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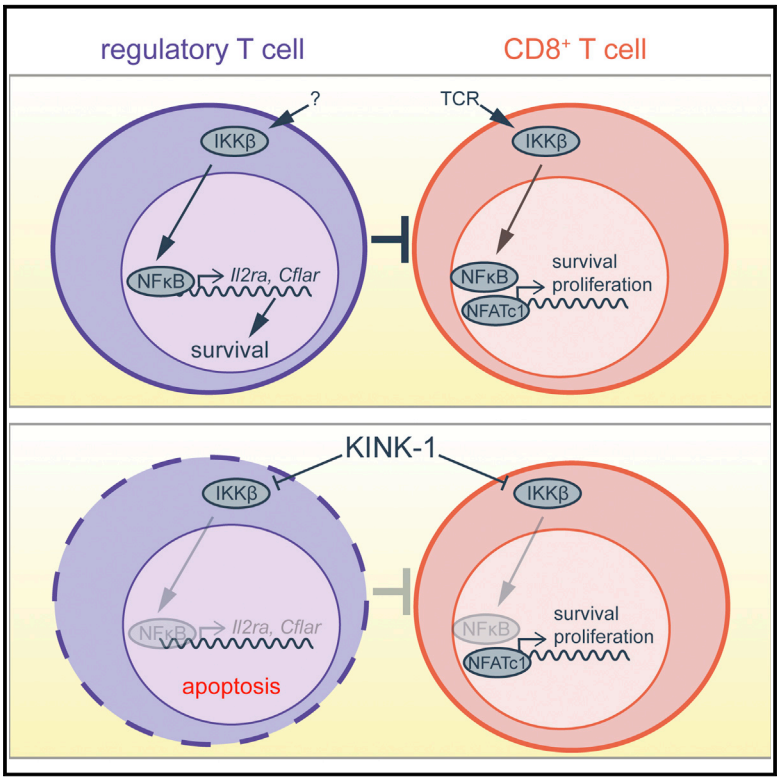
Prolonged IKKβ Inhibition Improves Ongoing CTL Antitumor Responses by Incapacitating Regulatory T Cells

Graphical Abstract



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In Brief

FoxP3+ regulatory T cells prevent autoimmunity but often incapacitate antitumor immunity. Heuser et al. show that IKKβ deficiency or inhibition preferentially decimated these cells but not cytotoxic T cells in vivo. IKKβ inhibition after tumor vaccination improved antitumor immunity, identifying IKKβ as a potential druggable checkpoint.

Highlights

- Deleting IKKβ from Tregs causes severe autoimmunity and auto-inflammation in mice
- Mature Tregs require IKKβ signaling for CD25 and c-Flip expression and survival
- CD8+ T cells use additional pathways (e.g., NFATc1) for survival and proliferation
- Prolonged IKKβ inhibition after tumor vaccination improves CTL antitumor responses

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Prolonged IKKβ Inhibition Improves Ongoing CTL Antitumor Responses by Incapacitating Regulatory T Cells

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SUMMARY

Regulatory T cells (Tregs) prevent autoimmunity but limit antitumor immunity. The canonical NF-κB signaling pathway both activates immunity and promotes thymic Treg development. Here, we report that mature Tregs continue to require NF-κB signaling through IκB-kinase β (IKKβ) after thymic egress. Mice lacking IKKβ in mature Tregs developed scurfy-like immunopathology due to death of peripheral FoxP3$^+$ Tregs. Also, pharmacological IKKβ inhibition reduced Treg numbers in the circulation by ~50% and downregulated FoxP3 and CD25 expression and STAT5 phosphorylation. In contrast, activated cytotoxic T lymphocytes (CTLs) were resistant to IKKβ inhibition because other pathways, in particular nuclear factor of activated T cells (NFATc1) signaling, sustained their survival and expansion. In a melanoma mouse model, IKKβ inhibition after CTL cross-priming improved the antitumor response and delayed tumor growth. In conclusion, prolonged IKKβ inhibition decimates circulating Tregs and improves CTL responses when commenced after tumor vaccination, indicating that IKKβ represents a druggable checkpoint.

INTRODUCTION

IκB-kinase β (IKKβ) is a central element of the canonical NF-κB signaling pathway that mediates the development and activation of immune cells (Gerondakis et al., 2014; Hayden and Ghosh, 2012; Vallabhapurapu and Karin, 2009). For instance, mice lacking IKKβ in T cells lose cytotoxic T lymphocyte (CTL) function and succumb to otherwise spontaneously rejected tumors (Barnes et al., 2015). Thus, the NF-κB pathway is generally considered to promote inflammation, and IKKβ inhibitors are currently being tested for the treatment of inflammatory diseases (Gasparini and Feldmann, 2012; Ziegelbauer et al., 2005). They are also considered for therapy of certain tumors whose growth depends on IKKβ. However, potential adverse effects on antitumor immunity raised concerns that they might worsen disease (Zhang et al., 2017).

Regulatory T cells (Tregs) are critical to maintain self-tolerance by inhibiting autoreactive T and B cells (Gavin et al., 2007; Gotot et al., 2012; Sakaguchi et al., 2008). NF-κB induces the forkhead/winged-helix box P3 (FoxP3) transcription factor during thymic development of Tregs (Gavin et al., 2007; Gerondakis et al., 2014), and IKKβ deletion in thymocytes prevents the development of Tregs (Schmidt-Supprian et al., 2003). Also, genetic FoxP3 loss prevents Treg development, resulting in severe autoimmunity and auto-inflammation, referred to as immunodysregulation, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome (Powell et al., 1982) in humans and scurfy phenotype in mice (Godfrey et al., 1991). The role of IKKβ in peripheral Tregs (i.e., after thymic maturation) is incompletely understood (Gerondakis et al., 2014).

Tregs also suppress antitumor responses and thus limit the success of tumor immunotherapies. Tregs consume IL-2 via the high-affinity IL-2 receptor and thereby deprive cytotoxic CD8$^+$ T cells (CTLs) of mitogenic signals (Savage et al., 2013). CTLs require IKKβ for the post-thymic induction of the IL-7

receptor (Silva et al., 2014) that enables STAT5- and Bcl-2-dependent survival. T cell receptor-mediated activation of CTLs rewires their survival signaling toward calcineurin-dependent Bcl-xL expression and calcineurin-independent repression of Bcl-2 (Koenen et al., 2013). This model suggests that activated CTLs lacking calcineurin signaling will die, hinting at a role of the transcription factor NFATc1, which is regulated by calcineurin (Klein-Hessling et al., 2017). NFATc1 can also be induced by NF-κB (Hock et al., 2013), yet IKKβ-deficient CTLs were able to expand in a tumor model (Barnes et al., 2015). The precise roles of NFATc1 and NF-κB in CTL survival thus requires further analysis.

In contrast to the principal role in immune activation, recent studies using cell type-specific and conditional deletion approaches revealed anti-inflammatory functions of certain NF-κB components: IKKβ deletion in non-immune cells such as keratinocytes promoted inflammation (Pasparakis, 2009) and stimulated IL-1β production in myeloid cells (Greten et al., 2007). Deletion of TGF-β-activated kinase 1 (TAK1), which is upstream of IKKβ, reduced Treg numbers in mice and caused mild autoimmunity (Chang et al., 2015). Also dendritic cells (DCs) require NF-κB, both to induce immunity and to maintain immune tolerance (Baratin et al., 2015; Dissanayake et al., 2011). Thus, NF-κB has both pro- and anti-inflammatory functions, but which of them prevails in vivo is unclear.

We recently observed that prolonged treatment with the IKKβ inhibitor KINK-1 (kinase inhibitor of NF-κB-1) surprisingly aggravated a T helper (Th) cell-mediated kidney disease model (Gotot et al., 2016). Given that NF-κB activation promotes FoxP3 expression (Ruan et al., 2009; Schuster et al., 2012; Zheng et al., 2010) and that CD25 signaling promotes Treg survival (Furtado et al., 2002), we hypothesized that mature Tregs may require IKKβ for expansion, maintenance, and/or recruitment. We investigated this hypothesis using genetic and pharmacological approaches and found an unexpected pro-inflammatory effect of sustained IKKβ inhibition that may be exploited to invigorate CTL responses after tumor vaccination.

RESULTS

Mice Lacking IKKβ in Tregs Develop a Phenotype Identical to Scurfy Mice

We recently noted lower Treg numbers after prolonged IKKβ inhibition in a kidney disease model (Gotot et al., 2016). To investigate whether Tregs require cell-intrinsic IKKβ, we crossed $IKK\beta^{fl/fl}$ mice (Park et al., 2002) with $FoxP3^{Cre}$ mice (Rubtsov et al., 2008) to generate mice whose FoxP3-expressing cells lacked IKKβ (termed $FoxP3^{\Delta IKK\beta}$ mice). Strikingly, already 2–4 weeks after birth, these mice were severely compromised and died before reaching adulthood. On day 21, they displayed reduced agility, their skin was scaly, and their tails showed padded rings (Figure 1A). This phenotype was identical to that of FoxP3-deficient and of scurfy mice, which lack Tregs because of the absence or a mutation of the *Foxp3* gene, respectively, and which die at young age of unrestrained systemic inflammation (Gavin et al., 2007; Godfrey et al., 1991). Like these mice, $FoxP3^{\Delta IKK\beta}$ mice showed enlarged lymph nodes and spleens (Figure 1B) and severe mononuclear infiltration and tissue damage in spleen, lung, and skin (Figure 1C). Peripheral organs of $FoxP3^{\Delta IKK\beta}$ mice contained hardly any $FoxP3^+$ Tregs (Figures 1D, 1E, and S1A–S1C), and most splenic T cells displayed an activated $CD44^+CD62L^-$ phenotype (Figure S1D). By contrast, the thymi of $FoxP3^{\Delta IKK\beta}$ mice showed unaltered architecture (Figure S1E) and contained normal Treg frequencies (Figures 1F and S1C), indicating intact thymic Treg generation. RT-PCR detected an IKKβ signal in $FoxP3^+$ thymocytes but not in the remaining $FoxP3^+$ splenocytes of $FoxP3^{\Delta IKK\beta}$ mice, indicating that the IKKβ message must have been excised after thymic exit (Figure S1F). This is consistent with studies showing that FoxP3 is upregulated late during thymic Treg generation (Gerondakis et al., 2014). When we transferred IKKβ-competent wild-type Tregs into newborn $FoxP3^{\Delta IKK\beta}$ mice, these animals developed normally (Figure 1G), demonstrating that Treg-intrinsic IKKβ was sufficient to prevent the scurfy phenotype of $FoxP3^{\Delta IKK\beta}$ mice.

Tregs Require IKKβ for Peripheral Homeostasis, Not for Suppressive Functionality

To investigate whether the peripheral survival of IKKβ-deficient Tregs was compromised, we pooled the splenocytes from several $FoxP3^{\Delta IKK\beta}$ or $FoxP3^{Cre}$ mice and transferred them into IKKβ-competent $RAG1^{-/-}$ mice. After 7 days, we noted that the few remaining YFP^+ Tregs from $FoxP3^{\Delta IKK\beta}$ mice had undergone homeostatic proliferation, like Tregs from IKKβ-competent $FoxP3^{Cre}$ control mice (Figure 2A). However, only the IKKβ-competent Tregs accumulated, whereas the deficient ones were lost from the $RAG1^{-/-}$ recipients (Figure 2B). No difference in the survival of YFP^-CD4^+ T cells from $FoxP3^{\Delta IKK\beta}$ and control mice was evident (Figure 2C). Both IKKβ-competent and IKKβ-deficient Tregs suppressed the response of cocultured activated Th cells by 70% and 90%, respectively (Figure 2D). Thus, functional Tregs were generated in the thymi of $FoxP3^{\Delta IKK\beta}$ mice but were lost after release into the circulation, explaining the scurfy phenotype.

Based on these findings, we predicted that pharmacological IKKβ inhibition might induce the death of wild-type $FoxP3^+$ Tregs. We tested this notion by injecting the IKKβ inhibitor KINK-1 into $FoxP3^{LuciDTR}$ mice, which express a FoxP3-luciferase reporter to allow noninvasive in vivo semiquantification of Tregs through luminescence imaging (Suffner et al., 2010). After 7–14 days of KINK-1 treatment every second day, the FoxP3 signal was reduced by ~40% (Figure 2E). Flow cytometric analysis after 15 days showed almost 50% less splenic $FoxP3^+$ Tregs, whereas Th cells, CTLs (Figure 2F), and total numbers of circulating leukocytes (data not shown) remained normal, indicating that systemic IKKβ inhibition acted preferentially on Tregs. DCs and macrophages showed elevated signs of activation (Figure S2B), presumably because of the loss of Tregs, consistent with our previous study on the effects of KINK-1 in myeloid cells (Gotot et al., 2016). Scurfy symptoms were not observed under KINK-1 treatment, consistent with the reduction of Tregs by only 50%. Daily or twice daily injection of KINK-1 did not significantly improve Treg depletion and higher KINK-1 doses impaired also the CTL response (Figure S2C).

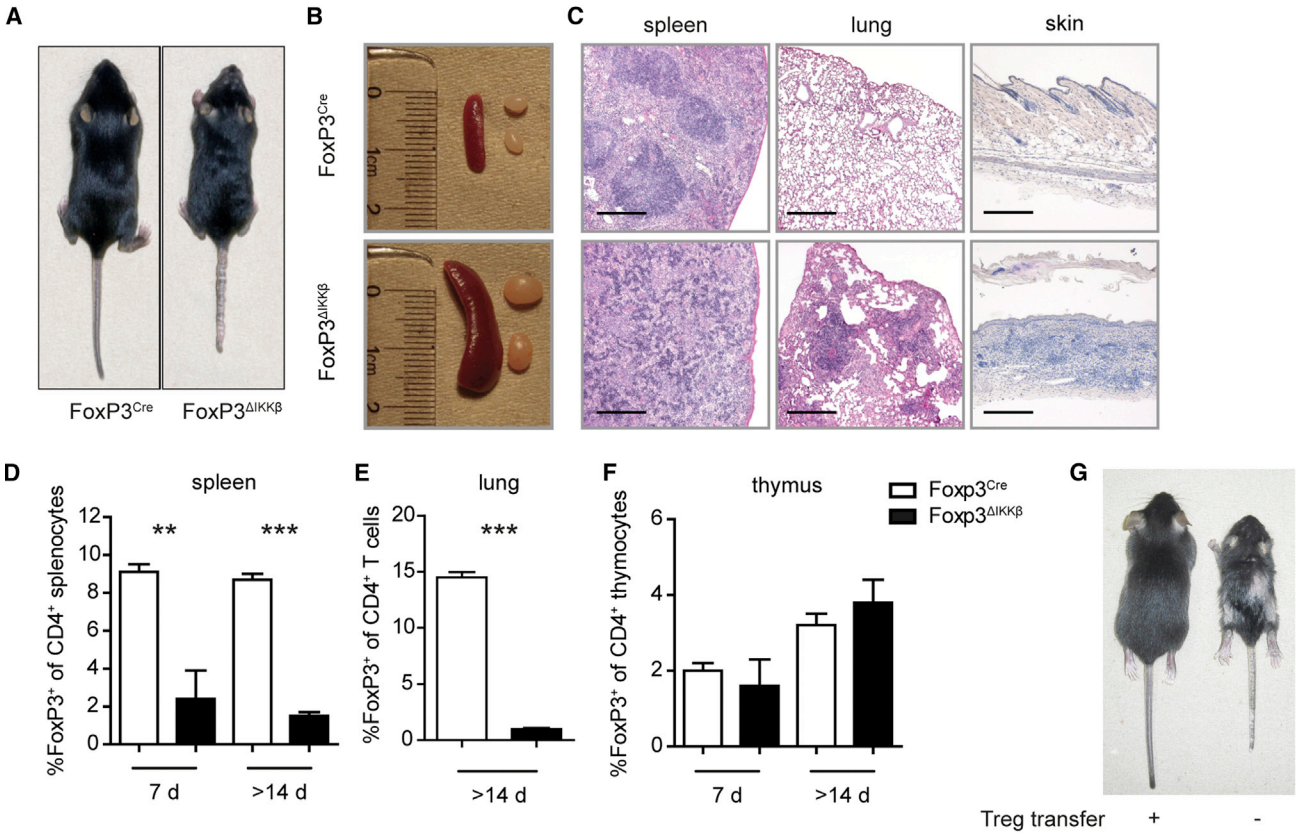

Figure 1. Treg-Specific Deletion of IKKβ Leads to Scurfy-like Immunopathology Because of a Lack of Peripheral Tregs

(A–C) Physical appearance (A), size of spleen and lymph nodes (B), and H&E staining of lung, spleen, and skin sections (C) of FoxP3$^{\Delta IKK\beta}$ mice and FoxP3^{Cre} mice on day 14 after birth. Scale bar represents 200 μm.

(D–F) Frequency of FoxP3^{+} T cells among CD4^{+} T cells in spleen (D), lung (E), and thymus (F) of FoxP3$^{\Delta IKK\beta}$ versus FoxP3^{Cre} mice on day 7 or after more than 14 days after birth.

(G) Physical appearance of FoxP3$^{\Delta IKK\beta}$ mice 14 days after transfer of wild-type Tregs or no transfer at 3 days after birth.

Data are represented as mean ± SEM. See also Figure S1.

Activated CTLs Are Less Dependent on IKKβ Than Tregs Because of Additional Signaling Pathways

In vivo KINK-1-treatment reduced not only the FoxP3 reporter signal (Figure 2E) but also CD25 expression on wild-type Tregs (Figure 3A). CD25 and FoxP3 levels were even more reduced in the few Tregs of FoxP3$^{\Delta IKK\beta}$ mice (Figure 3B), and more of them were caspase-3^{+} (Figure 3C), demonstrating that they were undergoing apoptosis in vivo. To study a potential link between IKKβ, FoxP3, CD25, and survival in Tregs, we cultured wild-type Tregs in the presence of KINK-1. This reduced dose-dependently FoxP3 and CD25 expression and STAT5 phosphorylation (Figures 3D and S3A) and caused more Tregs to undergo apoptosis in vitro (Figures 3E–3G). As IL-2-induced CD25 signaling prompts CD25 and FoxP3 expression and maintains survival of Tregs (Furtado et al., 2002), we added IL-2 to the culture. This restored FoxP3 and CD25 expression and STAT5 phosphorylation in KINK-1-treated Tregs (Figure 3D), suggesting that IKKβ inhibition might deprive Tregs from sensing IL-2 as a survival signal. By contrast, treatment with an IKKα inhibitor had only mild effects on Treg numbers and their FoxP3 expression (Figure S3B), indicating that Treg survival required IKKβ.

After 18 hr of culture with KINK-1, less than 60% of the Tregs had survived, whereas the survival of Th cells and CTLs and of vehicle-treated Tregs was unaffected (Figure 3H), indicating a preferential effect of KINK-1 on Tregs. We therefore asked why effector T cells were resistant to KINK-1 treatment, although they also use NF-κB signaling (Hayden and Ghosh, 2012). The transcription factor NFATc1 has been reported to mediate survival of CTLs but not of Tregs (Vaeth et al., 2012). To clarify whether effector T cells require this factor, we cultured NFATc1-deficient CTLs, Tregs, and Th cells with KINK-1. Although Th cells survived independent of IKKβ, NFATc1, and both, CTL indeed started to die when both IKKβ and NFATc1 were incapacitated (Figure 3H). This indicated that CTLs, but not Th cells, used NFATc1 for survival when IKKβ was blocked. The survival of Tregs was not further compromised when NFATc1 was absent (Figure 3H).

When we examined CTL expansion, they were less abundant when they lacked NFATc1 or when KINK-1 was present throughout the culture period. Importantly, CTLs could expand when KINK-1 was added 24 hr after their activation, unless NFATc1 was deleted (Figure 3I), indicating that CTLs required

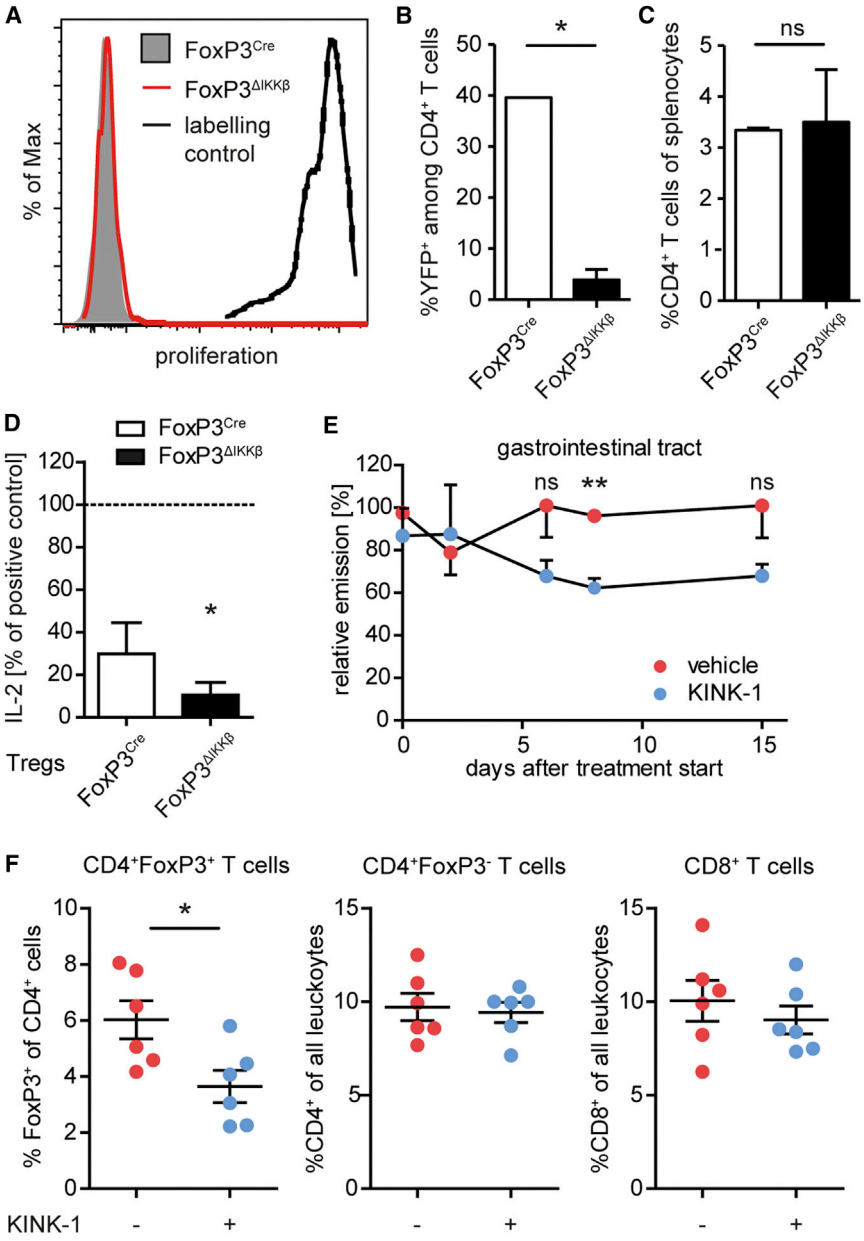

Figure 2. Tregs Require IKKβ for Peripheral Homeostasis, Not for Suppressive Functionality

(A–C) Splenocytes from FoxP3$^{\Delta IKK\beta}$ or FoxP3^{Cre} mice were transferred into RAG1$^{-/-}$ mice, and spleens of recipient mice were analyzed 7 days later. (A) CellTrace violet dilution profile of transferred Tregs, (B) frequency of FoxP3$^+$ Tregs among splenic CD4$^+$ T cells, and (C) frequency of Th cells among splenocytes.

(D) IL-2 concentration in the supernatants of CD3/28-bead-activated T cells co-cultured with Tregs from FoxP3$^{\Delta IKK\beta}$ or FoxP3^{Cre} mice for 3 days. The dashed line indicates IL-2 production in the absence of Tregs.

(E and F) Photon emission from the gastrointestinal tract (E) and frequency of FoxP3$^+$ cells among CD4$^+$ T cells and CD4$^+$ and CD8$^+$ T cells among total blood leukocytes from FoxP3^{LuciDTR} mice treated with KINK-1 every other day for 15 days (F). Data are represented as mean ± SEM. See also Figure S2.

IKKβ only during activation, but used NFATc1 for survival and expansion after day 1.

IKKβ Inhibition after CTL Priming Improves Protection in a Tumor Vaccination Model

Tregs can hamper antitumor immunity by inhibiting CTLs (Savage et al., 2013). Our findings above suggested that the ability of KINK-1 to target Tregs but not that CTLs might improve the defense against tumors. We tested this hypothesis in the B16-OVA melanoma mouse model. To exclude that KINK-1 might act directly on the tumor, we engineered IKKβ-deficient melanoma cells (Figures S4A and S4B) and established that these grew in vivo like wild-type melanoma cells (Figure S4C). On day 9 after implanting IKKβ-deficient tumor cells, we treated the mice with KINK-1 and/or a Treg-blocking/depleting antibody. However, tumor growth was unaltered (Figure S4D), potentially because reducing Tregs to 50% did not sufficiently enhance the endogenous antitumor response. Therefore, we decided to boost this response by vaccinating with a tumor antigen (Figure 4A), in order to boost cross-priming of antitumor CTLs (Kurts et al., 2010). Tumor vaccination alone delayed tumor growth somewhat in a CD8$^+$ cell-dependent manner (Figures 4B and S4E). In view of our observations above (Figures 3F and 3G), we commenced KINK-1 treatment on day 3 after vaccination (i.e., after the CTL activation phase). Indeed, KINK-1 delayed tumor growth even more (Figure 4B) and noticeably enhanced the tumor antigen-specific CTL response (Figure 4C). KINK-1-treated mice survived significantly longer than vehicle-treated mice (Figure 4D), confirming that prolonged IKKβ inhibition after tumor vaccination can enhance the antitumor defense.

Already after two KINK-1 applications, STAT5 signaling in Tregs was impaired both in secondary lymphatic organs and within the tumor (Figure 4E), indicating that Tregs had sensed less IL-2 survival signals. To investigate whether KINK-1 treatment can improve CTL recruitment into the tumor, we injected activated OT-I cells into tumor-bearing, vaccinated mice. Indeed, more OT-I cells were detected under KINK-1 treatment within the tumor, but not in secondary lymphatic organs (Figure 4F). Thus, higher CTL recruitment may have contributed to their superior antitumor response resulting from KINK-1 treatment after tumor vaccination.

Finally, despite unchanged numbers of intratumoral macrophages (vehicle, 3.4 ± 1.4 × 10^5; KINK-1, 3.6 ± 0.6 × 10^5 cells

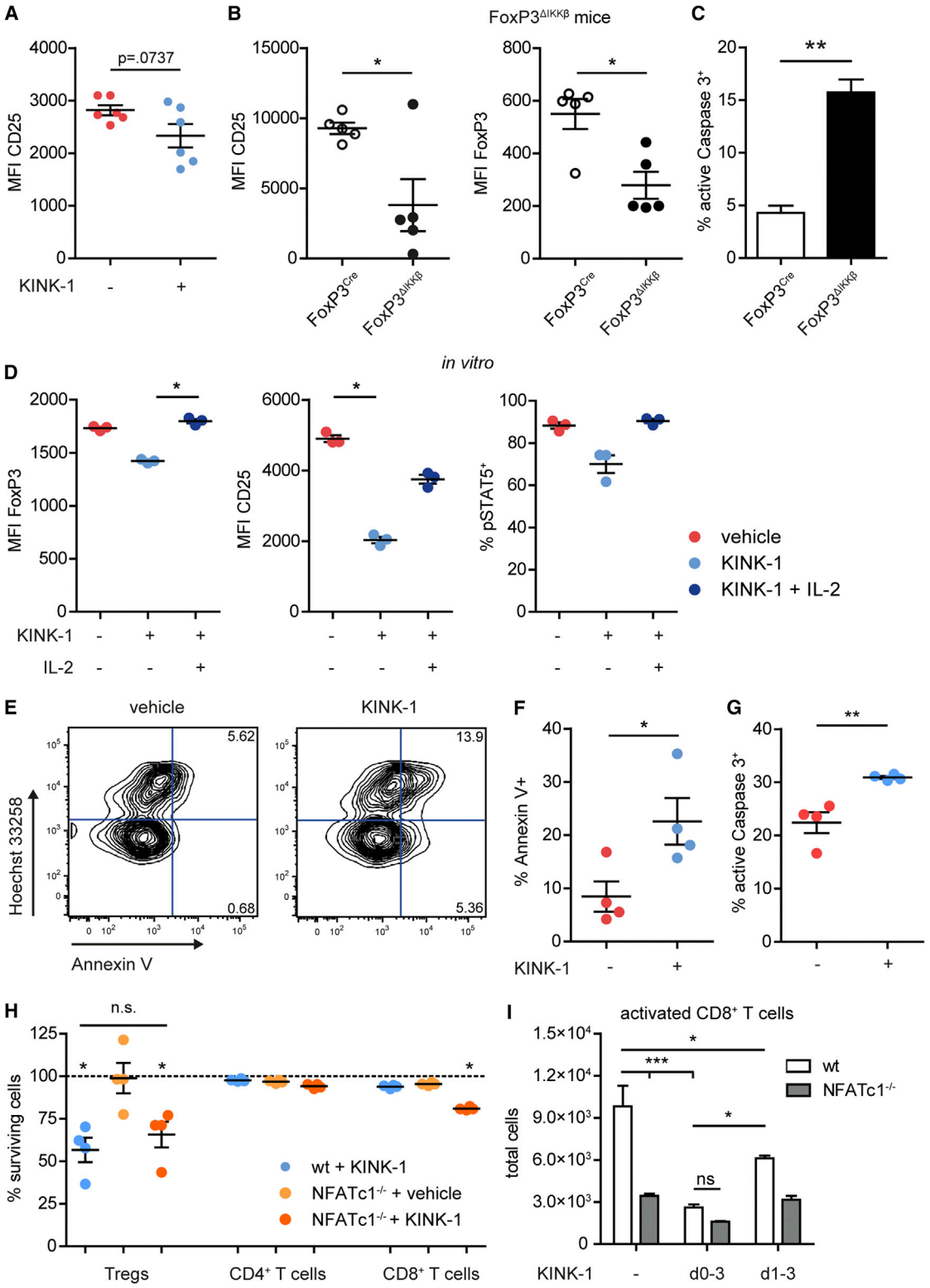

Figure 3. The Loss of IKKβ Signals Is Compensated in CTLs by NFATc1-Directed Signaling

(A) Flow-cytometric analysis of CD25 expression by FoxP3$^+$ Tregs from Figure 2G on day 12 after KINK-1 treatment start.

(B and C) CD25 and FoxP3 expression on FoxP3$^+$ Tregs (B) and frequency of cleaved caspase-3$^+$ FoxP3$^+$ Tregs from FoxP3$^{\Delta IKK\beta}$ or FoxP3^{Cre} mice on day 7 after birth (C).

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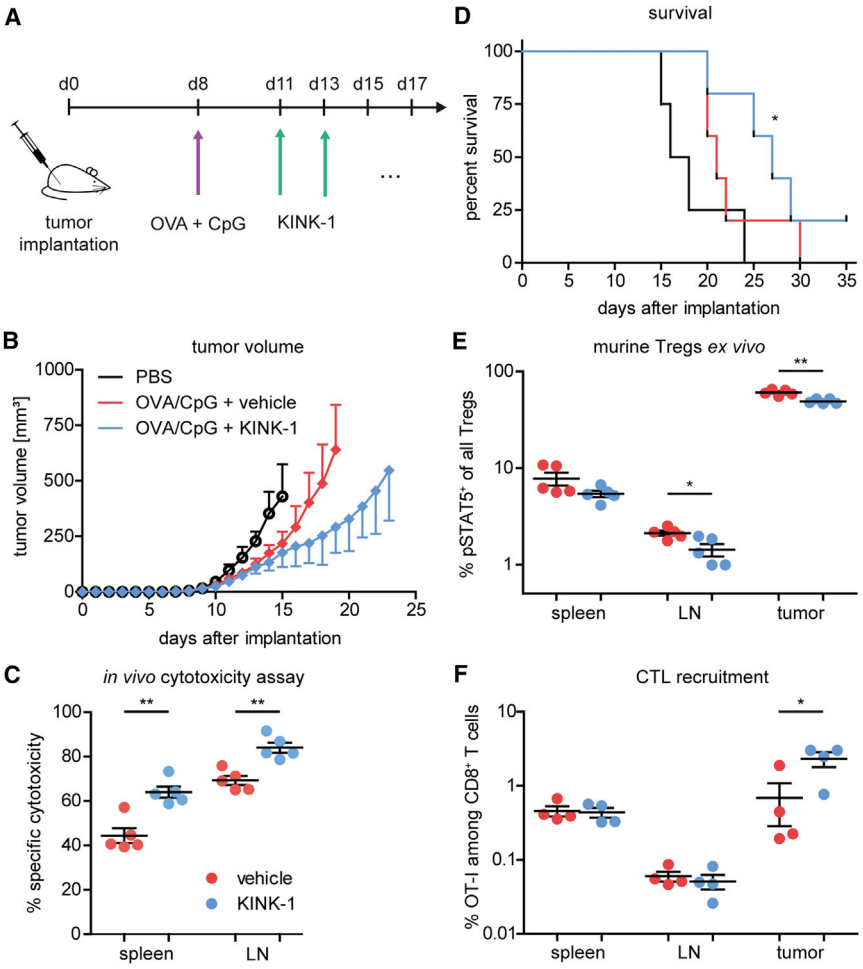

Figure 4. Pharmacological Inhibition of IKKβ Improves the Efficacy of Tumor Vaccination

(A–E) Mice received 4 × 10^5 IKKβ$^{-/-}$ B16-OVA cells s.c. on day 0, OVA/CpG s.c. on day 8, and vehicle or KINK-1 every other day from day 11 onward. The tumor volume (B) was monitored for >21 days after tumor implantation (A). (C) Specific cytotoxicity on day 14 after tumor implantation, (D) survival of the mice shown in (B), and (E) phosphorylation of STAT5 in FoxP3$^+$CD4$^+$ T cells on day 14 after implantation. (F) On day 13, 1 × 10^6 activated OT-I T cells were transferred i.v., and their frequency among CD8$^+$ T cells was determined 24 hr later.

Data are represented as mean ± SEM. See also Figure S4.

per tumor; n = 4), we noted a slight shift from intratumoral M2 to M1 polarization (Figure S4F). Moreover, more eosinophils were detected in the tumor (Figure S4G) under KINK-1 treatment. Both of these have been reported to occur after depleting Tregs in FoxP3^{LuciDTR} mice bearing melanoma (Carretero et al., 2015), suggesting that they might have contributed to the superior antitumor response resulting from KINK-1 treatment after tumor vaccination.

DISCUSSION

NF-κB is critical for both the activation and the activity of immune cells, including DCs and T cells (Hayden and Ghosh, 2012), and IKKβ inhibitors are currently tested for their therapeutic potential to prevent unwanted immune responses. In the present study, we discovered that mice lacking IKKβ in Tregs developed full scurfy-like immunopathology due to the absence of peripheral Tregs and succumbed at 3–5 weeks of age. Previous studies had shown that NF-κB was required for thymic Treg development. In contrast, in our model, Tregs matured in the thymus, because the IKKβ gene was excised late during their development. Instead, they subsequently died after release from the thymus when IKKβ was incapacitated, resulting in a scurfy-like phenotype. IKKβ may be linked to the anti-apoptotic gene c-FLIP, whose deletion in Tregs has recently been shown to cause a scurfy-like phenotype as well (Plaza-Sirvent et al., 2017). The few Tregs in FoxP3$^{\Delta IKK\beta}$ mice were functional, as they could suppress effector T cells, and they did so somewhat more potently than wild-type Tregs. This is consistent with the finding that NF-κB over-activation in Tregs impaired their suppressive capacity (Long et al., 2009). In contrast, the canonical NF-κB component RelA was recently shown to be important for activation and effector function of Tregs but not homeostasis (Messina et al., 2016; Vasanthakumar et al., 2017). Our findings show that Treg-intrinsic IKKβ mediated the survival of Tregs in the circulation.

These findings suggested that pharmacological IKKβ inhibition should reduce Treg numbers in vivo, and this was verified in mice treated with KINK-1 for more than 1 week. Also in vitro, KINK-1 caused the death of Tregs, and this was preceded by

(D) Flow-cytometric analysis of FoxP3, CD25 expression, and phosphorylation levels of STAT5 in CD4$^+$FoxP3$^+$ Tregs after ex vivo culture with 1 μM KINK-1 or vehicle and with exogenous IL-2 for 18 hr.

(E–G) Flow cytometric analysis of Treg death from the experiment shown in (D) assessed by annexin V and Hoechst 33258 staining (E), with annexin V$^+$Hoechst$^\pm$ cells quantified in (F) or by staining for active caspase-3 (G).

(H) Flow cytometric analysis of survival of CD4$^+$FoxP3$^+$ Tregs, CD4$^+$FoxP3$^-$ Th cells and CTLs among bulk splenocytes from wild-type (WT) and CD4$^{\Delta NFATc1}$ mice normalized to vehicle-treated cells from WT mice after ex vivo culture with 1 μM KINK-1 or vehicle for 18 hr.

(I) Proliferation of anti-TCRβ/CD28-stimulated CTLs from WT and CD4$^{\Delta NFATc1}$ mice after IKKβ inhibition.

Data are represented as mean ± SEM. See also Figure S3.

the loss of FoxP3 and CD25 expression and of STAT5 signaling. This may be explained by the presence of functional binding sites for the NF-κB transcription factors in both the *Foxp3* and *Il2ra* genes (encoding CD25) (Ballard et al., 1988; Long et al., 2009; Ruan et al., 2009; Schuster et al., 2012; Zheng et al., 2010). Supplementation of IL-2 partially restored FoxP3 and CD25 expression and Treg survival, consistent with the known ability of IL-2 to maintain CD25 and FoxP3 expression, to counteract pro-apoptotic activities of FoxP3 (Tai et al., 2013) and hence to support Treg survival (Furtado et al., 2002). Thus, IKKβ inhibition likely compromised Treg homeostasis by downregulating FoxP3 and CD25 expression, so that they could no longer sense the survival signal IL-2.

These findings explain our recent observation that inhibiting IKKβ a few days after inducing a Th cell-mediated murine glomerulonephritis model aggravated the symptoms (Gotot et al., 2016). Importantly, we had noted reduced intrarenal Treg numbers, which can now be explained by compromised Treg survival. These findings suggest that caution is warranted in therapeutic attempts to treat inflammatory diseases by IKKβ inhibition, because the loss of Tregs might aggravate rather than attenuate inflammation.

At the same time, our findings uncovered an unexpected opportunity to boost weak adaptive immune responses. CTL responses against tumors are often curtailed by Tregs, and checkpoint blockade that targets Tregs can overcome this problem (Sharma and Allison, 2015). Therefore, we tested our protocol in a melanoma tumor model. However, the endogenous CTL response could not be invigorated sufficiently by IKKβ inhibition. This may be explained by the reduction of Treg numbers by only 50% using our protocol, which a previous tumor study found to be insufficient (Li et al., 2010). This reduction, however, sufficed to enhance antitumor CTL cross-priming induced by vaccination with a tumor antigen, resulting in slower melanoma growth and longer survival of tumor-bearing animals. Thus, IKKβ may indeed represent a pharmacologically relevant antitumor therapy target. As our model was designed to rule out effects of IKKβ blockade on the tumor itself, it may underestimate its effectivity in situations in which tumor cells also use IKKβ for survival (Zhang et al., 2017), which needs to be tested in future studies.

The IKKβ dependence of Tregs is consistent with the importance of NF-κB for immune cell activation, but it raised the question why CTLs, which also use NF-κB, were unaffected by KINK-1 treatment. A dose effect is certainly involved, as higher KINK-1 doses were able to suppress CTLs in our hands. This is consistent with a previous study showing that the complete loss of IKKβ signaling, achieved by genetic ablation, abrogated the antitumor response against fibrosarcoma (Barnes et al., 2015). We found that CTLs were more resistant than Tregs to IKKβ inhibition, because they used the transcription factor NFATc1 that promoted CTL but not Treg survival (Klein-Hessling et al., 2017; Vaeth et al., 2012). Although Tregs were unaffected by the absence of NFATc1, CTLs started to die in vitro when in addition to IKKβ also NFATc1 was blocked. This is consistent with a previously proposed model that NF-κB signals induce NFATc1 components in effector T cells, including CTLs, which then switch to NFATc1-controlled signaling (Hock et al., 2013; Koenen et al., 2013). Our findings support this model, and indicate that Tregs do not undergo this switch.

In summary, we found that mature Tregs rely on the canonical NF-κB pathway and that genetic deletion or pharmacological inhibition of IKKβ suspends Treg-mediated suppression of activated effector T cells, which use other pathways to counteract apoptosis. It was important to commence IKKβ inhibition after tumor vaccination, because earlier KINK-1 application prevented CTL activation, thereby abolishing antitumor immunity. Hence, on a systemic level, sustained IKKβ inhibition acts predominantly pro-inflammatory on ongoing immune responses, uncovering an immune checkpoint that may be exploited for invigorating tumor vaccination.

EXPERIMENTAL PROCEDURES

Mice and Reagents

FoxP3^{Cre} mice carry a yellow fluorescent protein (YFP)-Cre recombinase transgene in the *Foxp3* locus. RAG1$^{-/-}$ mice are devoid of B and T cells. FoxP3^{LuciDTR} express eGFP, human diphtheria toxin receptor, and click beetle luciferase from the endogenous *Foxp3* locus. In CD4^{Cre} × NFATc1$^{fl/fl}$ mice (designated CD4$^{\Delta NFATc1}$ mice), NFATc1 is excised in T cells in the thymic double-positive stage (Klein-Hessling et al., 2017). Mice were bred at the animal facilities of the University Hospitals Bonn and Hamburg-Eppendorf under specific-pathogen-free (SPF) conditions. We used sex- and age-matched mice for all experiments. Animal experiments were approved by governmental committees (Behörde für Gesundheit und Verbraucherschutz Hamburg and Landesamt für Natur, Umwelt und Verbraucherschutz NRW).

Pharmacological Inhibition of IKKβ

KINK-1 (also known as Bay65-1942 and CpdA) (Ziegelbauer et al., 2005) is a highly selective ATP-competitive inhibitor of IKKβ (K_i = 2 nM). Off-target activity (>40% inhibition at 1 μM) has been observed for PIM3, ERK8, CAMK1, SGK1, and CDK2-Cyclin A (International Centre for Kinase Profiling, MRC Protein Phosphorylation Unit, University of Dundee; http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors). KINK-1 (Gotot et al., 2016; Ziegelbauer et al., 2005) was dissolved in 10% Kolliphor EL (Sigma-Aldrich) as vehicle, and 5 mg/kg body weight was given subcutaneously (s.c.) at the time points indicated in the figures and their legends.

Isolation and Adoptive Transfer of Tregs

Tregs were isolated from spleens of donor mice using a Treg isolation Kit (Miltenyi). On day 3 after birth, 1.3 × 10^6 viable Tregs were injected intraperitoneally (i.p.) in FoxP3$^{\Delta IKK\beta}$ mice. For transfer of IKKβ-deficient cells into RAG1$^{-/-}$ mice, 10^5 viable splenocytes from FoxP3$^{\Delta IKK\beta}$ or from control FoxP3^{Cre} mice were labeled with CellTrace violet (Life Technologies) and injected i.v.

In Vitro Suppression Assay

CD4$^+$ YFP$^+$ Tregs and CD4$^+$ YFP$^-$ responder T cells were purified from spleens of 7-day-old Foxp3^{Cre} and FoxP3$^{\Delta IKK\beta}$ mice by flow cytometry. Cells were cultured at a 1:2 ratio in 96-well plates and activated with anti-CD3/28 beads (Invitrogen) for 72 hr. Supernatant IL-2 concentration was measured by ELISA.

Bioluminescence Imaging

FoxP3^{LuciDTR} mice were imaged 5 min after i.p. injection of 4.5 mg d-luciferin (SynChem) using the IVIS 100 Imaging System and Living Image software (Xenogen) as previously described (Tittel et al., 2012).

In Vitro Inhibitor Treatment

Bulk splenocytes (1 × 10^5) were seeded into 96-well plates in X-VIVO 15 medium (Lonza); incubated with KINK-1, the IKKα inhibitor BAY11-7082 (Schön et al., 2008), or the respective vehicle for 18 hr with 10 ng/mL IL-2, when indicated; and analyzed using flow cytometry.

CD8α^+ T cells (5×10^4) were isolated by negative magnetic selection (Miltenyi) and seeded into 96-well flat-bottom plates coated with 1.25 μg/mL anti-TCRβ and 5 μg/mL anti-CD28 antibody.

Tumor Experiments

Mice were injected with 4×10^5 IKKβ$^{-/-}$ B16-OVA cells s.c. into the flank. When tumors reached 25 mm^3 (day 8 or 9 after implantation), mice received 50 μg ovalbumin (Sigma-Aldrich) + 10 nmol CpG 1668 (TIB Molbiol Berlin) s.c. as previously described (Klages et al., 2010). The tumor volume is given as (width × width × length)/2.

In Vivo Cytotoxicity Assay

Splenocytes were pulsed for 20 min at 37°C with SIINFEKL (2 μg/mL) and labeled with 1 μM CFSE ($CFSE_{hi}$) or were not pulsed and labeled with 0.1 μM CFSE ($CFSE_{lo}$) and injected as a 1:1 mix i.v. After 5 hr, target cells were enumerated by flow cytometry. Specific lysis was calculated using the following formula: percentage specific cytotoxicity = 100 − [100 × ($CFSE_{hi}$/$CFSE_{lo}$) primed/($CFSE_{hi}$/$CFSE_{lo}$) control].

In Vitro Activation of OT-I Cells

Splenocytes from OT-I mice were pulsed with 10 μM SIINFEKL at 37°C for 1 hr and cultured with 10 ng/mL rIL-12. After 2 days, the medium was replaced with fresh medium containing 20 ng/mL rIL-2 (both Peprotech), and cells were cultured for 5 days.

Statistical Analysis

Differences were compared using the Kruskal-Wallis test with post hoc analysis using the Mann-Whitney test, one-way ANOVA with post hoc Bonferroni test, or the log rank test (GraphPad Prism). Statistical significance was defined as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.09.082.

AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, J.G., C.H., C.K., and F.T.; Investigation, J.G., C.H., E.C.P., J.L.S.-B., C.J.F.C., M.-S.P., M.S.O., and L.G.; Resources and Validation, A.H., P.A.K., C.E., E.S., N.G., V.H., and F.T.; Writing – Original Draft, C.H., J.G., and C.K.; Writing – Review & Editing, C.H., J.G., and C.K.; Supervision, C.K.; Funding Acquisition, C.K., C.H., and F.T.

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