro orbital sinus of recipient *Rag2*^{-/-} mice under isoflurane-induced anesthesia.

EAE

Mice were injected subcutaneously in each flank with 50 μ g of MOG₃₅₋₅₅ peptide (Smart Biosciences SB023) emulsified in 100 μ L of complete Freund adjuvant (Sigma) supplemented with 243 μ g of heat-killed Mycobacterium tuberculosis H37Ra (Difco-BD Bioscience 2311). Bordetella pertussis toxin (200 ng/injection, Enzo) was injected intravenously at the time of immunization and two days later. Disease severity was evaluated every other day following an established scoring system: 0, no clinical sign; 1, limp tail; 2, limp tail, impaired righting reflex, and paresis of one limb; 3, hindlimb paralysis; 4, complete hindlimb and partial forelimb paralysis; to 5, moribund/death. A score of 5 was permanently attributed to dead animals. After 20 days, mice were anesthetized with a mixture of ketamine and xylazine and perfused with ice-cold PBS1X. Draining (brachial) lymph nodes, brain and spinal cord were harvested in ice cold NaCl solutions.

Preparation of Cell Suspensions

Single cell suspensions from LN and spleens were obtained by mechanical dilaceration in FACS Buffer (PBS 1X + 2% FBS, 2mM EDTA) with glass slides, strained, washed in complete RPMI and enumerated.

Brains and spinal cords were cut into small pieces and digested in RPMI 1640 (Gibco) supplemented with 1 mg/mL collagenase type IV (Sigma), 500 µg/mL DNase I (Sigma) for 30 minutes at 37 °C followed by mechanical desegregation. Reactions were stopped by the addition of 15 mL PBS1X containing 5 mM EDTA. The solution was passed through a 70 µm cell strainer and residual solid pieces were mechanically disrupted. After centrifugation, cell pellets were resuspended in 8 mL of Percoll 40% (Sigma) and then laid on 4 mL Percoll 80% in a 15 mL polypropylene tube. Tubes were spun at 2500 rpm for 20 minutes at RT. Mononuclear cells were collected from the interface of the 40:80% Percoll gradient, washed in complete RPMI and enumerated.

Colons were extensively washed and fragments were incubated in 5 mL of cell dissociation solution (Ca²⁺- and Mg²⁺-free containing 5 mM EDTA and 10 mM HEPES) and incubated for 20 minutes at 37°C under slow rotation. Supernatant was discarded and fragments were minced and digested in 5 mL PBS containing 5% FBS, 1 mg/mL of Collagenase and 400 µg/mL DNase I for 20 minutes at 37°C under slow rotation. Digestion was stopped by adding 15 mL PBS1X supplemented with EDTA 5 mM. Lamina propria leukocytes (LPL) cells were passed through a 70 µm cell strainer. Mononuclear cells were isolated by a Percoll gradient as above, washed in complete RPMI and enumerated.

In vitro T-cell proliferation and polarization assays

Naïve CD4⁺ T cells were negatively isolated using the CD4⁺ T Cell Isolation Kit (Miltenyi). Cells were labeled using the CellTrace Violet Cell Proliferation Kit (CTV; Invitrogen). Cells were cultured in 120 µL of complete IMDM (10% FBS, 25,000 U Penicillin/Streptomycin, 10 mM sodium pyruvate, non-essential amino acids and 50 µM 2-Mercaptoethanol) (Gibco).

To study proliferation and inflammatory cytokine production, 3x10⁴ cells were stimulated with 1.5x10⁴ T cell-depleted mitomycin C-treated Ly5.1 splenocytes, 0.5 µg/mL soluble anti-CD3ε (clone 145-2C11, BioXcell) with or without 10 ng/mL mIL-2 (Miltenyi). For TH polarization assays, cells were either stimulated as above, or 5x10⁴ cells were stimulated with coated anti-CD3ε 1 µg/mL (BioXcell) and anti-CD28 1 µg/mL (clone 37.51, BioXcell). The culture medium was supplemented as follows: TH0: 10 ng/mL mIL-2; TH1: 20 ng/mL mIL-12 (Peprtech), 10 ng/mL mIL-2 (Miltenyi), 2 µg/mL anti-mouse IL-4 (clone 11B11 BD Biosciences); TH2: 30 ng/mL mIL-4 (Peprtech), 2 µg/mL anti-mouse IFNγ (clone XMG1.2 BD Biosciences), 2 µg/mL anti-mouse IL-12 (clone C17.8 BD Biosciences); iTreg: 5 ng/mL hTGF-β (R&D Systems); 10 ng/mL mIL-2; TH17: 1 or 5 ng/mL hTGF-β, 20 ng/mL mIL-6 (Peprtech), 2 µg/mL anti-mouse IFNγ, 2 µg/mL anti-mouse IL-4, with or without 5 ng/mL mIL-1β (Peprtech); 5 ng/mL hIL-23 (Peprtech) and 2 µg/mL anti-mouse IL-2 (clone JES6-1A12, Biolegend).

After 4 days of culture, supernatants were collected and stored at -80°C until further use. Proliferation and subset-specific transcription factors and cytokine expression were assessed by FACS.

Flow cytometry

For cytokine analysis by FACS, single cell suspensions were stimulated for 4 hours at 37°C with 50 ng/mL PMA (Sigma), 1 µg/mL ionomycin (Sigma) and 1X Protein Transport Inhibitor containing Brefeldin A (BD GolgiPlug). Cells were washed in PBS1X and stained with a viability marker for 10 minutes at RT in the dark. Cells were washed in PBS1X, and incubated with 50 µL surface marker antibody mix for 20 minutes at 4°C in the dark. Cells were then washed in PBS1X and fixed and permeabilized using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to manufacturer's instructions. Cells were washed and incubated with the intracellular marker antibody mix for 20 minutes at 4°C in the dark, washed in permeabilization buffer and resuspended in FACS Buffer. The complete list

of antibodies is attached. Acquisition was performed on a LSR Fortessa (BD Biosciences) or an Aurora spectral cytometer (Cytex). Data were analyzed with FlowJO software (Tree Star)

Histology

Colons were fixed in 4% formaldehyde solution (Sigma) for 1 day and stored at 4°C in EtOH 70%. They were then embedded in paraffin, sectioned and stained with hematoxylin/eosin (Sigma Aldrich). Intestinal inflammation was scored in a blinded fashion using a scoring system based on various criteria: colon length, inflammatory cell infiltrate (severity and extent), crypt hyperplasia, and presence of neutrophils within the crypts, presence of crypt abscesses, erosion, granulation tissues and villous blunting.

ELISA

ELISA were performed using “ELISA MAX™ Deluxe Set Mouse IL-2”, and “ELISA MAX™ Deluxe Set Mouse GM-CSF” (Biolegend) following manufacturer’s instructions.

Statistics

Statistics were performed using GraphPad Prism Software. Unless mentioned otherwise, two-tailed Mann-Whitney t test was used to calculate statistical significance. P values <0.05 were considered significant. ns: non-significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

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Author contributions

SG and YGB conceptualized the project. GL, RL and YGB designed experiments. GL, RL, AV, JT, PS and MPN performed experiments. GL, RL, AV, RI, SS and YGB analyzed the data. JM, MV and UK provided reagents. YGB wrote the paper and secured funding.

Competing interests

The authors declare no competing interests.

Data availability

Flow cytometry and other raw data will be made available upon request.

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Figure legends

Figure 1. Conditional ablation of *Relb* in T cells does not impair their homeostasis at steady-state. Thymus, spleen and peripheral lymph nodes (LN) from CD4^{cre} (control) and CD4^{cre}Relb^{F/F} mice were analyzed by flow cytometry. **(A)** Quantification of live TCR- β ⁺CD4⁺ and CD8⁺ cells. **(B)** Proportion of Ki67⁺ in CD4⁺Foxp3⁻ (Tconv) cells. **(C)** Representative dot plots in the spleen and cumulative data of CD44 and CD62L expression in Tconv cells. T_{CM}: T central memory; T_{EM}: T effector memory. **(D)** Cytokine expression by Tconv cells following restimulation with PMA/ionomycin. Each dot represents an individual mouse from 5 experiments. Mean +/- SEM is shown. *ns*: non-significant.

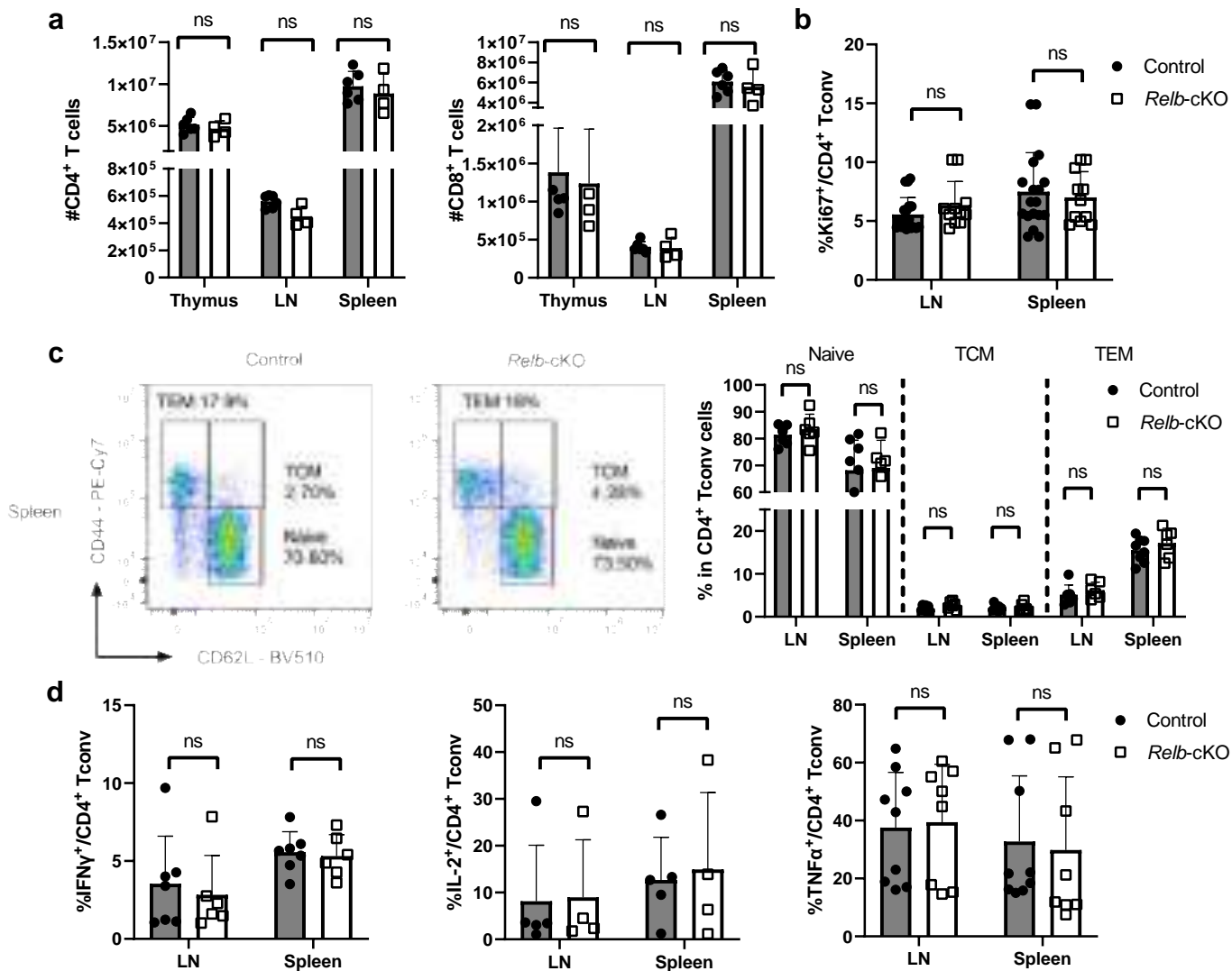
Figure 2. Normal proliferative capacity and cytokine expression of *Relb*-deficient T cells *in vitro*. Cell Tracer Violet-labeled naïve Tconv cells were stimulated with soluble anti-CD3+mitomycin C-treated feeders. **(A, B)** After 4 days of culture, cells were restimulated with PMA/ionomycin and analyzed by flow cytometry. **(A)** Representative CTV profile and cumulative proliferation index of live cells. **(B)** Cytokine expression following restimulation with PMA/ionomycin. **(C)** ELISA analysis of supernatants after 24 hours of culture. Each dot represents an individual mouse from 5 experiments. Mean +/- SEM is shown. *ns*: non-significant.

Figure 3. Thelper polarization profile is maintained in the absence of RelB. CTV-labeled naïve Tconv cells were stimulated in TH1, TH2, iTreg-polarizing conditions **(A-C)** or with different TH17 stimulation protocols **(D)** as described in the *Methods* section. After 4 days of culture, cells were restimulated with PMA/ionomycin and analyzed by flow cytometry. **(A)** Percentage of T-bet⁺ and IFN γ ⁺ cells in the TH1 condition, **(B)** GATA3⁺ cells in the TH2 condition, and **(C)** Foxp3⁺ cells in the iTreg condition. **(D)** Percentage of ROR γ t⁺ and IL-17a⁺ cells in various TH17 conditions. *ns*: non-significant.

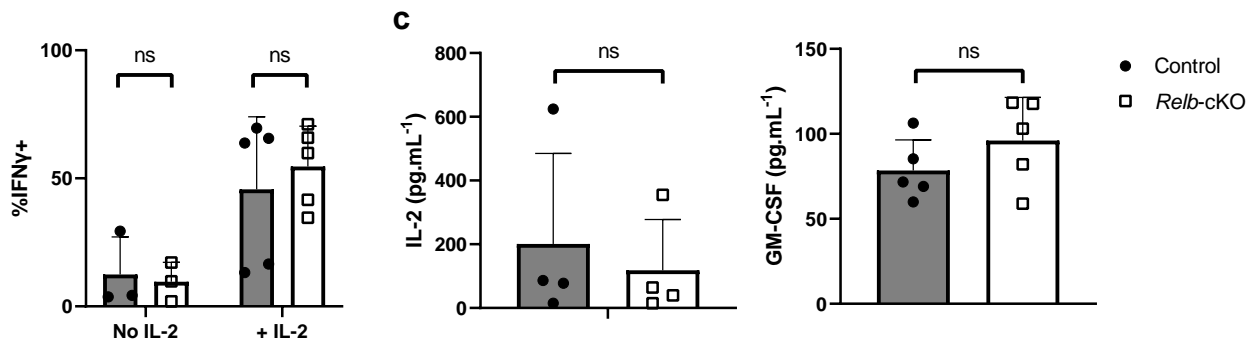
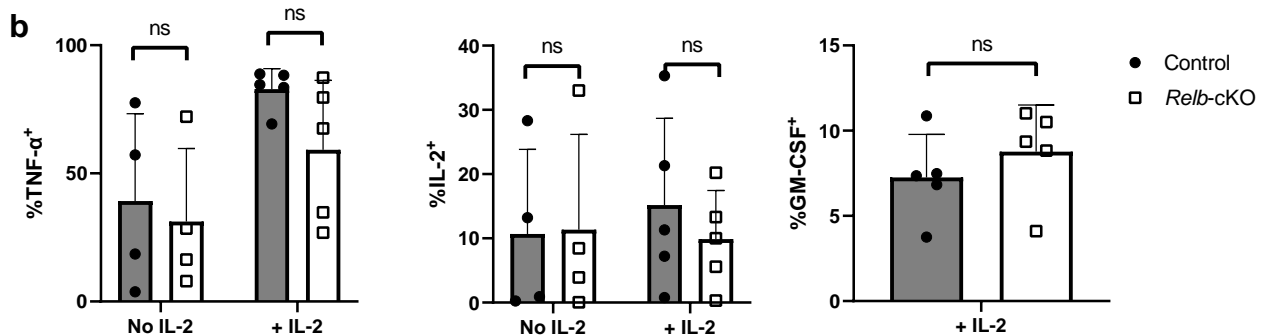
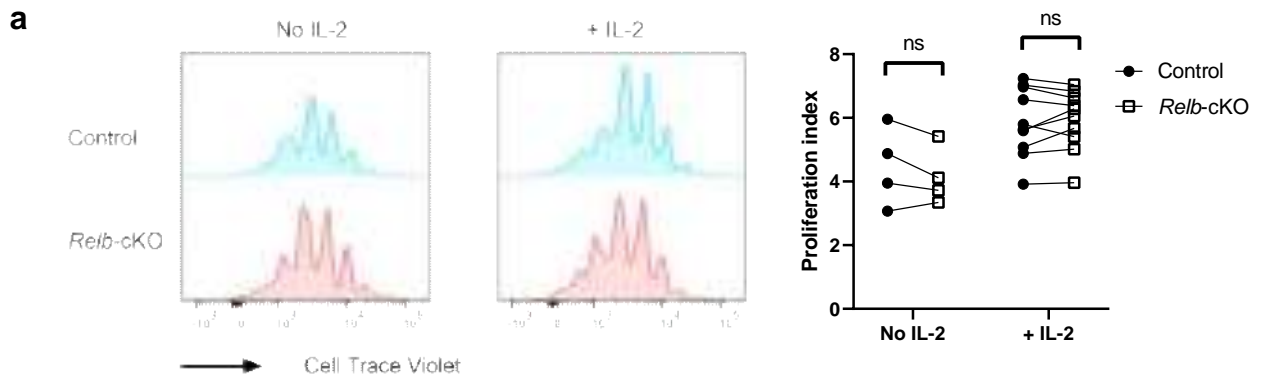
Figure 4. The colitogenic potential of Tconv cells is maintained in the absence of RelB. Naïve Tconv cells were transferred to sex-matched RAG2^{-/-} recipients. **(A)** Weight curves presented according to the % of initial body weight. **(B-C)** Histological analysis of colon sections, 6 weeks after transfer. **(B)** Representative section of colon stained with H/E. Bars: original magnification 10X (left), 50X(right). **(C)** Double-blinded histological score. **(D-G)** 6 weeks after transfer, spleens, mesenteric lymph nodes (mLN) and colon lamina propria leukocytes (LPL) were processed for FACS analyses following restimulation with PMA/ionomycin. Each dot represents a mouse from 3 independent experiments; mean +/- SEM is shown. **p* < 0.05, *ns*: non-significant.

Figure 5. T-cell autonomous RelB is required for optimal pathogenicity in EAE. Active EAE was induced as described in the *Methods*. **(A)** Cumulative incidence of detectable disease (score > 0), **(B)** mean clinical scores and **(C)** maximal scores of each mouse during the course

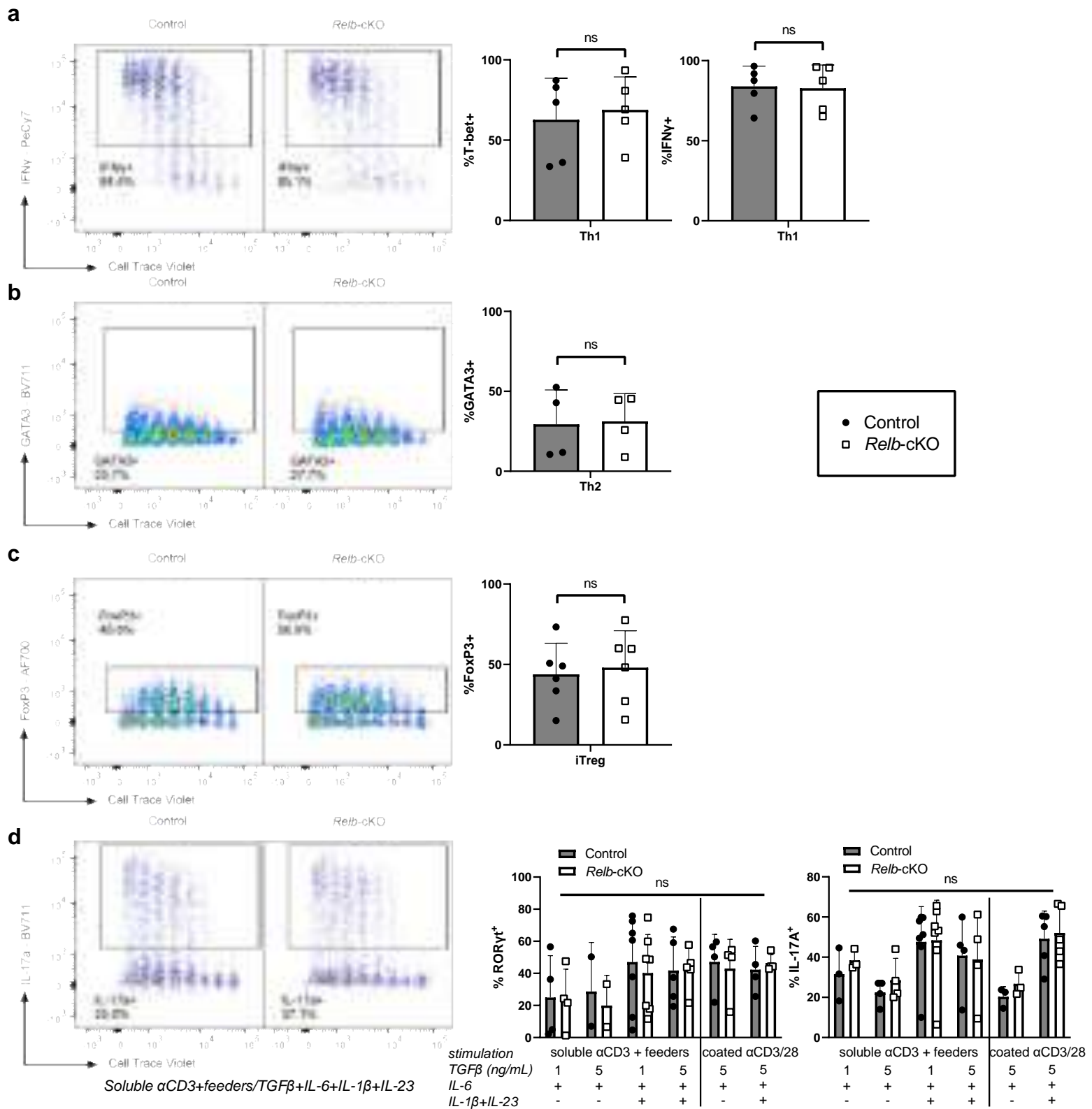
of disease were recorded. **(D-H)** 21 days after transfer, tissues were processed for FACS analyses following restimulation with PMA/ionomycin. **(D)** Representative TCR β and CD45 staining in gated live brain cells. **(E)** Absolute numbers of CD4⁺ T cells (live CD45⁺TCR β ⁺CD4⁺CD8⁻) in the indicated tissues. **(F-H)** Percentage of Tconv cells (live CD45⁺TCR β ⁺CD4⁺Foxp3⁻) expressing the indicated markers. **(I)** Representative GM-CSF staining in brain Tconv cells, and cumulative proportion and number of live CD45⁺TCR β ⁺CD4⁺Foxp3⁻GM-CSF⁺ Tconv cells. Each dot represents a mouse from 3 independent experiments; mean +/- SEM is shown. *p < 0.05, **p < 0.005, ns: non-significant.



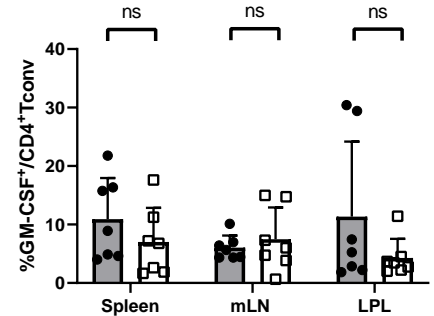
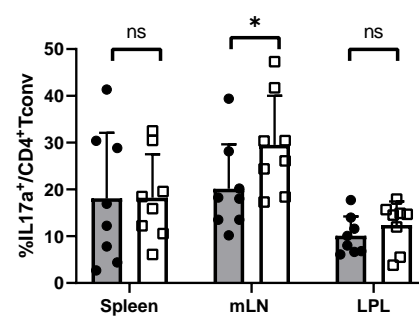
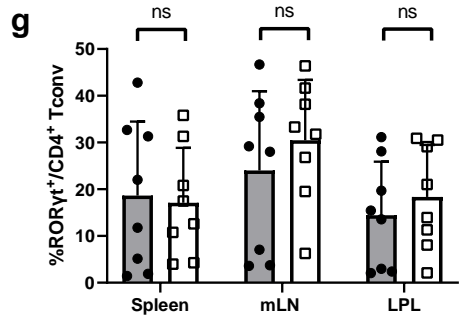
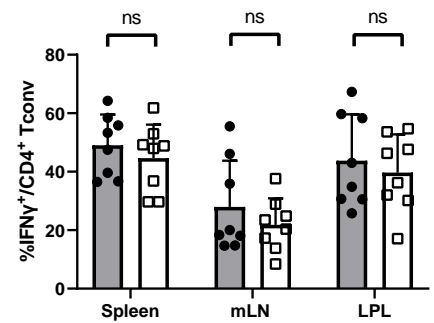
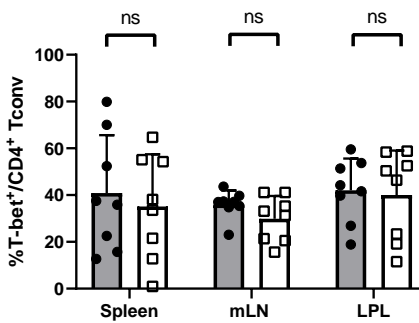
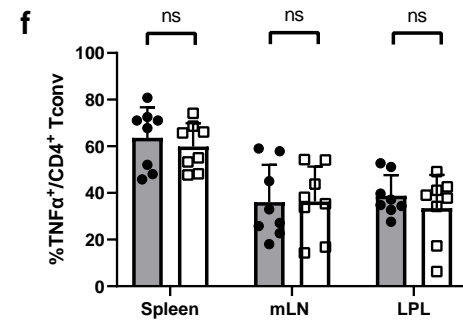
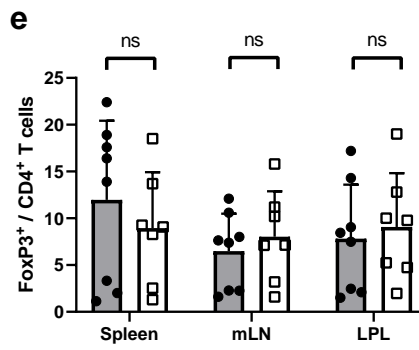
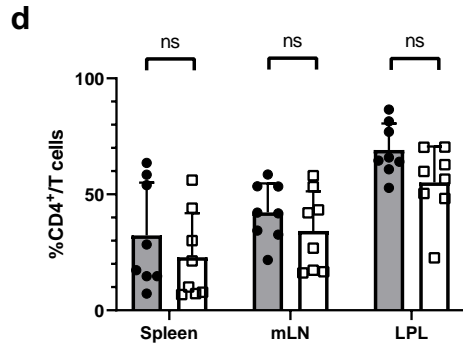
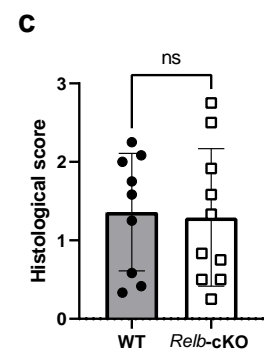
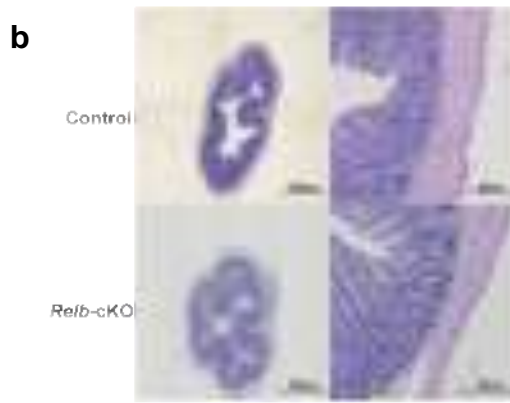
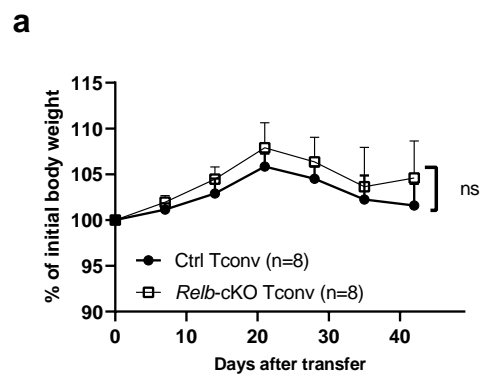
Lalle et al., Figure 1



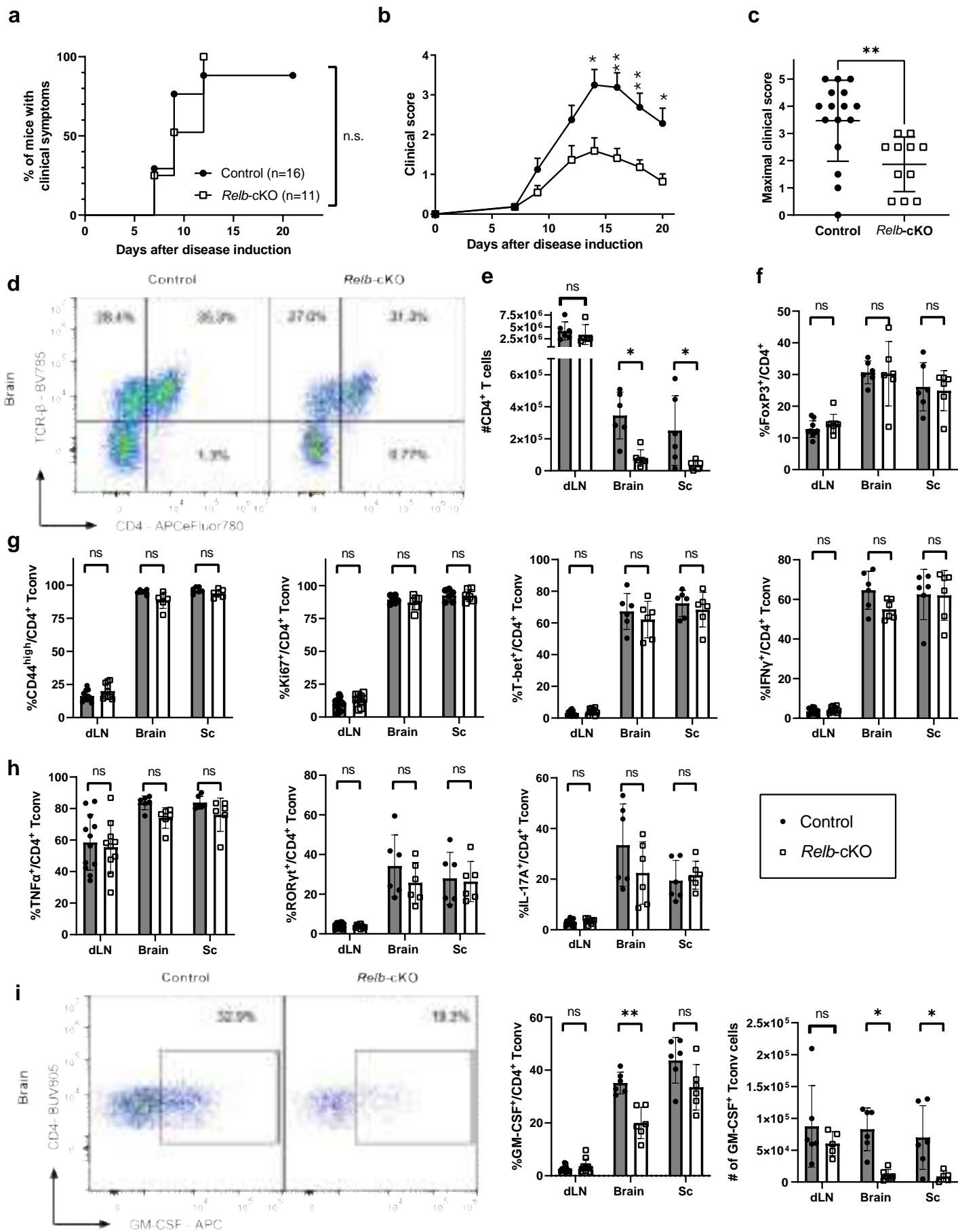
Lalle et al, Figure 2



Lalle et al, Figure 3



Lalle et al, Figure 4



Lalle et al, Figure 5