CORRESPONDENCE

A serine protease inhibitor induces type 1 regulatory T cells through IFN-γ/STAT1 signaling

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Type 1 regulatory T cells ($CD4^+Foxp3^-LAG3^+CD49b^+$), or Tr1 cells, have a remarkable capacity to suppress autoimmune inflammation through IL-10.[1] Although the signals that lead to Tr1-cell differentiation are not completely elucidated, it is known that IL-27 plays a major role in this phenomenon.[2] However, committed inflammatory Th17 cells are resistant to IL-27,[3] making their effectiveness in the clinic limited. Thus, there is an unmet need to identify new drugs that stimulate Tr1-cell development. We have shown that a soybean-derived serine protease inhibitor, Bowman-Birk inhibitor (BBI), dramatically suppressed experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis (MS), by inducing IL-10.[4] However, the immunomodulatory mechanism by which BBI induces IL-10 and suppresses EAE has not been elucidated.

We first investigated whether BBI could induce IL-10 at similar levels as IL-27 by activating $CD4^+$ T cells in the presence or absence of these compounds and measuring IL-10 levels in culture supernatants. We found that both BBI and IL-27 increased IL-10 levels similarly (Fig. 1a). Because IL-10 is closely associated with $Foxp3^+$ regulatory T cells, we analyzed Foxp3 expression in cells activated with BBI and found that BBI did not induce Foxp3 expression in $IL\text{-}10^+CD4^+$ T cells; however, it did induce T-bet and IFN-γ expression (Fig. 1b). Th1 cells that produce IL-10 have been previously described,[5] and coexpression of LAG3 and CD49b identifies Tr1 cells and distinguishes them from Th1 cells in humans and mice.[1,6] We found that BBI significantly increased the coexpression of LAG3 and CD49b on total $CD4^+$ T cells and in $IL\text{-}10^+CD4^+$ T cells (Fig. 1c). Interestingly, pretreatment with BBI significantly reduced the pathogenicity of $MOG_{35\text{-}55}$-reactive cells (Fig. 1d) by inducing $Foxp3^-LAG3^+CD49b^+$ Tr1 cells (not shown). These findings show that BBI induces the development of $IL\text{-}10^+CD4^+$ T cells with a phenotype consistent with that of Tr1 cells.

Although IL-27 potently induces Tr1 cells,[2] we found that BBI induced IL-10 and IFN-γ production in WT and $IL\text{-}27R^{-/-}$ $CD4^+$ T cells (Fig. 1e). BBI did not increase the expression of IL-27 subunits (p28 and EBI-3) (data not shown). These data show that BBI induces IL-10 in an IL-27-independent manner. Additionally, Stat-1 is downstream of IL-27R signaling and induces IL-10- and Tr1-cell differentiation.[2] Moreover, it is known that $T\text{-bet}^{-/-}$ and $Stat\text{-}1^{-/-}$ cells produce more IL-10 than WT cells under homeostatic conditions.[7,8] We found that BBI failed to upregulate IL-10 production in $Stat\text{-}1^{-/-}$ cells (Fig. 1f). In $T\text{-bet}^{-/-}$ cells, BBI induced IL-10 but at significantly lower levels than in WT cells (Fig. 1f).

BBI increased IFN-γ production, and the lack of Stat-1 abrogated the IFN-γ and IL-10 production stimulated by BBI (Fig. 1f). To address whether IFN-γ production is upstream of IL-10 production, $CD4^+$ T cells from WT and $IFN\text{-}\gamma^{-/-}$ mice were stimulated with or without BBI, and IL-10 production was assessed. We found that, while WT $CD4^+$ T cells had increased IL-10 levels, this effect was hampered in $IFN\text{-}\gamma^{-/-}$ $CD4^+$ T cells (not shown). We then examined whether IFN-γ plays a role in the therapeutic effect of BBI in WT and $IFN\text{-}\gamma^{-/-}$ EAE-mice-fed BBI. The lack of IFN-γ significantly decreased the therapeutic effect of BBI in treated groups (Fig. 1g). Moreover, BBI increased $IL\text{-}10^+CD4^+$ T cells among the CNS-infiltrating cells in WT but not in $IFN\text{-}\gamma^{-/-}$ mice (Fig. 1h). Together, these findings show that IFN-γ and Stat1 are fundamental signals in Tr1-cell differentiation and in BBI-induced EAE suppression.

To verify whether human cells can phenocopy the effect of BBI treatment we observed in the mouse system, peripheral blood mononuclear cells (PBMCs) from healthy donors were collected and cultured in the presence or absence of BBI. Our results showed that BBI induced a significant increase in $LAG3^+CD49b^+CD4^+$ T cells compared to PBS treatment (Fig. 1i). Moreover, we measured IL-10 production by BBI-treated PBMCs obtained from healthy donors and untreated MS patients. We found that BBI significantly increased IL-10 production by cells from healthy donors and untreated MS patients (Fig. 1j). Collectively, our results show that Tr1 cells are efficiently induced by BBI in an IFN-γ/Stat-1-dependent manner. Moreover, cells from untreated MS patients produced high amounts of IL-10 after BBI treatment, suggesting that BBI may be effective in suppressing chronic inflammation in MS and other autoimmune diseases.

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ADDITIONAL INFORMATION

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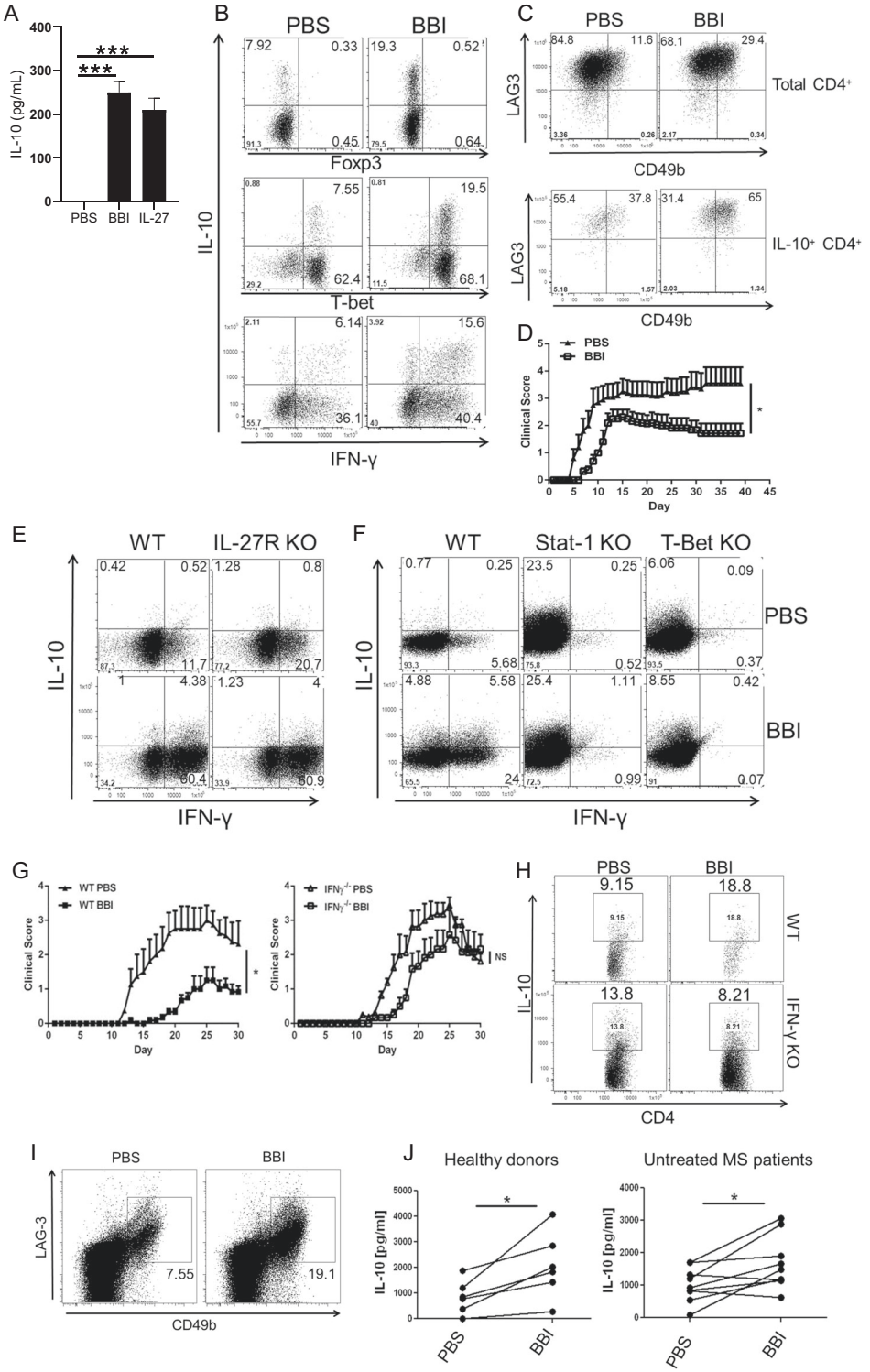

Fig. 1 BBI induces Tr1 cells. a ELISA of IL-10 production by purified $CD4^+$ T cells stimulated with anti-CD3/CD28 (1 µg/mL each), plus PBS, BBI (0.5 mg/mL), or IL-27 (10 ng/mL). One-way ANOVA with Bonferroni post-test where values of $p < 0.001$ were considered significant (***). **b** Flow cytometry of cells stimulated as in **a**, then restimulated with PMA (50 ng/mL), ionomycin (500 ng/mL), and GolgiPlug (1 µg/mL) for 4 h. **c** Cells were stimulated as in **a**, and the expression of CD49b and LAG3 in total $CD4^+$ T cells and in IL-10^+CD4^+ T cells was analyzed by flow cytometry. **d** Cells from the lymph nodes of MOG_{35-55}-immunized C57BL/6 mice were reactivated in the presence of IL-12 (10 ng/mL) plus PBS or BBI (0.5 mg/ml) for 72 h before adoptive transfer to naïve mice. The area under the curve was used to calculate p-values (*$p < 0.05$). **e**, **f** Cells were stimulated as in **a**, and the expression of IL-10 and IFN-γ in $CD4^+$ T cells was analyzed by flow cytometry. **g** WT and IFN-$\gamma^{-/-}$ mice were immunized with MOG_{35-55} and fed 3 mg/day BBI or PBS ($n = 5$/group) from the day of immunization until the end of the experiment. The area under the curve was used to calculate p-values (*$p < 0.05$). **h** EAE mice were euthanized, and cells from the CNS were isolated and analyzed by flow cytometry. **i** Flow cytometry of $CD4^+$ T cells from PBMCs of healthy donors after activation in the presence or absence of BBI. **j** ELISA of IL-10 production by PBMCs from healthy donors ($n = 6$) and untreated MS patients ($n = 7$) stimulated with PBS or BBI for 72 h. Paired *t*-test was used to calculate p-values. (*$p < 0.05$).

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