Poster No. NDM05

Inhibition of Bruton's tyrosine kinase prevents inflammatory macrophage differentiation: a potential role in multiple sclerosis

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INTRODUCTION

- Bruton's tyrosine kinase (BTK) mediates B-cell receptor (BCR) and Fc receptor (FcR) signaling (**Figure 1**).[1]

Figure 1. Evobrutinib dual mechanism of action

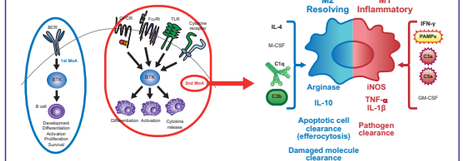


Figure adapted under terms of the Creative Commons Attribution License (CC BY 3.0) from Bohlson SS, et al. *Front Immunol* 2014;5:402.[2]

BCR, B-cell receptor; **BTK**, Bruton's tyrosine kinase; **FcεRI**, Fc receptor I; **GM-CSF**, granulocyte–macrophage colony-stimulating factor; **GPCR**, G-protein coupled receptor; **IFN**, interferon; **IL**, interleukin; **iNOS**, inducible nitric oxide synthase; **M1**, pro-inflammatory macrophage; **M2**, anti-inflammatory macrophage; **M-CSF**, macrophage colony-stimulating factor; **MoA**, mechanism of action; **PAMPs**, pathogen-associated molecular patterns; **TLR**, toll-like receptor; **TNF**, tumor necrosis factor.

- BTK inhibitors (BTKis) inhibit B cells and prevent innate immune activation via FcR, suggesting that they may be beneficial for treating autoimmune diseases involving B cells.
- BTK has also been implicated in signal mediation for certain chemokine and cytokine receptors.[3]
- The highly selective, irreversible BTKi evobrutinib inhibits disease development in an experimental autoimmune encephalomyelitis model that is not amenable to B-cell inhibition by anti-cluster of differentiation (CD)20 antibody, indicating that efficacy is mediated by effects beyond BCRs.
- Activated monocytes are abundant in multiple sclerosis (MS) lesions and play an important role in MS pathogenesis.[4]

OBJECTIVE

- The aim of this preclinical study was to investigate the effect of BTK inhibition (using evobrutinib and other BTK tool inhibitors) on the differentiation and activation of monocytes and macrophages, which may contribute to MS disease activity and progression, as a possible secondary mechanism of action of evobrutinib.

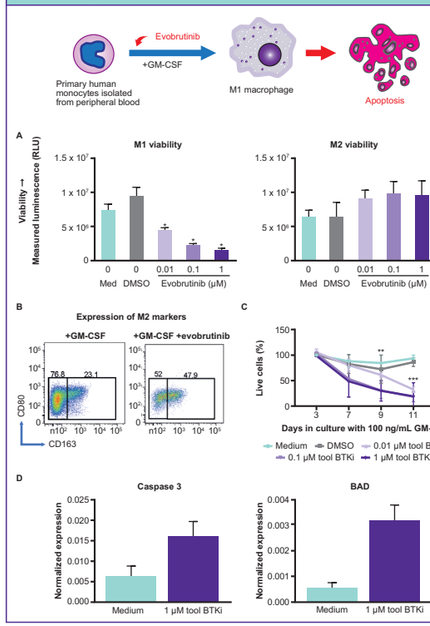
METHODS

- Monocytes were isolated from the peripheral blood of healthy volunteers.
- BTK activation was analyzed by Western blot after a 30-minute BTKi treatment followed by a granulocyte–macrophage colony-stimulating factor (GM-CSF, 100 ng/mL) stimulation time course.
- Survival of GM-CSF–differentiated pro-inflammatory (M1) macrophage cells was analyzed by flow cytometry following Annexin V/propidium iodide staining.
- Expression of interleukin (IL)-1β and IL-10 was determined by quantitative polymerase chain reaction following 48 hours of GM-CSF stimulation and BTKi treatment.
- Tumor necrosis factor (TNF)-α levels in cell culture supernatants were measured by enzyme-linked immunosorbent assay after overnight lipopolysaccharide (LPS) stimulation and BTKi treatment.
- Uptake of apoptotic neutrophils by anti-inflammatory (M2) macrophages (6 days of differentiation with 50 ng/mL macrophage colony-stimulating factor) after 30 minutes of coculture was analyzed by flow cytometry.

RESULTS

- BTK inhibition with evobrutinib triggers M1 macrophage apoptosis and skews their phenotype toward the M2 lineage (**Figure 2**).

Figure 2. Triggering M1 macrophage apoptosis through evobrutinib

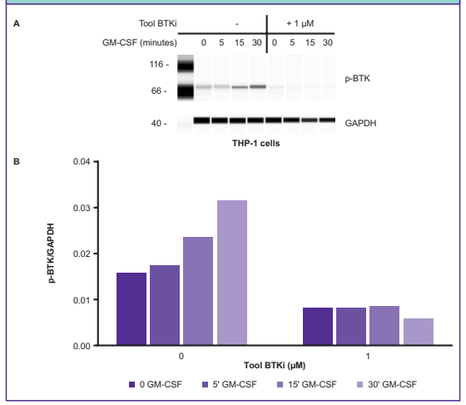


(A) Primary human monocytes were differentiated to M1 cells in the presence of 100 ng/mL GM-CSF and different concentrations of evobrutinib for 11 days in culture. Primary human monocytes were differentiated to M2 cells in the presence of 50 ng/mL M-CSF and evobrutinib for 6 days in culture. Cell survival was measured by luminescence-aided measurement of ATP in the supernatant (CellTiter-Glo by Promega, Madison, WI) at Day 11. Two-way ANOVA *p<0.05, compared with DMSO control.
(B) CD163 and CD80 expression on M1 cells was measured after 11 days of culture by flow cytometry. Cells were treated with 0.1 μM evobrutinib.
(C) Primary human monocytes were differentiated to M1 cells in the presence of 100 ng/mL GM-CSF and varying concentrations of a tool BTKi for 11 days in culture. Cells were harvested at different days during culture and cell survival was assessed by Annexin V/propidium iodide staining, followed by flow cytometry. Two-way ANOVA **p<0.01 ***p<0.001, compared with DMSO control.
(D) Primary human monocytes were differentiated to M1 cells in the presence of 100 ng/mL GM-CSF and varying concentrations of a tool BTKi for 11 days in culture. Cells were harvested at different days during culture and qPCR was performed to analyze the expression of apoptosis-related genes. Data shown were collected on Day 2 of the culture and are representative of later time points.

ANOVA, analysis of variance; **ATP**, adenosine triphosphate; **BAD**, B-cell lymphoma 2–associated death promoter; **BTKi**, Bruton's tyrosine kinase inhibitor; **Caspase**, cysteine–aspartic protease; **CD**, cluster of differentiation; **DMSO**, dimethyl sulfoxide; **GM-CSF**, granulocyte–macrophage colony-stimulating factor; **M1**, pro-inflammatory macrophage; **M2**, anti-inflammatory macrophage; **M-CSF**, macrophage colony-stimulating factor; **Med**, medium; **qPCR**, quantitative polymerase chain reaction; **RLU**, relative light unit.

- BTK is activated downstream of the GM-CSF receptor (**Figure 3**).
- BTK inhibition with evobrutinib dampens monocyte inflammatory response (**Figure 4**).
  - Monocytes express less IL-1β and upregulate expression of anti-inflammatory genes such as IL-10.

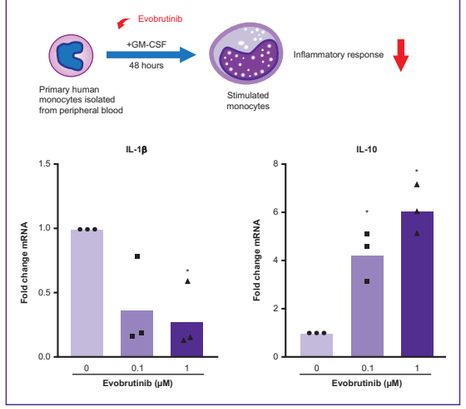
Figure 3. Activation of BTK downstream of GM-CSF receptor



(A) THP-1 cells were treated with a tool BTKi for 30 minutes. Following treatment, GM-CSF (100 ng/mL) stimulation time course was performed. Cells were lysed subsequently and BTK phosphorylation at Y223 was analyzed by Western blot.
(B) Phosphorylated BTK signal was normalized to GAPDH for quantification.

BTKi, Bruton's tyrosine kinase inhibitor; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **GM-CSF**, granulocyte–macrophage colony-stimulating factor; **p-BTK**, phosphorylated BTK.

Figure 4. Dampening monocyte inflammatory response through evobrutinib

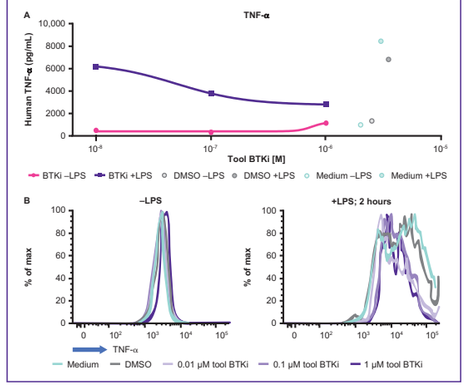


Primary human monocytes were stimulated with 100 ng/mL GM-CSF and treated with evobrutinib for 48 hours. Gene expression was measured subsequently by qPCR. Cells were treated with 0.1 and 1 μM evobrutinib. Student t-test *p<0.05, compared with GM-CSF control.

GM-CSF, granulocyte–macrophage colony-stimulating factor; **IL**, interleukin; **mRNA**, messenger RNA; **qPCR**, quantitative polymerase chain reaction.

- BTK inhibition reduces TNF-α secretion by both monocytes and M1 cells upon LPS stimulation (**Figure 5**).
- BTK inhibition enhances apoptotic cell uptake by M2 macrophages (**Figure 6**).

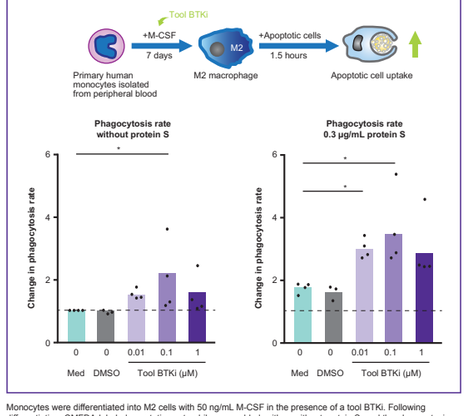
Figure 5. Dampening monocyte and M1 cell inflammatory response through BTK inhibition



(A) Primary human monocytes were stimulated with 50 ng/mL LPS and 100 ng/mL GM-CSF, and treated with varying concentrations of a tool BTKi for 16 hours. TNF-α secretion was subsequently measured by ELISA.
(B) Primary human monocytes were differentiated to M1 cells in the presence of 100 ng/mL GM-CSF and varying concentrations of a tool BTKi for 11 days in culture, and subsequently stimulated with 50 ng/mL LPS for 2 hours. TNF-α production was measured by intracellular TNF-α staining and flow cytometry.

BTKi, Bruton's tyrosine kinase inhibitor; **DMSO**, dimethyl sulfoxide; **ELISA**, enzyme-linked immunosorbent assay; **GM-CSF**, granulocyte–macrophage colony-stimulating factor; **LPS**, lipopolysaccharide; **M1**, pro-inflammatory macrophage; **TNF**, tumor necrosis factor.

Figure 6. Dampening monocyte inflammatory response through BTK inhibition



Monocytes were differentiated into M2 cells with 50 ng/mL M-CSF in the presence of a tool BTKi. Following differentiation, CMFDA-labeled apoptotic neutrophils were added, with or without protein S, and the phagocytosis rate was measured by flow cytometry. Kruskal–Wallis non-parametric test with Dunn's multiple comparison post-test *p<0.05, compared with medium control (medium = 1; dashed line).

BTKi, Bruton's tyrosine kinase inhibitor; **CMFDA**, 5-chloromethylfluorescein diacetate; **DMSO**, dimethyl sulfoxide; **M2**, anti-inflammatory macrophage; **M-CSF**, macrophage colony-stimulating factor; **Med**, medium.

CONCLUSIONS

- **BTK is activated downstream of the GM-CSF receptor.**
- **BTK inhibition with evobrutinib hinders M1 macrophage differentiation and directs monocytes towards an anti-inflammatory M2 phenotype, while enhancing apoptotic cell uptake by M2 cells.**
- **BTK inhibition with BTKi tool compounds increases the rate of efferocytosis by M2 cells in vitro, contributing to the mitigation of inflammatory responses.**
- **BTKis, such as evobrutinib, could have additional benefit in the treatment of autoimmune diseases such as MS by simultaneously targeting B cells and myeloid cells.**

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DISCLOSURES

Y-BA, **PH**, and **JB** are employees of Merck KGaA, Darmstadt, Germany. **RG** and **AB** are employees of EMD Serono Research and Development Institute, Inc.,* Billerica, MA, USA.

Evobrutinib is currently under clinical investigation and has not been approved by any regulatory authority. Status: May 2019.

*A business of Merck KGaA, Darmstadt, Germany.