frontiers in Immunolog

anergy, generation of Treg cells and production of IL-10 and transforming growth factor (TGF)- (4, 11–13). In addition, immature DCs can support Treg cell di erentiation in vivo through presentation of low levels of antigen in major histocompatibility complex (MHC)-II (14–16). erapeutic strategies that augment numbers and/or function of Treg cells, immature DCs, or both, represent a way to enhance mucosal tolerance by limiting T cell activation.

e Escherichia colheat-labile enterotoxin is a hetero-oligomeric AB_5 toxin composed of a toxic enzymatic A subunit and ve identical non-toxic B subunits (EtxB) (17). In the context of infection, the B subunit mediates cellular entry of the A subunit into the cytoplasm by binding to GM1 ganglioside receptor, which is ubiquitously expressed by all somatic cells (18). Several studies haerajor hi).026 Tw (m)4(e26 Tw (m)4(e2631A1opl s)-8-8.9(co)12(m)]TJ 0(io)121 0 0)-4EtxB) (

gentle pipetting to disrupt tissue. Lung lymphocytes were isolated by nely mincing the lung tissues and digesting with 2 mg/ml

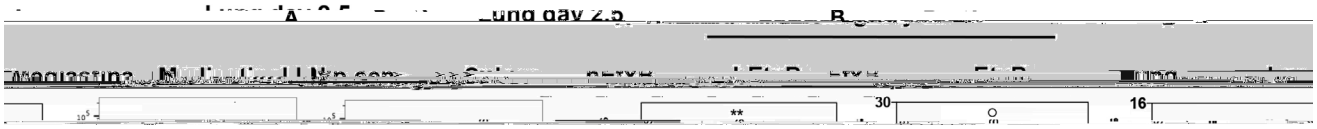

FIGURE 1 | Intranasal treatment of EtxB increases the proliferation of Treg cells. C57BL/6 mice were administered, EtxB (100 g/mouse), heat inactivated EtxB (hEtxB, 100 g/mouse) or PBS on three consecutive days. On day 2.5 following the last dose, spleen, mediastinal lymph node, and lung were removed and analyzed by ow cytometry. Flow cytometric contour plots (A) and dot plots (B) show the frequency of Foxp3regulatory T (Treg) cells in CD4B220 cells. The frequency of Ki67+ proliferating Treg cells or CD4 Foxp3 T cells (C,D) and frequency of live (Annexin VDAPI) CD4+ CD25+ cells (E,F) in the lung. Results are from

status and/or suppressive capacity of Treg cells. CTLA-4 is a key

FIGURE 2 | Continued
EtxB increases the suppressive function of Treg cells. Expression of CTLA-4 (A,B) or Neuropilin-1 (C,D) on lung B220 CD4+ Foxp3+ Treg cells from C57BL/6 mice treated intranasally with 100 g/mouse EtxB or heat-EtxB (hEtxB) for three consecutive days, and analyzed by ow cytometry at 2.5 days after the nal treatment. (E,F) Intranasal administration of 100 g/mouse EtxB or hEtxB to ITIB mice. Day 2.5 posttreatment, lungs were removed and analyzed by ow cytometry for the proportion of IL-10+ (CCF-4 blue) in B220 CD4+ Foxp3+ Treg cells. (G,H) Groups of 10 C57BL/6 mice were treated intranasally with 100 g/mouse EtxB or hEtxB for three consecutive days. On day 2.5 posttreatment, lungs were removed and CD4+CD25+ Treg cells were ow sorted and were co-cultured with splenic CD4+ CD25

FIGURE 3 | EtxB does not alter mature dendritic cell (DC) subsets. C57BL/6 mice were treated intranasally with 100 g/mouse EtxB or heat-EtxB (hEtxB) for 3 days. On day 2.5 posttreatment, lung and mediastinal lymph node (mLN) were removed and analyzed, ow cytometric contour plots (A) and dot plots show the proportion of DCs subsets in the lung (B) and mLN (C). Subsets of DC include plasmacytoid DC (pDC, CD11c^{low}CD11b^{-} CD8^{-} MHC-II^{+}), CD8^{+} and CD8^{+} conventional DCs (CD8^{+} or CD8^{-} cDC, CD11c^{high}CD11b^{+}CD8$^{-/+}$ MHC-II^{+}). Results are from one experiment representative of six mice in each group from three independent experiments In dot plots, the mean and SD are represented and each circle represents one mouse: white dots hEtxB-treated animals, black dots EtxB-treated animals.

FIGURE 4 | Continued

FIG

In this study, we have addressed the question of how administration of EtxB induces an immunoregulatory microenvironment in the lung. Our results suggest that EtxB is able to promote Treg cells and "immature" DCs. In both cases, there is a marked increase in IL-10 production. Interestingly, IL-10 is able to support the induction of both immature DCs and Treg cells (59). Previous studies have shown increased expression of Il10 transcript in epithelial cells and CD11b cells following EtxB treatment (25,26). Taken together, IL-10 production a er EtxB administration is likely one of the key mechanisms supporting the increase in Treg cells and immature DCs. Interestingly, ERK1/2 pathway is one of the signaling cascades that is activated in macrophages and DCs that results in IL-10 expression (60). Of note, Polumuri et al shown that TLR4 engagement in murine innate cells activates the PI3-kinase/Akt pathway and promotes IL-10 production that is reversed by PI3-kinase inhibition (61). Because EtxB induces PI3-kinase and MAPK/ERK kinase signaling cascades in B cells, it would be interesting to assess the potential link between theses pathways and IL-10 production in Treg and DCs following EtxB administration. Also, how EtxB directly or indirectly promotes Treg cells and immature DCs able to produce IL-10 warrants further study.

Taken together, our study demonstrates that EtxB exerts its e ects in vivo mainly at mucosal surfaces. It limits T cell activation through two mechanisms: rst through increasing "immature" IL-10$^+$ DCs that cannot activate T cells and second through increasing the proportion and function of Treg cells that limit

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