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Interleukin-35-mediated induction of a novel regulatory T cell population

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Abstract

Regulatory T cells (T_{regs}) play a critical role in the maintenance of immunological self-tolerance. Naïve human or murine T cell treatment with the inhibitory cytokine IL-35 induces a regulatory population, termed iT_R35, that mediates suppression via IL-35, but not IL-10 or TGFβ, neither express nor require Foxp3, are strongly suppressive in five *in vivo* models, and exhibit *in vivo* stability. T_{reg}-mediated suppression induces iT_R35 generation in an IL-35- and IL-10-dependent manner *in vitro*, and in inflammatory conditions *in vivo* in *Trichuris*-infected intestines and within the tumor microenvironment, where they appear to contribute to the regulatory milieu. iT_R35 may constitute a key mediator of infectious tolerance, may contribute to T_{reg}-mediated tumor progression, and *ex vivo* generated iT_R35 may possess therapeutic utility.

Regulatory T cells (T_{regs}) are a unique subset of $CD4^+$ T cells that are essential for maintaining peripheral tolerance, preventing autoimmunity, and limiting chronic inflammatory diseases. However, they also prevent beneficial anti-tumor responses and

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AUTHOR CONTRIBUTIONS

L.W.C. designed (with help from D.A.A.V) and executed all mouse experiments, analyzed data and wrote the manuscript; V.C. performed human experiments; A.L.H. (with L.W.C) performed B16 tumor experiments; J.B. performed MC38 tumor experiments; P.R.G. carried out *Trichuris muris* infections; C.G. did confocal microscopy analyses; D.F. performed Affymetrix analyses; K.F. and S.A.B (with C.J.W) generated and screened anti-IL35 mAbs; C.J.W. coordinated anti-IL35 mAb development, testing and purification, and aided in figure preparation; M.L.J. generated and purified mEbi3 protein for immunizations and mAb development; J.N. provided key reagents and information; J.E.R. created and performed histological analyses of *Foxp3*$^{-/-}$ mice; D.A. designed *Trichuris muris* experiments and provided input on interpretation; M.J.T provided training in the B16 tumor model, and provided input to the research design and interpretation; and D.A.A.V. conceptualized the research, directed the study, and edited the manuscript.

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sterilizing immunity against certain chronic infections. Consequently, the modulation of T_{reg} activity or generation of T_{regs} *ex vivo* are important goals of immunotherapy. Naturally-occurring, thymus-derived $CD4^+$ T_{regs} (nT_{regs}) express the lineage specific transcription factor Foxp3 (forkhead box P3), which is required for their development, homeostasis and function[1–4]. Despite their limited numbers (5–10% of $CD4^+$ T cells), T_{regs} play a pivotal role in immune homeostasis. Indeed, it has been suggested that the suppressive milieu is potentiated by *in vivo* conversion of non-T_{regs} into suppressive cells, a process termed 'infectious tolerance'. This contagious spread of suppression is thought to be a primary mechanism underlying transplantation tolerance[5] and modulating autoimmune and inflammatory diseases, such as experimental allergic encephalomyelitis (EAE)[6] and asthma[7]. While the mechanisms that mediate infectious tolerance remain obscure, both TGFβ and IL-10 have been implicated.

Induced regulatory T cell populations (iT_R) can be generated in the periphery, or *in vitro*, from conventional $CD4^+Foxp3^-$ T cells (T_{conv})[8–10]. There is substantial interest in the therapeutic potential of iT_R as it has been shown that antigen-specific regulatory populations can be generated that are potently inhibitory *in vivo*[11, 12]. Two types of iT_R have been described based on the cytokines that induce them; TGFβ- and IL-10-iT_R. TGFβ-iT_R are generated following T cell activation in the presence of TGFβ with or without retinoic acid and IL-2. Both types of iT_R are potently suppressive both *in vitro* and *in vivo*[11, 13, 14], but possess distinct molecular signatures. While TGFβ iT_R express Foxp3 and primarily secrete TGFβ, IL-10 iT_R cells remain $Foxp3^-$ following conversion and are defined by high IL-10 secretion.

T_{reg}-based approaches to treating inflammatory conditions such as allergy, autoimmune diseases, and graft-versus-host responses have great potential, but also have limitations [reviewed in [12]]. The therapeutic potential of human T_{regs} is limited by their polyclonal specificity, poorly defined markers for enrichment, and reduced proliferative capacity which limits *ex vivo* expansion. Antigen-specific iT_R (IL-10 iT_R or TGFβ iT_R) can be generated *ex vivo* but their utility is restricted by technical complexities in their generation, limited potency and/or ambiguity regarding stability and longevity *in vivo*. Thus, the identification of a well-defined population of T_{regs} which can be readily generated *ex vivo*, and are stable and potently inhibitory *in vivo* is a critical goal for effective cell-based immunotherapy.

We have recently described a novel T_{reg}-specific cytokine, IL-35, that is required for maximal regulatory activity of murine T_{regs} *in vitro* and *in vivo*[15]. In this study, we show that IL-35, like IL-10 and TGFβ, can generate human and murine iT_R and address four questions: (1) what is their *in vivo* efficacy and stability, (2) can they be generated by nT_{regs}, (3) are they generated at inflammation sites, and (4) what is their physiological contribution to the regulatory milieu established by nT_{regs}?

RESULTS

Human IL-35 treated T_{conv} acquire a regulatory phenotype

Human IL-35 can suppress the proliferation of umbilical cord-derived human $CD4^+$ T_{conv} cells to a degree similar to that seen by activated T_{regs} (see Supplementary Information and Supplementary Figs. 1 and 2). T_{conv} cells activated with anti-CD3- + anti-CD28-coated latex beads (αCD3/CD28) in the presence of IL-35 dramatically upregulated *EBI3* and *IL12A* mRNA, the two constituents of IL-35 (Ebi3 and p35, respectively) (Fig. 1a), but not IL-10 or TGFβ (Supplementary Fig. 3). Single cell analysis by both intracellular cytokine staining (Fig. 1b) and confocal microscopy (Fig. 1c) suggests that IL-35, but not control protein, treatment induces homogeneous expression of IL-35 in human $CD4^+$ T_{conv} cells. Similarly, $CD4^+CD45RA^+CD25^-$ T_{conv} cells from adult peripheral blood expressed *EBI3* and *IL12A*,

but not *TGFB* or *IL10* mRNA following activation in the presence of IL-35 (Supplementary Fig. 3i,j and data not shown).

We next assessed if IL-35-treated cells assumed the functional phenotype of iT_R. T_{conv} cells activated in the presence of IL-35 but not control were hyporesponsive to secondary restimulation (Fig. 1d). To determine whether IL-35-pretreated T_{conv} cells had acquired regulatory capacity, they were co-cultured as potential suppressors with freshly purified responder T_{conv}. While control-treated cells lacked any suppressive capacity, IL-35 treated cells were strongly suppressive (Fig. 1e). Human IL-35, but not control-treated, T_{conv} cells also suppressed responder T_{conv} cell proliferation across a permeable membrane, in the absence of direct cell contact, supporting a role for cytokine-mediated suppression (Fig. 1f). Moreover, neutralizing mAbs to IL-35, but not IL-10 or TGFβ, blocked their suppressive capacity (Fig. 1g, Supplementary Information and Supplementary Fig. 3). Taken together, these data suggest that IL-35 can convert human T_{conv} into a homogeneous population of iT_R cells that suppress via IL-35.

IL-35 treated murine T_{conv} acquire a regulatory phenotype *in vitro*

Given that human IL-35 can mediate iT_R generation, we then asked if murine IL-35 possessed a similar capacity (see Supplementary Information and Supplementary Fig. 4). Analysis of T_{conv} cells activated in the presence of murine IL-35 upregulated both *Ebi3* and *Il12a*, but not *Il10* or *Tgfb* mRNA (Fig. 2a, Supplementary Information and Supplementary Fig. 5). Immunoprecipitation and western blot analysis demonstrated that only IL-35 treated cells secrete IL-35, which was equivalent to the amount of IL-35 produced by natural T_{regs}. Both control-treated T_{conv} cells and IL-35-treated *Ebi3*$^{-/-}$ T_{conv} cells did not secrete IL-35 (Fig. 2b). We next assessed if IL-35-treated murine cells, like their human counterparts, assumed an iT_R phenotype. Consistent with earlier reports[16], previously activated T_{conv} cells proliferated well in response to secondary re-stimulation (Fig. 2c). IL-10 and IL-27 pre-treated T_{conv} also proliferated strongly in response to re-stimulation (note that short-term IL-10 treatment alone, in the absence of DCs, is insufficient to mediate IL-10 iT_R conversion[14]) (Supplementary Fig. 6). However, both IL-35 and TGFβ pretreated T_{conv} cells were hyporesponsive to re-stimulation, albeit to a lesser degree than freshly purified nT_{regs}. To determine whether these cytokine-pretreated T_{conv} cells had acquired regulatory capacity, they were co-cultured as potential suppressors with freshly purified responder T_{conv} cells (Fig. 2d and Supplementary Fig. 6). Whereas the control, IL-10- and IL-27-treated T_{conv} cells had no effect on responder proliferation, TGFβ-treated T_{conv} cells suppressed responder T cell proliferation[13]. As seen with human T cells, murine T_{conv} cells pretreated with IL-35 were also capable of suppressing responder T cell proliferation. Furthermore, IL-35- but not control-treated T_{conv} could suppress T cell proliferation in a contact-independent manner, across a permeable membrane, implicating soluble suppressive mediators (Fig. 2e). Using an IL-35 sandwich ELISA we also showed that approximately 500–700pg/ml IL-35 is required to mediate the induction of *Ebi3* and *Il12a* expression and of the suppressive phenotype (Supplementary Fig. 4).

To determine the mechanism of suppression, we first showed that IL-35-pretreated *Il10*$^{-/-}$ (which cannot make IL-10), but not *Ebi3*$^{-/-}$ (which cannot make IL-35), T_{conv} could suppress responder T cells (Fig. 2f). In addition, TGFβR.DN T_{conv} that are unable to respond to TGFβ were fully suppressed by IL-35-treated T_{conv} (see Supplementary Information and Supplementary Fig. 7). Using cytokine neutralizing mAbs, we then showed that IL-35-pretreated T_{conv} mediated suppression via IL-35 but not IL-10 or TGFβ (Fig. 2g,h [note that the anti-Ebi3 mAb used neutralizes IL-35 but not IL-27; Supplementary Fig. 8]). Collectively, these data suggest that murine IL-35 converts T_{conv} into an iT_R population that appears to mediate suppression exclusively via IL-35.

IL-35-treated murine T_{conv} exhibit a highly restricted genetic signature

Given that IL-35 can convert proliferative, Foxp3$^-$ T_{conv} cells into hypo-responsive, strongly suppressive iT_R, we next sought to define their genetic signature. Interestingly, Foxp3 is neither induced nor required for the generation of IL-35-iT_R. While natural T_{regs} and TGFβ-iT_R express Foxp3, neither control nor IL-35 treated T_{conv} cells express Foxp3 (Supplementary Fig. 9). Moreover, *Foxp3*$^{-/-}$ T_{conv} cells could be converted to IL-35 iT_R which expressed IL-35 and mediated suppression in a manner indistinguishable from their wild type counterparts (Supplementary Fig. 9c). In addition, iT_R35 cells do not express Foxp3 following *in vivo* inoculation as demonstrated by utilizing iT_R35 generated from Foxp3^{gfp} mice in an *in vivo* model of homeostatic expansion. Seven days post-transfer into *Rag1*$^{-/-}$ mice, iT_Rcontrol, wild-type iT_R35 and *Ebi3*$^{-/-}$ iT_R35 were purified based on congenic Thy1 markers and Foxp3 expression assessed by flow cytometric analysis of GFP expression. No induction of Foxp3 was seen in any of the transferred iT_R (Supplementary Fig. 9d).

We next compared the global gene expression of IL-35- and control-treated T_{conv} cells using Affymetrix GeneChip microarrays. Prior to analyses, we verified that the IL-35-iT_R used for analysis expressed *Ebi3* and *p35* mRNA, secreted IL-35 and suppressed responder T_{conv} cells (Supplementary Fig. 10). While clear differences were observed between nT_{reg} and T_{conv}, no genes appear to be significantly up- or down-regulated between IL-35- and control-treated T_{conv} (>3-fold; Supplementary Information and Supplementary Fig. 11). Nevertheless, the IL-35-iT_R cells generated from five independent experiments expressed *Ebi3* and *Il12a* mRNA, secreted IL-35 and mediated potent *in vitro* suppression. The minimalistic genetic alteration observed in IL-35- compared to control-treated T_{conv} was further supported by analysis of T cell activation and co-stimulatory molecule expression and cytokine production. While the expression of most of the proteins/cytokines examined was indistinguishable between IL-35- and control-treated T_{conv}, reductions in the secretion of GM-CSF, IFNy, and IL-4 were observed, albeit not statistically significant (Supplementary Fig. 12). In addition, surface molecules such as CLTA-4 and CD25, which have been previously described as mediators of nT_{reg} suppression, were similarly upregulated in both control and IL-35-treated T_{conv}, cells, arguing against an exclusive role in the latter. Furthermore, the percentage of CTLA4$^+$ IL-35-iT_R was relatively small (<15%). Taken together, these results suggest that IL-35 treatment mediates surgical rather than global gene expression changes.

Given that IL-35 is central to both the generation and suppressive function of IL-35-iT_R, we refer to this novel iT_R population as iT_R35 (for induced T regulatory population making IL-35) [Control-treated T_{conv}, which do not acquire a suppressive phenotype, are referred to as iT_Rcontrol]. While our data suggest that iT_R35 have a highly restricted *Cd4*$^+$/*Foxp3*$^-$/*Ebi3*$^+$/*Il12a*$^+$/*Il10*$^-$/*Tgfb*$^-$ genetic signature, we cannot rule out the possibility that there are some molecular changes that might distinguish IL-35- from control-treated T_{conv} that were not revealed by this analysis, or that this signature may apply to an as yet undefined non-regulatory population (See Supplementary Information).

iT_R35 are potently suppressive *in vivo*

The regulatory capacity of iT_R35 was tested in five different *in vivo* models. We first assessed whether iT_R35 could restore immune homeostasis and prevent lethal autoimmunity in *Foxp3*$^{-/-}$ mice[17, 18]. iT_R35 and various control populations were transferred into newborn (2–3 day old) *Foxp3*$^{-/-}$ mice. Approximately 25 days later, an external clinical score, splenic and lymph node CD4$^+$ T cell numbers, and a histological score (lungs, liver and skin) were determined (see Supplementary Information)[19]. As expected, nT_{regs} and TGFβ-iT_R, but not control-treated T_{conv} (iT_Rcontrol) were able to restore immune

homeostasis and prevent autoimmunity (Fig. 3a-d and Supplementary Fig. 13). Interestingly, iT_R35 were as effective as nT_{regs} at restoring immune homeostasis and preventing autoimmunity in *Foxp3*$^{-/-}$ mice. Importantly, neither *Ebi3*$^{-/-}$ nor *p35*$^{-/-}$ iT_R35 could restore immune homeostasis demonstrating the necessity for IL-35 production *in vivo* by iT_R35.

Second, T_{regs} can control the homeostatic expansion of T_{conv} cells in lymphopenic recombination activating gene 1 (*Rag1*)$^{-/-}$ mice[15, 20, 21]. Purified wild-type Thy1.1$^+$ T_{conv} cells, either alone or in the presence of control- or IL-35-treated Thy1.2$^+$ T cells were adoptively transferred into *Rag1*$^{-/-}$ mice, and splenic responder (Thy1.1$^+$) and suppressor (Thy1.2$^+$) T cell numbers determined 7 days later[19]. Thy1.2$^+$ iT_Rcontrol expanded significantly and failed to block the expansion of Thy 1.1$^+$ responder T_{conv} cells (Fig. 3e, f). In contrast, Thy1.2$^+$ T_{conv} iT_R35, but not *Ebi3*$^{-/-}$ iT_R35, had low proliferative capacity and significantly limited proliferation of Thy 1.1$^+$ responder T_{conv} cells.

Third, experimental autoimmune encephalomyelitis (EAE) is a model for the human autoimmune disease multiple sclerosis and can be induced in C57BL/6 mice following immunization with $MOG_{35–55}$ peptide. Adoptively transferred nT_{regs} have been shown to reduce EAE disease severity[6, 23, 24]. To determine whether iT_R35 could slow or prevent EAE, 10^6 nT_{regs}, iT_Rcontrol or iT_R35 cells were transferred into mice prior to EAE induction[19]. Consistent with previous reports, clinical scores were reduced in nT_{regs} recipients, while disease course in mice receiving the iT_Rcontrol cells or saline alone was unaffected (Fig. 3g). Strikingly, the iT_R35-treated mice were completely protected from EAE, while mice receiving *Ebi3*$^{-/-}$ iT_R35 were indistinguishable from saline control mice, suggesting that IL-35 production by iT_R35 *in vivo* is required for protection.

Fourth, T_{regs} can prevent anti-tumor CD8$^+$ T cell responses against the poorly-immunogenic B16 melanoma[25, 26]. Wild type naïve CD4$^+$CD25$^-$ and CD8$^+$ T cells alone or in combination with nT_{regs} or iT_R35 cells were adoptively transferred into *Rag1*$^{-/-}$ mice followed by i.d. injection of B16 melanoma cells and tumor size monitored daily[19]. As expected, tumor size was reduced in CD4$^+$/CD8$^+$ T cell recipients lacking T_{regs} compared with the untreated *Rag1*$^{-/-}$ mice (Fig. 3h). In contrast, transfer of either nT_{regs} or iT_R35 cells completely blocked the anti-tumor response resulting in more aggressive tumor growth that was comparable to untreated *Rag1*$^{-/-}$ mice. Surgical excision of the primary tumor and subsequent secondary tumor challenge at a distal site demonstrated that concomitant tumor immunity was also prevented by both nT_{regs} and iT_R35 (Supplementary Fig. 13).

Fifth, inflammatory bowel disease (IBD) is initiated by the adoptive transfer of naïve CD4$^+$CD45RB^{hi}CD25$^-$ T cells into *Rag1*$^{-/-}$ recipient mice and disease assessed by weight loss and colonic histopathology[22]. After mice developed clinical symptoms of IBD (˜4 weeks post T cell transfer), they received iT_Rcontrol or iT_R35 and were monitored daily19. Recovery from disease, marked by weight gain (Fig. 3i) and decreased histopathology (Fig. 3j and Supplementary Fig. 13), was observed in mice that received iT_R35 but not the iT_Rcontrol cells. We also used this model to further demonstrate that TGFβ is not required for the *in vivo* suppressive capacity of iT_R35 (see Supplementary Information and Supplementary Fig. 14).

Finally, we further assessed the importance of IL-35 production by iT_R35 *in vivo* using a unique Ebi3-specific mAb that neutralized IL-35 but not IL-27 (Supplementary Fig. 8). Administration of this mAb, but not an isotype control, blocked the suppressive capacity of iT_R35 in an *in vivo* model of homeostatic expansion (Supplementary Fig. 13g), consistent with our observations using *Ebi3*$^{-/-}$ or *p35*$^{-/-}$ iT_R35 in three *in vivo* models (Fig. 3). Taken together, these data clearly demonstrate that iT_R35 have potent suppressive capacity in a

wide variety of *in vivo* models and that this activity is dependent on IL-35 production *in vivo*.

iT_R35 are stable *in vivo*

Some have suggested that *ex vivo* generated iT_R cells are unstable *in vivo.* Although our five *in vivo* transfer experiments suggest that iT_R35 have some degree of stability, we used two approaches to assess this question directly. First, CD45.2$^+$ iT_R35 or TGFβ-iT_R were generated *in vitro* and adoptively transferred into CD45.1$^+$ C57BL/6 mice to monitor cell recovery and function over time (see Supplementary Information). Both iT_R35 and TGFβ-iT_R can be recovered from the spleen post-transfer, and retain expression of their signature genes, *Ebi3*/*Il12a* and *Foxp3/Tgfb*, respectively (Supplementary Fig. 15d). As *in vitro*, iT_R35 inoculated *in vivo* fail to induce Foxp3 expression suggesting that this critical nT_{reg} transcription factor is not required for iT_R35 maintenance and function. While 33% of the initial iT_R35 inoculum was recovered 3 weeks post-transfer, only 12% of TGFβ iT_R cells were recovered (Fig. 4a). In addition, purified iT_R35 cells still retained strong suppressive capacity, whereas the function of TGFβ-iT_R cells was reduced by approximately 50% (Fig. 4b). While this suggests that iT_R35 may be more stable *in vivo*, it does not exclude the possibility that iT_R35 and TGFβ-iT_R may home to different anatomical locations in the mouse, which could affect their recovery from the spleen. Second, we transferred nTregs, iT_R35 or TGFβ-iT_R into 2–3 day old *Foxp3*$^{-/-}$ mice and determined how long they could prevent the onset of a moribund state (clinical score ≥4). By 5 weeks post-transfer, all the TGFβ-iT_R recipients were moribund compared with 40% and 33% of nT_{regs} and iT_R35 recipients, respectively (Fig. 4c). Furthermore, survival of the remaining nT_{reg} and iT_R35 recipients was longer, with 100% moribund not being reached until 6.5 and 8 weeks, respectively. Although additional experiments will be required to fully evaluate the long-term stability of iT_R35 in homeostatic and inflammatory environments, these data suggest that they may be functionally stable *in vivo*.

T_{reg}-mediated suppression generates iT_R35

It has been suggested that T_{regs} can amplify their suppressive capacity by converting non-regulatory populations into suppressive cells, consistent with the concept of infectious tolerance, and that this process might be cytokine-mediated[27–29]. We have previously shown that nT_{regs} are a natural source of IL-35, which increases 5–10-fold upon contact with the target T_{conv} cells[15, 30]. Thus, we asked whether nT_{reg}-derived IL-35 could mediate iT_R35 conversion. We first purified T_{conv} cells that had been cultured with, and suppressed by, nT_{regs} for 3 days (which we refer to as suppressed T_{conv}) and found that expression of both *Ebi3* and *Il12a* (p35) mRNA was significantly up-regulated following co-culture, to a level comparable with nT_{regs} (Fig. 5a,b). Furthermore, suppressed T_{conv} generated by co-culture with wild type, but not *Ebi3*$^{-/-}$ nT_{regs}, secrete a significant amount of IL-35 (Fig. 5c). This demonstrates that IL-35 secretion by nT_{regs} is required to induce IL-35 secretion by co-cultured, suppressed T_{conv} cells. To determine whether suppressed T_{conv} express Foxp3, a prerequisite for mediating the regulatory activity of nT_{regs} and TGFβ-iT_R, we activated Thy1.2$^+$ *Foxp3*^{gfp} T_{conv} cells alone or in combination with Thy1.1$^+$ nT_{regs}. Unlike TGFβ-iT_R, but similar to iT_R35, suppressed T_{conv} remain Foxp3$^-$ following activation in the presence of T_{regs} suggesting that TGFβ may not mediate this conversion (Supplementary Fig. 16). These data raise the possibility that iT_R35 are generated within the suppressed T_{conv} population.

We next assessed whether suppressed T_{conv} gained the phenotypic characteristics of a regulatory population. Interestingly, suppressed T_{conv} were profoundly unresponsive to anti-CD3 stimulation and were potently suppressive *in vitro* (Fig. 5d, e). T_{regs} can secrete IL-10, TGFβ and IL -35 which may influence their ability to convert T_{conv} into suppressed T_{conv}.

Likewise, the same cytokines could be secreted by suppressed T_{conv} and contribute in an autocrine fashion to their conversion and/or their suppressive activity. To address these questions we first co-cultured T_{conv} and T_{regs} that were wild type or lacked the capacity to produce IL-35 (*Ebi3*$^{-/-}$ or *Il12a*$^{-/-}$) or IL-10 (*Il10*$^{-/-}$), or were unable to respond to TGFβ (*TGFβR.DN*). While the generation of suppressed T_{conv} that were hyporesponsive and possessed regulatory capacity did not require TGFβ-mediated signaling, the absence of both IL-35 and IL-10 in the nT_{reg}:T_{conv} co-culture blocked their development and/or function (Fig. 5d, e). Further analysis using nT_{reg}:T_{conv} co-cultures in which only one population was mutant revealed that both nT_{reg}-derived IL-10 and IL-35 was required for the generation of the regulatory suppressed T_{conv} population. Interestingly, suppressed T_{conv}-derived IL-35 was required for conversion as suggested by qPCR analysis of signature genes (see Supplementary Information and Fig. 15b).

Neutralizing antibodies were included during the conversion process or in the secondary suppression assay to further assess the role of IL-35, IL-10 and TGFβ (Fig. 5f). While anti-TGFβ had no effect at either stage, IL-10 neutralization partially blocked conversion but not the regulatory capacity of suppressed T_{conv}, suggesting that IL-10 is required for optimal conversion of suppressed T_{conv} into a regulatory population. In contrast, IL-35 neutralization prevented both the conversion and regulatory function of suppressed T_{conv}. Collectively, these data suggest that some or all of the suppressed T_{conv} are iT_R35. The precise contribution of IL-10 to T_{reg}–mediated conversion of iT_R35 remains to be fully elucidated, as IL-10 alone does not induce *Ebi3* and *Il12a* mRNA expression (Supplementary Fig. 5). However, the addition of low dose IL-10 appears to augment IL-35-mediated conversion, which may help offset the delayed production of IL-35 by nT_{regs} (see Supplementary Information and Supplementary Fig. 16c). Taken together, these data suggest that IL-35, either from a natural source (nT_{regs}) or supplemented exogenously, mediates iT_R35 conversion.

Next, the regulatory capacity of nT_{regs}-suppressed T_{conv} (nT_{regs}-induced iT_R35) was assessed *in vivo*. First, they were able to significantly suppress the homeostatic expansion of co-transferred naïve T_{conv} in *Rag1*$^{-/-}$ mice in a manner comparable to nT_{regs} and iT_R35 (Fig. 5g). However, suppressed T_{conv} generated from *Ebi3*$^{-/-}$ T_{conv} cultured with wild-type nT_{reg}, failed to suppress the expansion of co-transferred T_{conv}. Second, in the EAE model, peak clinical disease scores were decreased by suppressed to a level T_{conv} comparable with nT_{regs} (Fig. 5h). However, suppressed T_{conv} could not ameliorate EAE as effectively as iT_R35 suggesting either that only a proportion of this suppressed T_{conv} population are iT_R35 or that conversion *in vitro* is suboptimal due the time required for potentiation of IL-35 production by nT_{regs}[15, 30]. Nevertheless, these data support the notion that iT_R35 are generated from T_{conv}, to some degree, by nT_{regs} during suppression. In contrast, there is no evidence for the generation of IL-10-iT_R or TGFβ-iT_R in this setting.

T_{reg}-mediated induction of iT_R35 *in vivo*

We reasoned that iT_R35 generation *in vivo* would occur predominantly in inflammatory or disease environments where optimally stimulated nT_{regs} might be secreting high amounts of IL-35. Infection with *Trichuris muris*, an intestinal nematode, is known to promote T_{regs} responses at the site of infection, the large intestine[31]. Thus, we assessed whether iT_R35 could be detected following *Trichuris muris* infection, using the CD4$^+$/Foxp3$^-$/*Ebi3*$^+$/*Il12a*$^+$ iT_R35 signature. *Foxp*^{gfp} mice were infected with *Trichuris muris* and Foxp3$^+$ and Foxp3$^-$ T cells were purified from spleens, small intestines and large intestines 14 days post-infection. Both *Ebi3* and *Il12a* (p35) expression were dramatically increased in Foxp3$^+$ T_{regs}, in both the small and large intestines, compared with splenic T_{regs}, consistent with our previous observations that nT_{regs} increase IL-35 expression ~10-fold in the presence of T_{conv} cells[15] (Fig. 6a,b). While essentially no *Ebi3*/*Il12a* expression was observed in splenic

Foxp3^{-}CD4^{+} T cells, expression was substantial in comparable isolates from the small and, especially, the large intestines (the primary site of infection). Indeed, *Ebi3*/*Il12a* expression in Foxp3^{+} T_{regs} and Foxp3^{-}CD4^{+} T cells from the large intestines was statistically indistinguishable. It should be emphasized that we have never observed IL-35 expression by naïve, activated or memory CD4^{+} T cells[15], and Foxp3^{-}CD4^{+} T cells from the intestines or MLN of uninfected mice do not express *Ebi3*/*Il12a* (data not shown) raising the possibility that iT_R35 are being generated by T_{regs} within this inflammatory microenvironment.

The inflammation induced by solid tumors is known to attract T_{regs}[32–37]. Using B16 melanoma and MC38 colorectal adenocarcinoma as model systems[25, 38, 39], tumor cells were inoculated into *Foxp*^{gfp} mice, solid tumors resected 15–17 days (for B16) or 12 days (for MC38) post-transfer and Foxp3^{+} and Foxp3^{-} T cells purified from spleens and tumors. Interestingly, tumor-infiltrating Foxp3^{+} T_{regs} had substantially increased expression of both *Ebi3* and *Il12a* (Fig. 6c-f). Surprisingly, tumor-infiltrating Foxp3^{-} T cells also dramatically upregulated *Ebi3* and *Il12a* expression. It is noteworthy that comparable observations were made in two distinct tumor types. We further analyzed IL-35 secretion and its physiological relevance using the B16 melanoma system. While a moderate amount of IL-35 secretion by splenic Foxp3^{+} T_{regs} was observed *ex vivo*, a substantial and comparable amount of IL-35 was secreted by both Foxp3^{+} T_{regs} and Foxp3^{-}CD4^{+} tumor-infiltrating T cells (Fig. 6e). Lastly, we assessed whether tumor-infiltrating CD4^{+}/Foxp3^{-}/*Ebi3*$^{+}$/*Il12a*$^{+}$ T cells were able to suppress the proliferation of fresh responder T_{conv} *in vitro*. Although their suppressive capacity was not as potent as that of tumor-infiltrating Foxp3^{+} T cells, our results clearly demonstrate that tumor-derived Foxp3^{-}CD4^{+} T cells can mediate effective suppression *in vitro* in an IL-35-dependent manner (Fig. 6f).

iT_R35 contribute to the regulatory milieu *in vivo*

Finally, we assessed the physiological contribution of iT_R35 to the T_{reg}-induced regulatory milieu. We reasoned that if iT_R35 development within the tumor played a significant role in blocking anti-tumor immunity, then mice reconstituted with T_{conv} cells that lacked the ability to be converted into iT_R35 would develop smaller tumors. Therefore, *Rag1*$^{-/-}$ mice were reconstituted with wild type CD8 cells, with or without wild type T_{regs} plus either wild type or *Ebi3*$^{-/-}$ CD4^{+} T_{conv} cells. As expected, tumor size was reduced in CD4^{+}/CD8^{+} T cell recipients lacking T_{regs} (50–90mm^3) regardless of whether wild type or *Ebi3*$^{-/-}$ CD4 T_{conv} cells were transferred. Co-transfer of nT_{regs} with wild type CD4^{+}/CD8^{+} T cells blocked anti-tumor immunity resulting in aggressive tumor growth (470mm^3) (Fig. 7). Analysis of congenically-marked tumor-infiltrating CD4^{+} T cells confirmed high *Ebi3*/*Il12a* expression that was comparable to the T_{regs} (see Supplementary Information and Supplementary Fig. 17). Thus it should be noted that in this instance both IL-35-producing nT_{regs} and iT_R35 contribute to the suppressive milieu (Fig. 6, 7 and Supplementary Fig. 17). Strikingly, co-transfer of nT_{regs} and CD8^{+} T cells with *Ebi3*$^{-/-}$ CD4^{+} T_{conv} (which are unable to be converted to IL-35-producing iT_R35) only partially blocked anti-tumor immunity resulting in intermediate tumor growth (220mm^3). These results suggest that T_{reg}-mediated induction of iT_R35 development has a significant impact on tumor burden and is responsible for approximately half the regulatory milieu within the tumor microenvironment (as the tumor burden is reduced from 470mm^3 to 220mm^3). Furthermore, these data suggest that nT_{regs} mediate their suppressive activity, in part, by inducing iT_R35.

DISCUSSION

iT_R35 cells represent a new and unique member of the regulatory T cell family that are generated by, and mediate their suppression via, IL-35. They do not express Foxp3 and are quite distinct from the currently known induced regulatory populations, TGFβ iT_R and IL-10 iT_R (see Supplementary Information and Supplementary Fig. 18). The concept of infectious

tolerance, whereby T_{reg} confer a suppressive phenotype upon T_{conv} cells, has been previously described in both murine and human systems[27, 29]. It has been suggested that TGFβ may play a critical role in mediating infectious tolerance[40]. Since IL-35-secreting T_{regs} can convert T_{conv} cells into iT_R35, a suppressed T_{conv} population with regulatory potential, this raises the possibility that IL-35 and iT_R35 may represent additional, important mediators of infectious tolerance. Indeed, our data suggest that up to half the regulatory microenvironment within the tumor is mediated by T_{reg}-induced iT_R35. This also suggests that iT_R35 may contribute to tumor progression. While iT_R35 may play a significant physiological contribution to the regulatory milieu established by T_{reg}, further studies will be required to fully elucidate their contribution in diverse disease settings.

Whether a similar system operates in humans remains to be determined, but may be controversial given recent studies suggesting that human T_{regs} do not make IL-35[41, 42], findings that await further confirmation. Nevertheless, human iT_R35 can be generated and can suppress primary human T cell proliferation in an IL-35-dependent manner. The potential therapeutic application of *ex vivo* generated IL-10 iT_R and TGFβ iT_R is complicated by complexities in their generation, their short half-life and reversal of their suppressive capacity in time or by IL-2 (see Supplementary Information)[13, 43, 44]. Although additional experiments are needed to fully assess the clinical potential of iT_R35, our data suggest that they represent a new, stable iT_R population that may have significant therapeutic utility.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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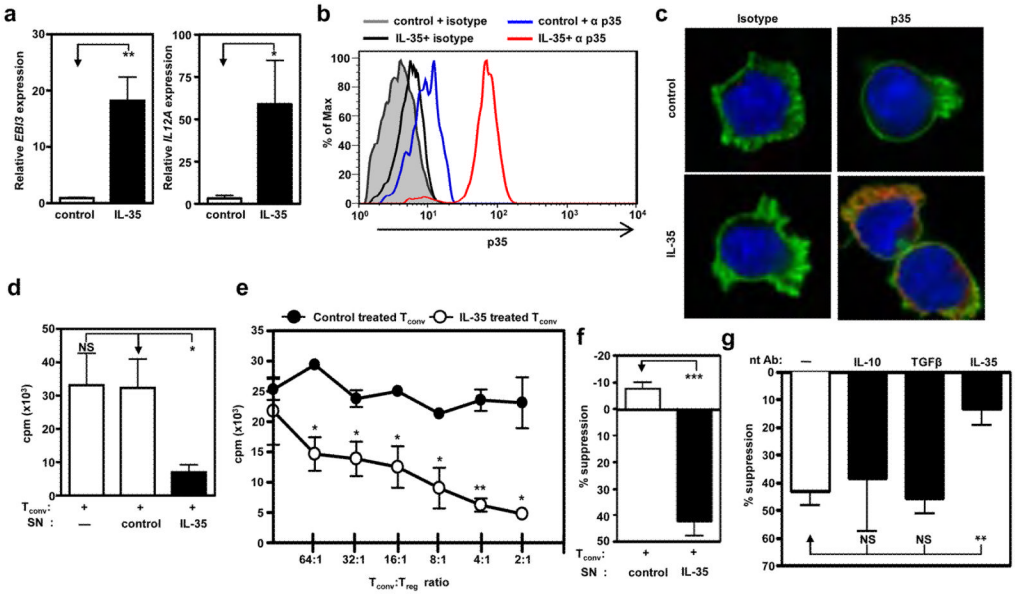

Figure 1. Human IL-35 treatment of T_{conv} induces autocrine IL-35 expression and confers a regulatory phenotype

T_{conv} purified by FACS from cord blood were treated with IL-35 or control at 25% of total culture volume for 9 days during activation (αCD3/CD28, and IL-2). (a) RNA was extracted, cDNA generated and qPCR performed. Relative *Ebi3* (left panel) and *Il12a* (right panel) mRNA expression. (b) Cytokine treated cells were re-purified and stained with an isotype or a p35-specific antibody following 4h activation with PMA and ionomycin. Intracellular staining of IL-35 was determined by FACS. (c) Microscopic analysis of p35 expression was similarly determined following 4h activation with PMA and ionomycin. Anti- p35 or isotype control antibodies (shown in red), phalloidin (shown in green) and DAPI (shown in blue). (d) Proliferation of cytokine treated cells was determined by [^{3}H]-thymidine incorporation (e) T_{conv} cells were mixed at indicated ratios (T_{conv}: suppressor) with control or IL-35 treated T_{conv}, hIL-2 and anti-CD3- + anti-CD28-coated latex beads for 9 days. Proliferation was determined by [^{3}H]-thymidine incorporation. The mean of 4 representative experiments with similar cpm is shown. (f) Control or IL-35 treated T_{conv} were cultured in the top chambers of a Transwell™ culture plate as indicated. Freshly purified responder T_{conv} were cultured in the bottom chamber of the 96-well flat bottom plates in medium containing hIL-2 and anti-CD3- + anti-CD28-coated latex beads. Top chambers were removed and [^{3}H]-thymidine was added directly to the responder T_{conv} cells in the bottom chambers of the original Transwell™ plate for the final 8 h of the 9 day assay. (g) T_{conv} cells were activated in the presence of IL-35 at 25% of total culture volume. Following conversion with cytokines, suppression assays were supplemented with neutralizing IL-10, TGFβ, or IL-35 to assess their requirement for indicated cytokines to mediate suppression. Counts per minute of T_{conv} cells activated alone, in the absence of control or IL-35, were 20,000–125,000 (f) and 25,000 – 570,000 (g). Data represent the mean ± SEM of (a)10 (b) 4, (c-g) independent experiments [* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, NS = not significant].

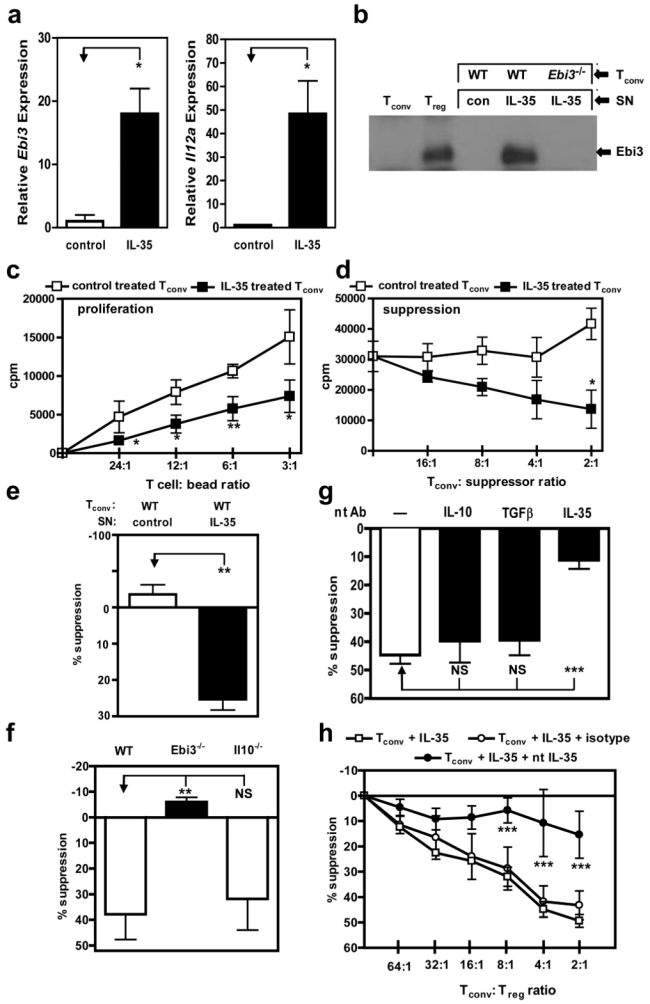

Figure 2. Murine IL-35 treatment of T_{conv} converts cells to an IL-35 producing suppressive population

T_{conv} purified by FACS from C57BL/6, *Ebi3*$^{-/-}$ or *Il10*$^{-/-}$ mice were treated with indicated cytokines for 72 h during activation (αCD3/CD28). (a) RNA was extracted and cDNA generated from T_{conv} following control or IL-35 treatment. Relative *Ebi3* (left panel) and *Il12a* (right panel) mRNA expression. (b) T_{conv} cells were cultured with control protein or IL-35 for 72 h. Cells were re-purified and cultured for an additional 24 h to facilitate IL-35 secretion. Culture supernatants from indicated cultures, or T_{conv} and T_{reg} as control, were immunoprecipitated with anti-p35 mAb, resolved by SDS-PAGE and probed with anti-Ebi3 mAb to identify IL-35 secretion. (c) Proliferative capacity, determined by [^{3}H]-thymidine incorporation, of T_{conv} treated with indicated cytokines for 72 h. (d) T_{conv} cells were mixed at indicated ratios (T_{conv}: suppressor) with control or IL-35 treated T_{conv} and anti-CD3- + anti-CD28-coated latex beads for 72 h. Proliferation was determined by [^{3}H]-thymidine incorporation (e) Control or IL-35 treated T_{conv} were cultured in the top chambers of a Transwell™ culture plate as indicated. Freshly purified wild-type responder T_{conv} were cultured in the bottom chamber of the 96-well flat bottom plates in medium containing anti-CD3- + anti-CD28-coated latex beads. After 60 h in culture, top chambers were removed and [^{3}H]-thymidine was added directly to the responder T_{conv} cells in the bottom chambers of the original Transwell™ plate for the final 8 h of the 72 h assay. (f) T_{conv} from C57BL/6, *Ebi3*$^{-/-}$ or *Il10*$^{-/-}$ mice were activated in the presence of IL-35 at 25% of total culture

volume, for 72 h to generate suppressive cells. Cells were re-purified and mixed at 4:1 ratio (T_{conv}: suppressor) and proliferation was determined. (g) Wild-type T_{conv} cells were activated in the presence of IL-35 at 25% of total culture volume. Following conversion with cytokines, suppression assays were supplemented with neutralizing IL-10, TGFβ, or IL-35 to assess their requirement for indicated cytokines to mediate suppression. Cells were cultured at a 4:1 ratio in suppression assays as described in e. (h) Suppressive capacity of IL-35 treated T_{conv} cells supplemented with titrations of isotype control or neutralizing IL-35 mAbs. Counts per minute of T_{conv} cells activated alone were 21,000–64,000 (e-h). Data represent the mean ± SEM of 4–8 independent experiments [* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, NS = not significant].

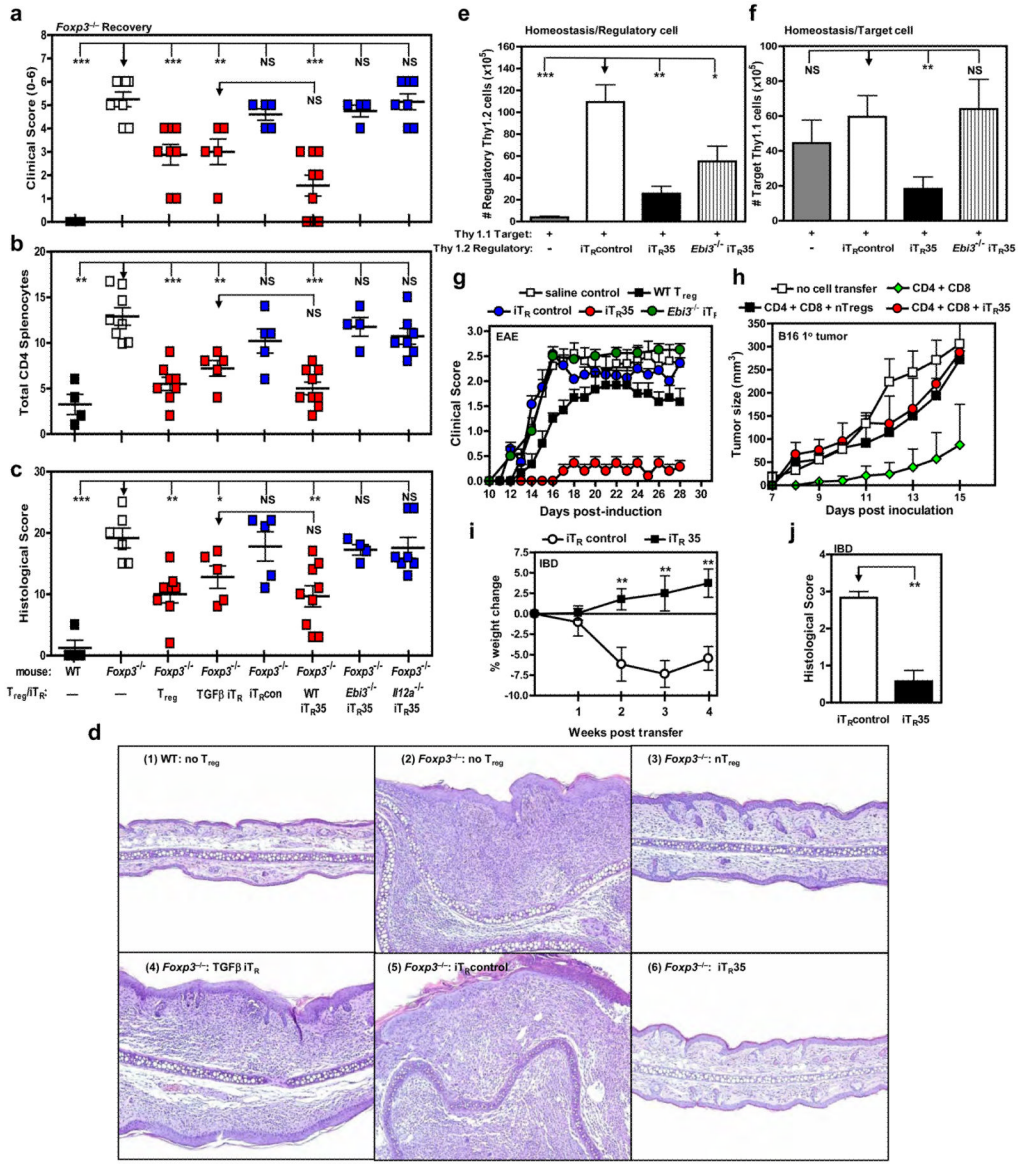

Figure 3. iT_R35 are suppressive *in vivo*

Control treated (iT_Rcontrol), IL-35 treated (iT_R35) or TGFβ treated (TGFβ iT_R) cells were generated from FACS purified T_{conv} from wild type C57BL/6 or *Ebi3*$^{-/-}$ mice. (a-c) Cells were injected i.p. into 2–3 day old *Foxp3*$^{-/-}$ mice. Recovery from disease was monitored and reported by reduction in (a) clinical score (b) splenic CD4$^+$ T cell number as determined by flow cytometric analysis and (c) combined histological score from lungs, liver and skin (d) Representative histopathology images of ear pinna following T cell transfer into *Foxp3*$^{-/-}$ mice. Panel 1: Wild type mouse receiving no T_{regs} is normal and has no inflammatory cells in the dermis. Panel 2: *Foxp3*$^{-/-}$ mouse receiving no T_{regs} is thickened with a dense inflammatory cell infiltrate causing distortion of the cartilage. Panel 3: *Foxp3*$^{-/-}$ mouse receiving nT_{regs} is mildly thickened with mild edema and a sparse inflammatory cell infiltrate. Panel 4: *Foxp3*$^{-/-}$ mouse receiving TGFβ iT_R cells is moderately thickened with inflammatory cells separated by edema fluid. Panel 5: *Foxp3*$^{-/-}$ mouse receiving iT_Rcontrol cells is markedly thickened with edema dispersed inflammatory cells causing distortion of the cartilage. Panel 6: *Foxp3*$^{-/-}$ mouse receiving iT_R35 cells is

mildly thickened with edema and a mild inflammatory cell infiltrate. (e-j) iT_Rcontrol or iT_R35 were generated from FACS purified T_{conv} from C57BL/6 or *Ebi3*$^{-/-}$ (Thy1.2) or B6.PL (Thy1.1) mice. (e) Homeostatic expansion was monitored by i.v. injection of Thy1.1$^+$ T_{conv} cells alone or with Thy1.2$^+$ iT_Rcontrol or iT_R35 cells (as regulatory cells) into *Rag1*$^{-/-}$ mice. Seven days after transfer, splenic T cell numbers were determined by flow cytometry. Thy1.2$^+$ regulatory T cell numbers (e). Thy1.1$^+$ target T_{conv} cell numbers (f). (g) EAE was induced by immunizing mice with MOG35–55 peptide in complete Freund's adjuvant followed by pertussis toxin administration. iT_Rcontrol, iT_R35 or nT_{reg} (10^6) were transferred i.v. into C57BL/6 mice 12–18 hours prior to disease induction. Clinical disease was monitored daily. (h) *Rag1*$^{-/-}$ mice received indicated cells via the tail vein on day –1 of experiment. On day 0, all were injected with 120,000 B16 cells i.d. in the right flank. Tumor diameter was measured daily for 15 days and is reported as mm^3. (i-j) IBD was induced by injecting *Rag1*$^{-/-}$ mice with T_{conv} cells via the tail vein. After 3–4 weeks, mice developed clinical symptoms of IBD and were given iT_Rcontrol or iT_R35 cells. Percentage weight change after iT_Rcontrol or iT_R35 cell transfer (i). (j) Colonic histology scores of experimental mice. Data represent the mean ± SEM of 8–12 mice per group from at least 2 independent experiments [* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, NS = not significant].

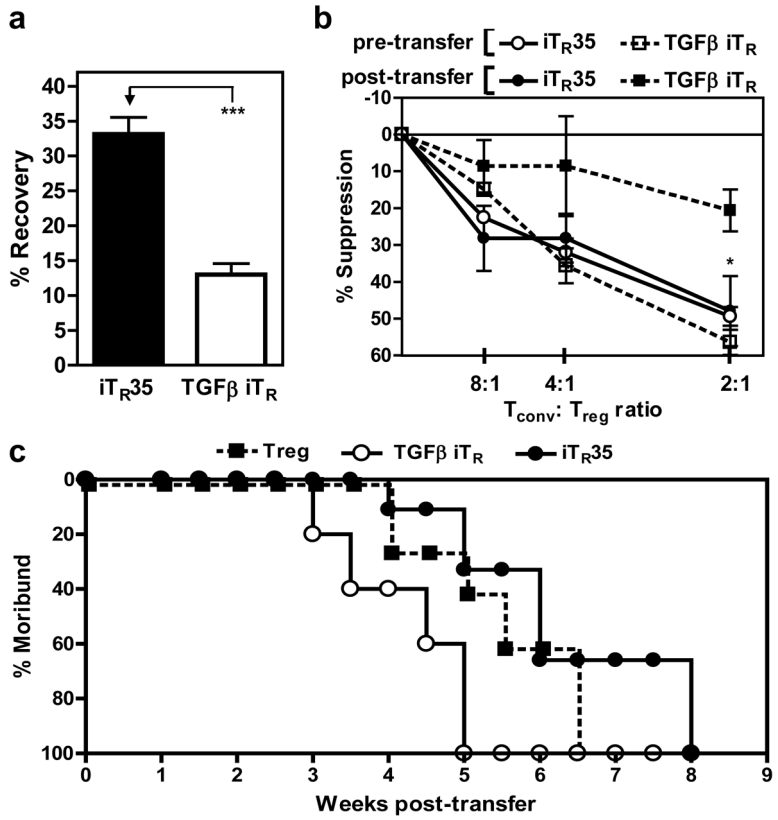

Figure 4. Comparative stability of iT_R35 and TGFβ iT_R *in vivo*

iT_R35 or TGFβ iT_R were generated *in vitro* with $CD45.2^+$ T_{conv} cells and adoptively transferred into $CD45.1^+$ C57BL/6 mice to assess stability. (a) Splenic iT_R cell number was determined by flow cytometric analysis of $CD45.2^+$ cells. Percentage of total cells injected that were recovered 25 days post-transfer. (b) T_{conv} cells were mixed at indicated ratios (T_{conv}: suppressor) with either freshly generated iT_R cells (pre-transfer) or iT_R cells recovered after *in vivo* resting (post-transfer) and anti-CD3- + anti-CD28-coated latex beads for 72 h. Proliferation was determined by $[^3H]$-thymidine incorporation. (c) Natural T_{regs} or iT_R were injected into 2–3 day old *Foxp3*$^{-/-}$ mice. Mice were monitored for clinical signs of sickness and scored accordingly. Mice obtaining a clinical score of 4 were considered moribund. Survival, based on longevity of mice, was significantly less in recipients of TGFβ iT_R when compared to nT_{regs}. Conversely, mice that received iT_R35 survived significantly longer than those that received nT_{regs} ($p < 0.005$). Counts per minute of T_{conv} cells activated alone were 18,000–44,000 (b). Data represent the mean ± SEM of 5–12 mice per group from at least 2 independent experiments [* $p < 0.05$, *** $p < 0.005$].

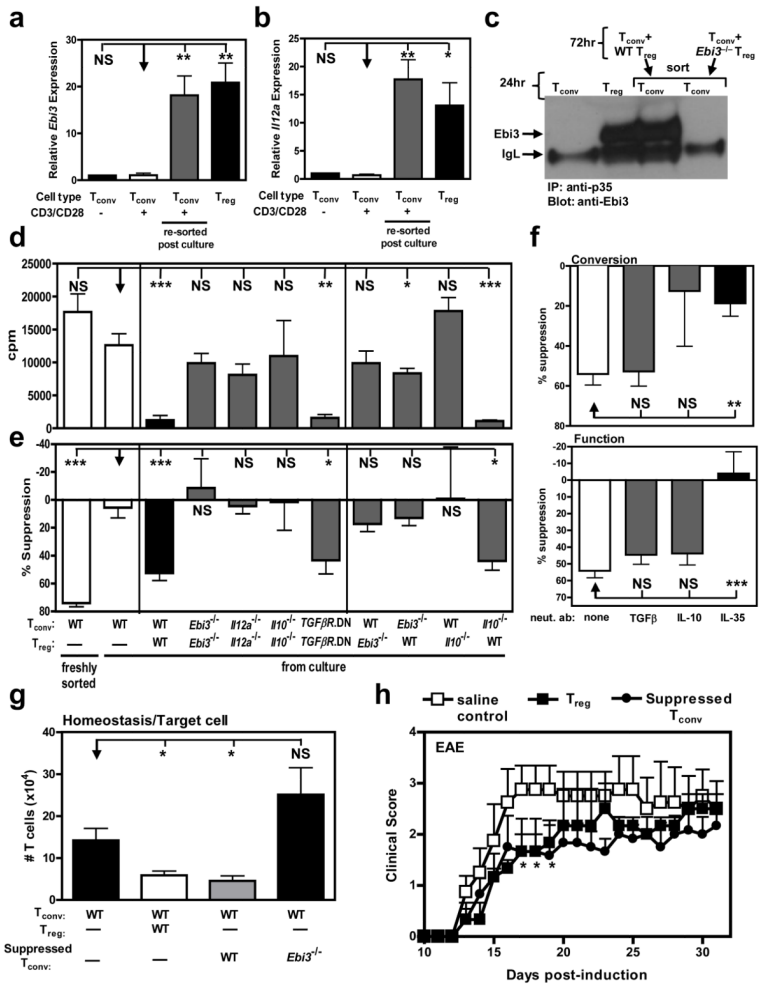

Figure 5. T_{regs} generate iT_R35 in an IL-35- and IL-10-dependent manner

T_{conv} were activated in the presence of T_{reg} at a 4:1 ratio (responder: suppressor) for 72 h. (a) RNA was extracted and cDNA generated from resting or activated T_{conv} cells or from T_{conv}:T_{reg} co-cultures (resorted based on differential Thy1 markers). *Ebi3* (a) and *Il12a* (b) expression of the populations indicated. (c) Following co-culture, suppressed T_{conv} were re-purified and cultured for an additional 24 h. Secretion of IL-35 was determined by IP/WB of culture supernatants and compared to that of freshly cultured T_{conv} and T_{reg}. (d) Following co-culture, suppressed T_{conv} were re-purified and activated (αCD3/CD28). Proliferative capacity was assayed by [^{3}H]-thymidine incorporation. (e) The suppressive capacity of suppressed T_{conv} upon fresh responder T_{conv} cells was determined by [^{3}H]-thymidine incorporation. (f) Anti-IL-10, anti-TGFβ, or anti-IL-35 neutralizing antibodies were added to co-cultures to inhibit cytokine driven "conversion" into suppressed T_{conv} (top panel) or added in secondary proliferation assays to inhibit cytokine driven suppression of "function" (bottom panel). (g) T_{conv} cells alone or with C57BL/6, *Ebi3*$^{-/-}$ suppressed T_{conv} (as regulatory cells) were injected into *Rag1*$^{-/-}$ mice. Seven days after transfer, splenic T cell numbers were determined by flow cytometry. (h) EAE was induced by immunizing mice with MOG35–55 peptide in complete Freund's adjuvant followed by pertussis toxin administration. 1×10^6 suppressed T_{conv} or natural T_{reg} were transferred i.v. into C57BL/6 mice 12–18 hours prior to disease induction. Clinical disease was monitored daily. Clinical score was statistically significant between mice receiving saline control and T_{reg} or

suppressed T_{conv} at days 16–18. Counts per minute of T_{conv} cells activated alone were 14,000–34,000 (e, f). Data represent the mean ± SEM of 8–12 mice per group from at least 2 independent experiments [* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, NS = not significant].

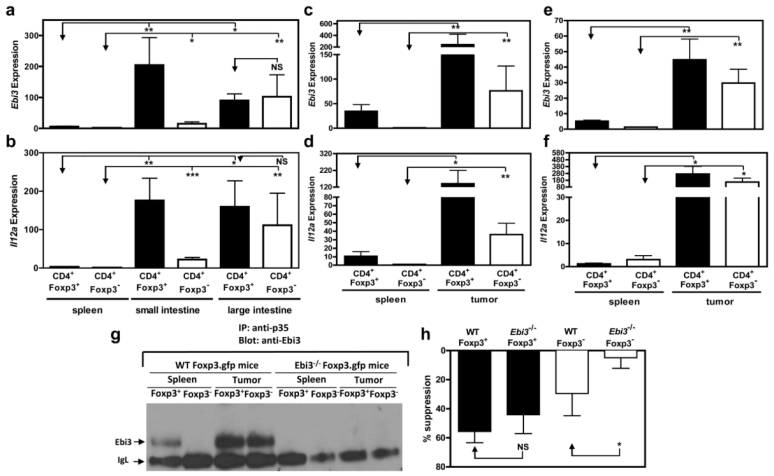

Figure 6. IL-35-producing Foxp3^{-} iT$_{R}$35 develop *in vivo*

Foxp3^{gfp} mice were infected with *Trichuris muris*. CD4^{+}Foxp3^{-} and CD4^{+}Foxp3^{+} cells were purified from spleen, small intestine IEL and LPL, or large intestine IEL and LPL by FACS, RNA extracted and cDNA generated. *Ebi3* (a) and *Il12a* (b) expression of the populations indicated. (c, d, g, h) *Foxp3*^{gfp} mice or *Ebi3*$^{-/-}$ *Foxp3*^{gfp} were injected with 120,000 B16 cells i.d. on the right flank. Tumors and spleens were excised after 15–17 days, CD4^{+}Foxp3^{-} and CD4^{+}Foxp3^{+} cells, purified by FACS, RNA extracted and cDNA generated. *Ebi3* (c) and *Il12a* (d) expression of the populations indicated. (e, f) *Foxp3*^{gfp} mice or *Ebi3*$^{-/-}$ *Foxp3*^{gfp} were injected with 2×10^{6} MC38 cells subcutaneously on the right flank. Tumors and spleens were excised after 12 days, CD4^{+}Foxp3^{-} and CD4^{+}Foxp3^{+} cells purified by FACS, RNA extracted and cDNA generated. *Ebi3* (e) and *Il12a* (f) expression of the populations indicated. (g) Following B16 cell inoculation, purified T cells from the spleen or tumor were cultured for 24 h to allow secretion of IL-35. Culture supernatants from indicated cultures were immunoprecipitated with anti-p35 mAb, resolved by SDS-PAGE and probed with anti-Ebi3 mAb to identify IL-35 secretion. No IL-35 secretion was seen in either the splenic or tumor-infiltrating lymphocytes from *Ebi3*$^{-/-}$ mice (g). Purified cells were assayed for regulatory capacity by mixing populations indicated at a 4:1 ratio with fresh responder T$_{conv}$ cells for 72 h. Proliferation was determined by [^{3}H]-thymidine incorporation. Counts per minute of T$_{conv}$ cells activated alone were 16,000–33,000 (h). Data represent the mean ± SEM of 8–10 mice per group from 2–3 independent experiments (B16) and 1 experiment (MC38) [* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, NS = not significant].

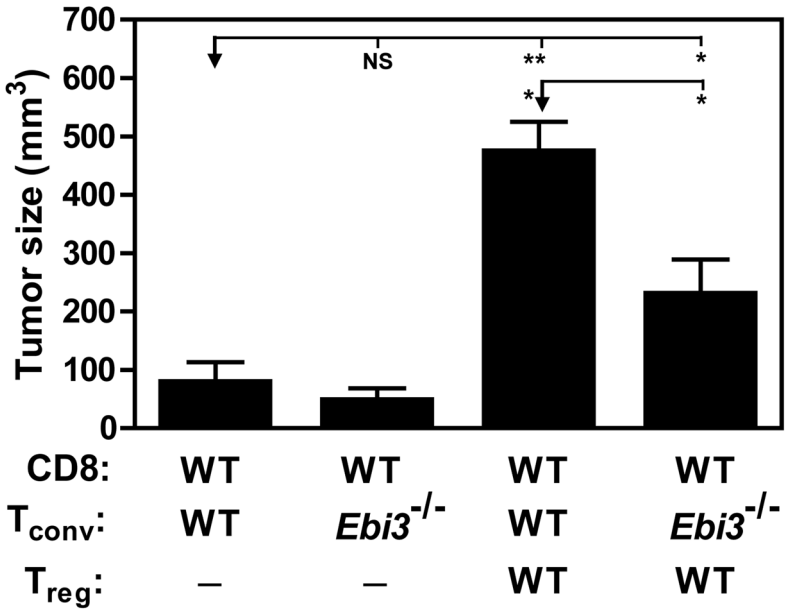

Figure 7. The suppressive T cell milieu in the tumor microenvironment is largely due to iT_R35

Rag1$^{-/-}$ mice were reconstituted with wild type C57BL/6 CD8$^+$ T cells and wild type or *Ebi3*$^{-/-}$ T_{conv} cells with or without wild type T_{regs}. The following day, all mice were injected with 120,000 B16 cells i.d. on the right flank. Tumors were excised on day 15 post-inoculation to facilitate analysis of tumor infiltrating lymphocytes and tumor diameter reported as mm^3. Data represent the mean ± SEM of 6–12 mice per group from at least 2 independent experiments [* $p < 0.05$, *** $p < 0.005$, NS = not significant].