*Inflammation, Vol. 42, No. 3, June 2019 (© 2019)*
DOI: 10.1007/s10753-019-00976-5

ORIGINAL ARTICLE

# MSU Crystals Enhance TDB-Mediated Inflammatory Macrophage IL-1β Secretion

**Kanu Wahi,[1,2] Kristel Kodar,[1,2] Melanie J. McConnell,[2,3] Jacquie L. Harper,[1] Mattie S. M. Timmer,[1,2,4] and Bridget L. Stocker[1,2,4]**

*Abstract*— The tumour microenvironment predominantly consists of macrophages with phenotypes ranging from pro-inflammatory (M1-like) to anti-inflammatory (M2-like). Trehalose-6,6′-dibehenate (TDB) displays moderate anti-tumour activity and stimulates M1-like macrophages *via* the macrophage inducible C-type lectin (Mincle) resulting in IL-1β production. In this study, we examined if monosodium urate (MSU), a known vaccine adjuvant, can boost IL-1β production by TDB-stimulated macrophages. We investigated the effect of MSU/TDB co-treatment on IL-1β production by GM-CSF (M1-like) and M-CSF/IL-4 (M2-like) differentiated mouse bone marrow macrophages (BMMs) and found that MSU/TDB co-treatment of GM-CSF BMMs significantly enhanced IL-1β production in a Mincle-dependent manner. Western blot analysis showed that increased IL-1β production by GM-CSF BMMs was associated with the induction of pro-IL-1β expression by TDB rather than MSU. Flow cytometry analysis showed that MSU/TDB co-stimulation of GM-CSF BMMs led to greater expansion of $CD86^{high}$/MHC $II^{high}$ and $CD86^{low}$/MHC $II^{low}$ subpopulations; however, only the latter showed increased production of IL-1β. Together, these findings provide evidence of the potential to use MSU/TDB co-treatment to boost IL-1β-mediated anti-tumour activity in M1-like tumour-associated macrophages.

**KEY WORDS:** trehalose dibehenate; monosodium urate crystals; macrophages; APC; adjuvant; M1-like.

## INTRODUCTION

The use of adjuvants in cancer vaccines has increased significantly due to their ability to trigger a robust innate immune response in the tumour microenvironment, enhance anti-tumour activity and overcome immune tolerance [1, 2]. A desirable attribute of a vaccine adjuvant is targeted activation of innate immune cells such as macrophages and dendritic cells in a manner that boosts innate immune responses, increases antigen presentation capacity and enhances adaptive immunity [1–4].

Macrophages account for more than half the population of tumour invading cells [5]. Macrophages in the tumour microenvironment display phenotypes on a spectrum

[1] School of Chemical and Physical Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand
[2] Centre for Biodiscovery, Victoria University of Wellington, PO Box 600, Wellington, New Zealand
[3] School of Biological Sciences, PO Box 600, Wellington, New Zealand
[4] To whom correspondence should be addressed at School of Chemical and Physical Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand. E-mails: mattie.timmer@vuw.ac.nz; bridget.stocker@vuw.ac.nz

**Abbreviations:** BMMs, Bone marrow macrophages; GM-CSF, Granulocyte macrophage-colony stimulating factor; M-CSF/IL-4, Macrophage-colony stimulating factor/interleukin-4; MSU, Monosodium urate; TDB, Trehalose dibehenate

ranging from pro-inflammatory (M1-like) to anti-inflammatory (M2-like) [6]. M1-like macrophages cause acute inflammation and are often associated with anti-tumour activity. Conversely, M2-like macrophages can be immunosuppressive, contribute to immune tolerance and promote tumour progression [7]. Hence, many current strategies in immunotherapy target either M1-like macrophages to increase the tumour immune response [8–10] or M2-like macrophages to dampen anti-inflammatory function and/or skew them towards a pro-inflammatory phenotype [11, 12].

Trehalose-6,6′-dimycolate (TDM) is a component of the mycobacterial cell wall [13] that induces a pro-inflammatory IL-1β response in macrophages *via* recognition by the macrophage inducible C-type lectin receptor (Mincle, Clec4e or Clecf9) [14–16]. TDM is also known to exhibit anti-cancer activity [17–19], as are related trehalose glycolipids [20, 21] including trehalose-6,6′-dibehenate (TDB) [22, 23], a synthetic analogue of TDM which induces inflammatory macrophage phenotypes in association with IL-1β production [15, 24, 25].

Monosodium urate crystals (MSU), a damage-associated molecular pattern (DAMP), are widely recognised as an innate adjuvant that boosts immune responses through the activation of the NLRP3 inflammasome to generate the active form of IL-1β, which is followed by release [26–28]. MSU has been shown to enhance the anti-tumour effects of *Mycobacterium smegmatis* in association with a local increase in IL-1β levels [29], while treatment with uric acid can enhance tumour rejection, potentially through the stimulation of antigen presenting cells (APCs) [30, 31]. Plasma uric acid levels also appear to be associated with a better prognosis in colon cancer patients [32, 33].

Previously, we have shown that TDB differentially modulates M1-like and M2-like macrophage phenotype and function [23]. In this study, we investigated the adjuvant effect of MSU co-stimulation with TDB on the phenotypes of GM-CSF (M1-like) and M-CSF/IL-4 (M2-like) macrophages *in vitro*. We found that MSU in combination with TDB significantly enhanced the pro-inflammatory profile of the M1-like macrophage phenotype, as illustrated by increased IL-1β release compared to MSU or TDB alone.

## MATERIALS AND METHODS

### Mice

All experimental mice were housed in the animal facility at the Malaghan Institute of Medical Research, Wellington, New Zealand. The mice used for the experiments were aged between 8 and 12 weeks. All experimental procedures were approved by the Victoria University Animal Ethics Committee in accordance with their guidelines for the care of animals (protocol nr 22371).

### Mouse Bone Marrow-Derived Macrophage Assay

Macrophages were isolated from the femurs and tibia of C57BL/6 wild-type or Mincle$^{-/-}$ mice as previously described [23]. BMMs were plated at a concentration of 250,000 cells/mL in complete RPMI media (RPMI (Gibco), 10% FBS, 100 units/mL penicillin-streptomycin). Macrophages were differentiated in the presence of 50 ng/mL GM-CSF (Peprotech) or 10 ng/mL M-CSF (Peprotech) with 10 ng/mL IL-4 (Peprotech) for 8 days (37 °C, 5% $CO_2$) [34, 35]. On day 8, media was removed and replaced with fresh complete RPMI media and cells were stimulated with either 40 μg/mL TDB (5 mg/mL stock in DPBS and 2% DMSO) or MSU (12 μg/mL or 25 μg/mL in DPBS) or both TDB and MSU together. TDB [36] and MSU [37] were prepared according to previously published protocols and determined to be endotoxin free (≤ 0.1 EU/mL) by using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific™). LPS (100 ng/mL) stimulation was used as a positive control. Supernatants and cell lysates were collected 6, 12, or 24 h after stimulation. Supernatants and cell lysates were stored at − 80 °C until further analysis.

### IL-1β Expression

IL-1β cytokine levels were measured in the supernatants using sandwich ELISA (R&D Systems) following manufacturer's instructions.

### Western Blot

Total protein was extracted from cell lysates (12 h post-stimulation) using 1% IGEPAL® lysis buffer (Sigma) containing protease inhibitor (Complete tablets, Roche) and phosphatase inhibitors (PhosSTOP, Roche). Proteins (25 μg protein/sample) were separated on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF; BioRad) membrane by wet blotting. The blots were probed with antibodies against IL-1β (mouse specific, D3H1Z from Cell Signalling) and β-actin (Cell Signalling). Protein expression was detected using anti-rabbit IgG-HRP (Cell Signalling) followed by development with SuperSignal

West Femto Substrate (Thermo Scientific) and imaged with Amersham Imager 600. Protein band intensities were quantified using the Amersham Imager software as well as ImageJ.

### Flow Cytometry

Macrophages were analysed 24 h post-stimulation by flow cytometry (BD FACSCANTO II). Non-specific epitopes were blocked with anti-mouse CD16/32 antibody (2.4G2). Fluorescently labelled antibodies were used to measure surface expression of I-A/I-E MHC II (BioLegend), CD11b, CD86 (BD Biosciences Pharmingen) and Mincle (MBL International) with Streptavidin-APC (BD Biosciences Pharmingen).

For intracellular staining of IL-1β, the GM-CSF BMMs were stimulated with MSU, TDB or MSU/TDB and treated with 1 μM GolgiPlug (BD) 4 h after stimulation to block the secretion of IL-1β. Cells were harvested at 24 h, stained for cell surface markers, fixed (formalin solution, neutral buffered; Sigma-Aldrich), permeabilized (0.1% Saponin buffer) and stained with anti-IL-1β antibody (R&D Systems).

### Statistics

Statistical analysis to measure significant differences was carried out using Prism v7 software (GraphPad) either by one-way ANOVA or two-way ANOVA with Bonferroni *post hoc* test.

## RESULTS AND DISCUSSION

### IL-1β Production Is Increased in GM-CSF BMMs Stimulated with MSU/TDB

Stimulation of mouse macrophages with TDB activates the NLRP3 inflammasome and induces the expression of IL-1β [24, 25]. The enhanced anti-tumour activity of MSU/*M. smegmatis* combined treatment is also reported to be associated with increased IL-1β expression in tumour-bearing mice [29]. We therefore tested the effect of MSU and TDB co-treatment on IL-1β production by GM-CSF and M-CSF/IL-4 differentiated BMMs. MSU/TDB co-treatment triggered a significant increase in IL-1β production by GM-CSF BMMs treated with MSU/TDB compared to either MSU or TDB alone at 12 h and 24 h (Fig. 1a). Neither MSU nor TDB, alone or in combination, induced IL-1β production by M-CSF/IL-4 BMMs (Fig. 1b). These results indicate that MSU and TDB together preferentially enhance IL-1β secretion from macrophages with a pro-inflammatory rather than anti-inflammatory phenotype.

Several current anti-cancer treatments aim to boost the pro-inflammatory macrophage phenotype and cause an increase in the production of pro-inflammatory cytokines such as IL-1β [10, 29, 38]. Accordingly, pro-inflammatory macrophages in tumours are an attractive target for cancer treatment and provide a mechanism for raising an anti-tumour immune microenvironment [7]. The enhanced effect of MSU/TDB on IL-1β production by GM-CSF BMMs could therefore indicate utility as a potential cancer

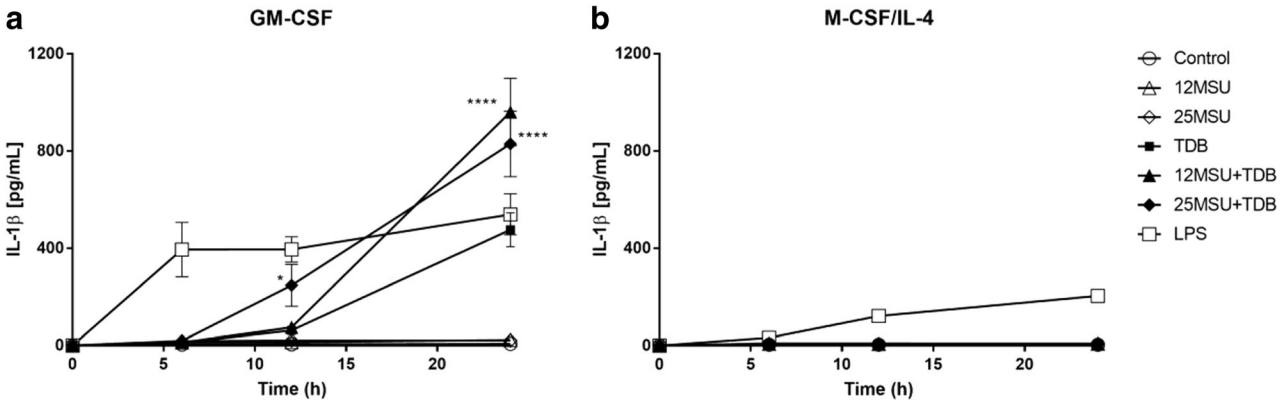


**Fig. 1.** MSU enhances IL-1β expression in TDB stimulated GM-CSF BMMs **a** GM-CSF and **b** M-CSF/IL-4 BMMs were stimulated with MSU (12 μg/mL or 25 μg/mL) or TDB (40 μg/mL) or both together and IL-1β was measured by ELISA after 6, 12, 24 h. Mean ± SEM for triplicate values from three independent experiments are shown. LPS was used as a positive control. One-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis (compared to TDB), where $*p < 0.05$, $****p < 0.0001$ were considered significantly different.

treatment by creating an acute pro-inflammatory tumour environment.

### Mincle-Dependent TDB Priming of GM-CSF BMMs for Enhanced MSU/TDB IL-1β Production

Classical IL-1β production typically involves a two signal process: a priming step usually initiated by pathogen-associated molecular pattern (PAMP) exposure and which generates pro-IL-1β; and a second stimulatory signal that activates the inflammasome to generate the active form of IL-1β [39]. As there is evidence that TDB activates the NLRP3 inflammasome [25], our observation that TDB leads to IL-1β expression is likely the result of TDB being able to induce both priming (pro-IL-1β production) and conversion of pro-IL-1β to IL-1β *via* inflammasome activation. To this end, we found that treatment of GM-CSF BMMs with either TDB or MSU/TDB induced pro-IL-1β expression, while MSU treatment alone did not alter pro-IL-1β expression in GM-CSF BMMs (Fig. 2a). There was no significant difference in the band intensities between TDB alone and TDB/MSU treatment.

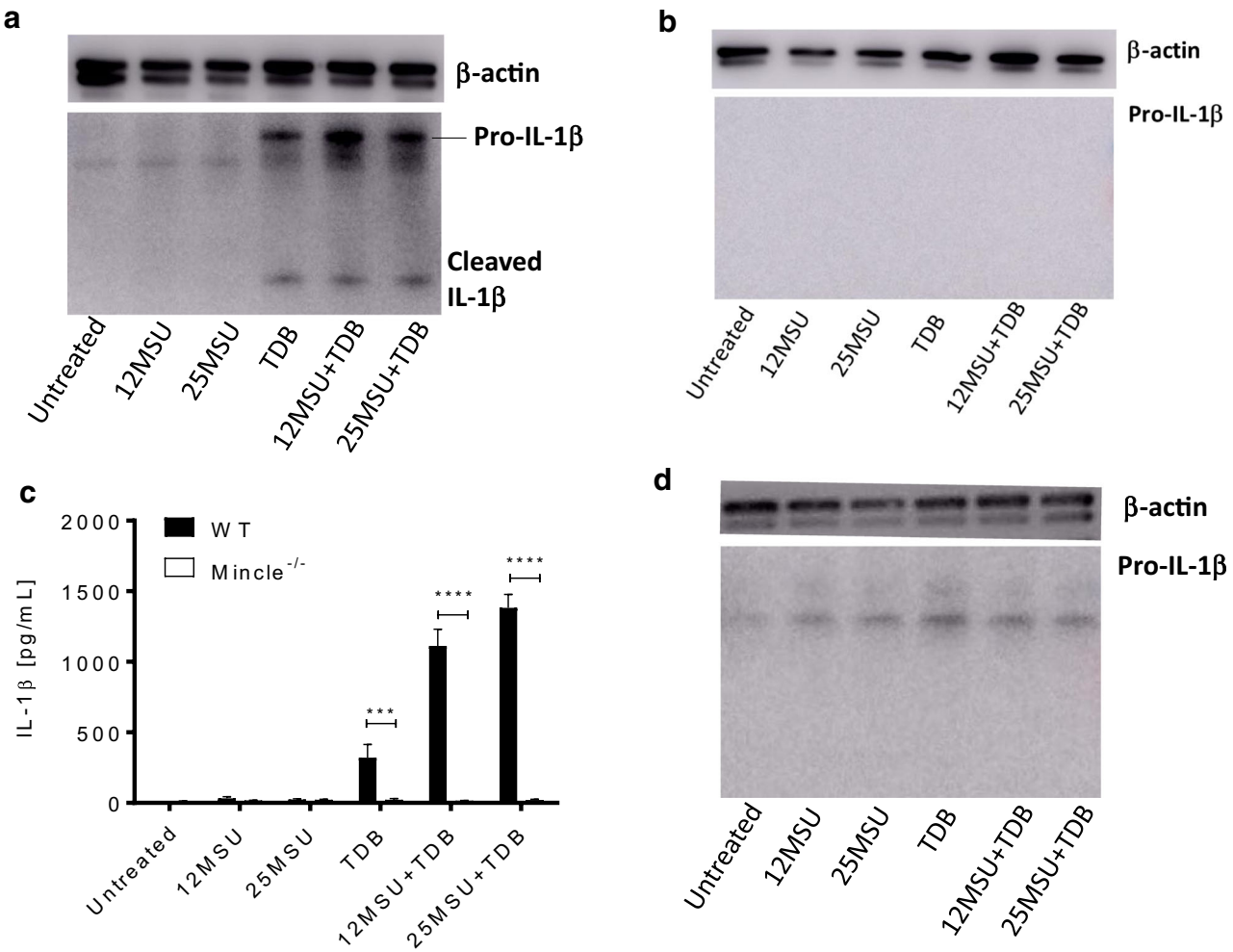


**Fig. 2.** TDB, but not MSU, primes GM-CSF BMMs for IL-1β expression in a Mincle-dependent manner. Pro-IL-1β and cleaved IL-1β detected by Western blot in cell lysates from **a** GM-CSF WT BMMs, **b** M-CSF/IL-4 WT BMMs stimulated with 12, 25 μg/mL MSU or 40 μg/mL TDB or together at 12 h. Representative graphs shown from three independent experiments **c** IL-1β expression in GM-CSF WT and Mincle$^{-/-}$ BMMs 24 h post-stimulation. Mean ± SEM for triplicate values from three independent experiments are shown. Two-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis, where ***$p < 0.001$ and ****$p < 0.0001$ were considered significantly different **d** GM-CSF Mincle$^{-/-}$ BMMs cell lysates analysed for pro-IL-1β expression by Western blot.

These results confirm that TDB and not MSU provides the priming signal that induces pro-IL-1β expression in GM-CSF BMMs. TDB did not induce pro-IL-1β expression in M-CSF/IL-4 BMMs (Fig. 2b).

Mincle, a C-type lectin receptor, is expressed on myeloid cells and recognises PAMPs, DAMPs and their analogues such as TDB [14–16]. We therefore compared pro- and active IL-1β production by GM-CSF BMMs derived from WT and Mincle knockout (Mincle$^{-/-}$) mice. GM-CSF Mincle$^{-/-}$ BMMs treated with TDB or MSU/TDB released significantly less IL-1β compared to the wild-type control (Fig. 2c). Consistent with this, Western

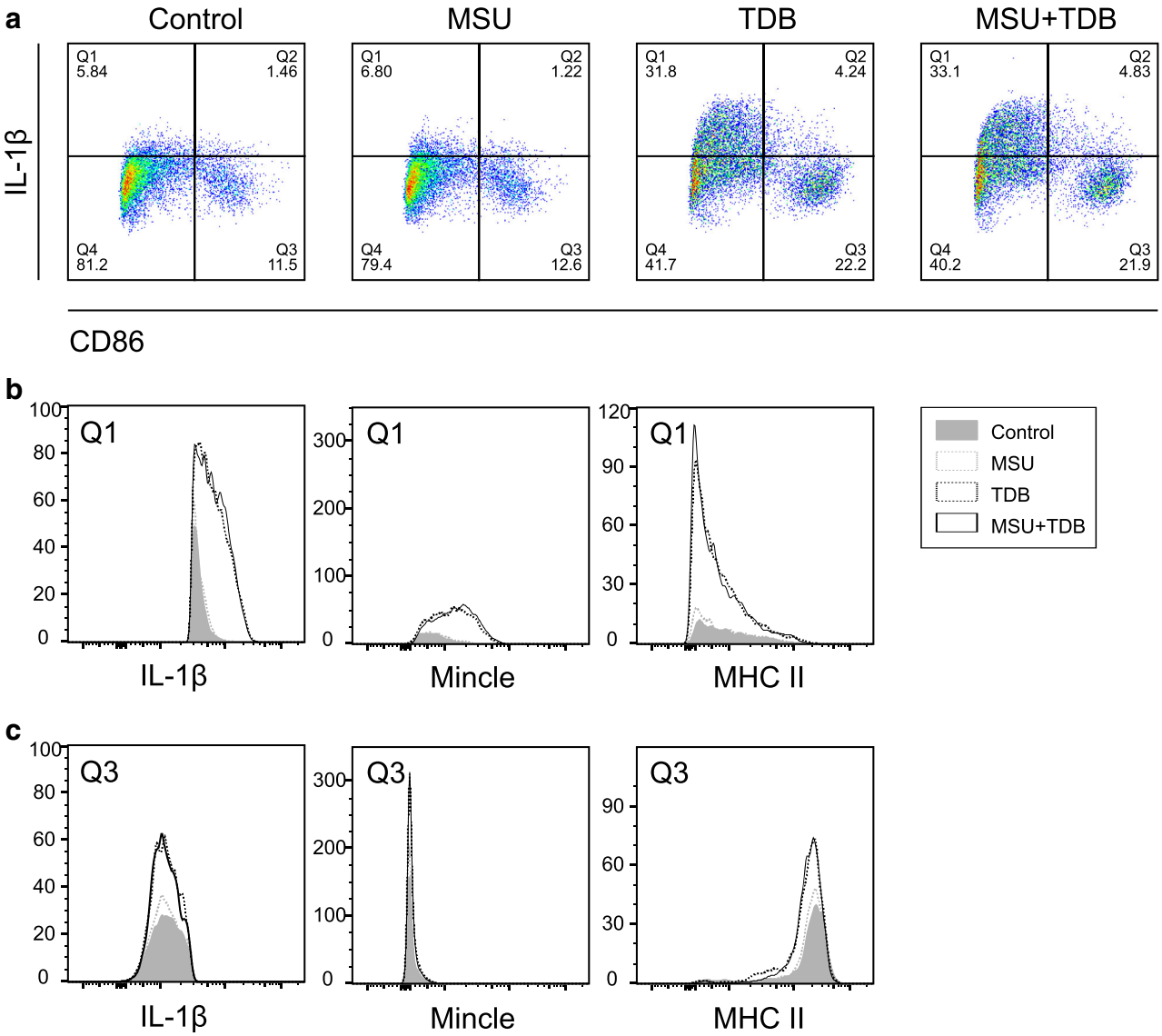


**Fig. 3.** MSU/TDB stimulation of GM-CSF BMMs leads to the expansion of CD86$^{high}$/MHC II$^{high}$/Mincle$^{low}$/IL-1β$^{low}$ and CD86$^{low}$/MHC II$^{low}$/Mincle$^{high}$/IL-1β$^{high}$ subpopulations. **a** Representative dotplots of the expression of intracellular IL-1β and cell surface CD86 by GM-CSF BMMs stimulated with either MSU (25 μg/mL), TDB (40 μg/mL) or MSU and TDB together for 24 h measured by flow cytometry. Histograms of the expression of intracellular IL-1β, cell surface Mincle and MHC II in Q1 (IL-1β$^{high}$/CD86$^{low}$) (**b**) and Q3 (IL-1β$^{low}$/CD86$^{high}$) (**c**). Representative graphs shown from two independent experiments run in triplicate.

blot analysis showed that Mincle$^{-/-}$ BMMs did not express pro-IL-1β following TDB treatment with or without the co-administration of MSU (Fig. 2d). These results indicate that the interaction between Mincle and TDB is required to prime the BMMs leading to the elevated IL-1β response observed after MSU treatment.

While previous work has investigated TDB-induced inflammasome activation, the macrophages were primed with LPS prior to TDB stimulation [25]. We have shown that TDB can prime pro-inflammatory murine macrophages to produce IL-1β, irrespective of the presence of MSU, and that Mincle is required to induce this priming effect by TDB. As TDB alone can trigger IL-1β release, it follows that TDB is capable of delivering both signal one and signal two required for IL-1β activation. The enhanced IL-1β production observed following combined MSU/TDB treatment, however, indicates that MSU triggers a significantly stronger second signal that boosts inflammasome activation and increases cleavage of pro-IL-1β.

### Effect of MSU/TDB Stimulation on Cell Surface Phenotype of GM-CSF BMMs

*In vitro* GM-CSF-derived BMMs have been described as being composed of a mix of dendritic cells (DCs) and macrophage populations that respond differentially to the inflammatory stimulus LPS [40, 41]. The activation of antigen presenting cells such as DCs is commonly associated with an increase in the expression of cell surface markers such as CD86 [42, 43]. Flow cytometry analysis of the GM-CSF BMMs treated with MSU, TDB or MSU/TDB identified two subpopulations of cells: CD86$^{low}$/IL-1β$^{high}$ and CD86$^{high}$/IL-1β$^{low}$ (Fig. 3a). The CD86$^{low}$/ IL-1β$^{high}$ subpopulation was Mincle$^{+}$ and MHCII$^{low}$ (Fig. 3b), whereas the CD86$^{high}$/IL-1β$^{low}$ subpopulation was found to be Mincle$^{-}$ and MHCII$^{high}$ (Fig. 3c). These results therefore show that stimulation of GM-CSF BMMs with MSU, TDB or MSU/TDB drives the development of DC and macrophage subpopulations with potentially two distinct functional phenotypes. A macrophage subpopulation (CD86$^{low}$/Mincle$^{+}$/MHCII$^{low}$/IL-1β$^{high}$) responsible for immune-boosting IL-1β production, alongside a population (CD86$^{high}$/Mincle$^{-}$/MHCII$^{hi}$/IL-1β$^{low}$) exhibiting a more classical APC phenotype.

In summary, this study demonstrates that TDB used in combination with MSU can enhance the acute inflammatory profile of a mixed population of GM-CSF differentiated macrophages and DC-like APCs. By activating the DC-like population alongside triggering elevated IL-1β production, MSU/TBD co-treatment has the potential to enhance the presentation of local tumour-associated antigens and anti-tumour immune responses. Previous work indicates that the induction of acute IL-1β-driven inflammation in the tumour microenvironment may also be beneficial in limiting tumour growth [10, 29, 38]. As such, the ability to enhance the proinflammatory profile of tumour macrophages through TDB/MSU co-treatment represents a potential mechanism for macrophage-mediated suppression of tumour growth.

## ACKNOWLEDGMENTS

We thank Professor Sho Yamasaki for kindly providing the Mincle$^{-/-}$ mice.

**Funding Information** This work was supported by the Cancer Society of New Zealand (2016/25) and the Health Research Council of New Zealand (Hercus Fellowship, BLS).

## COMPLIANCE WITH ETHICAL STANDARDS

All experimental procedures were approved by the Victoria University Animal Ethics Committee in accordance with their guidelines for the care of animals (protocol nr 22371).



## REFERENCES

1. Temizoz, B., E. Kuroda, and K.J. Ishii. 2016. Vaccine adjuvants as potential cancer immunotherapeutics. *International Immunology* 28: 329–338.
2. Bowen, W.J., K.S. Abhishek, L. Batra, H. Barsoumian, and H. Shirwan. 2018. Current challenges for cancer vaccine adjuvant development. *Expert Review of Vaccines* 17: 207–215.
3. Pasquale, A.D., S. Preiss, F.T. Da Silva, and N. Garcon. 2015. Vaccine adjuvants: From 1920 to 2015 and beyond. *Vaccines* 3: 320–343.
4. Zepp, F. 2016. Principles of vaccination. *Methods in Molecular Biology* 1403: 57–84.
5. van Ravenswaay Claasen, H.H., P.M. Kluin, and G.J. Fleuren. 1992. Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. *Laboratory Investigation* 67: 166–174.
6. Mosser, D.M., and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nature Reviews. Immunology* 8: 958–969.

7. Mantovani, A., F. Marchesi, A. Malesci, L. Laghi, and P. Allavena. 2017. Tumour-associated macrophages as treatment targets in oncology. *Nature Reviews Clinical Oncology* 14: 399–416.
8. Buhtoiarov, I.N., P.M. Sondel, J.M. Wigginton, T.N. Buhtoiarova, E.M. Yanke, D.A. Mahvi, and A.L. Rakhmilevich. 2011. Anti-tumour synergy of cytotoxic chemotherapy and anti-CD40 plus CpG-ODN immunotherapy through repolarization of tumour-associated macrophages. *Immunology* 132: 226–239.
9. Shi, Y., M.A.R. Felder, P.M. Sondel, and A.L. Rakhmilevich. 2015. Synergy of anti-CD40, CpG and MPL in activation of mouse macrophages. *Molecular Immunology* 66: 208–215.
10. Dewan, M.Z., C. Vanpouille-Box, N. Kawashima, S. DiNapoli, J.S. Babb, S.C. Formenti, S. Adams, and S. Demaria. 2012. Synergy of topical toll-like receptor 7 agonist with radiation and low-dose cyclophosphamide in a mouse model of cutaneous breast cancer. *Clinical Cancer Research* 18: 6668–6678.
11. Hussain, S.F., L.-Y. Kong, J. Jordan, C. Conrad, T. Madden, I. Fokt, W. Priebe, and A.B. Heimberger. 2007. A novel small molecule inhibitor of signal transducers and activators of transcription 3 reverses immune tolerance in malignant glioma patients. *Cancer Research* 67: 9630–9636.
12. Edwards, J.P., and L.A. Emens. 2010. The multikinase inhibitor Sorafenib reverses the suppression of IL-12 and enhancement of IL-10 by PGE2 in murine macrophages. *International Immunopharmacology* 10: 1220–1228.
13. Bloch, H., and H. Noll. 1954. Studies on the virulence of tubercle bacilli; the effect of cord factor on murine tuberculosis. *British Journal of Experimental Pathology* 36: 8–17.
14. Ishikawa, E., T. Ishikawa, Y.S. Morita, K. Toyonaga, H. Yamada, O. Takeuchi, T. Kinoshita, S. Akira, Y. Yoshikai, and S. Yamasaki. 2009. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *The Journal of Experimental Medicine* 206: 2879–2888.
15. Schoenen, H., B. Bodendorfer, K. Hitchens, S. Manzanero, K. Werninghaus, F. Nimmerjahn, E.M. Agger, S. Stenger, P. Andersen, J. Ruland, G.D. Brown, C. Wells, and R. Lang. 2010. Cutting edge: Mincle is essential for recognition and Adjuvanticity of the mycobacterial cord factor and its synthetic analog Trehalose-Dibehenate. *Journal of Immunology* 184: 2756–2760.
16. Braganza, C., T. Teunissen, M.S.M. Timmer, and B. Stocker. 2018. Synthetic Mincle ligands. *Frontiers in Immunology* 8: 1940.
17. Yarkoni, E., L. Wang, and A. Bekierkunst. 1974. Suppression of growth of Ehrlich ascites tumor cells in mice by trehalose-6,6′-dimycolate (cord factor) and BCG. *Infection and Immunity* 9: 977–984.
18. Yarkoni, E., E. Lederer, and H.J. Rapp. 1981. Immunotherapy of experimental cancer with a mixture of synthetic muramyl dipeptide and trehalose dimycolate. *Infection and Immunity* 32: 273–276.
19. Watanabe, R., Y.C. Yoo, K. Hata, M. Mitobe, Y. Koike, M. Nishizawa, D.M. Garcia, Y. Nobuchi, H. Imagawa, H. Yamada, and I. Azuma. 1999. Inhibitory effect of trehalose dimycolate (TDM) and its stereoisometric derivatives, trehalose dicorynomycolates (TDCMs), with low toxicity on lung metastasis of tumour cells in mice. *Vaccine* 17: 1484–1492.
20. Yamamoto, H., M. Oda, M. Nakano, N. Watanabe, K. Yabiku, M. Shibutani, M. Inoue, H. Imagawa, M. Nagahama, S. Himeno, K. Setsu, J. Sakurai, and M. Nishizawa. 2013. Development of Vizantin, a safe immunostimulant, based on the structure-activity relationship of trehalose-6,6′-dicorynomycolate. *Journal of Medicinal Chemistry* 56: 381–385.
21. Pimm, M.V., R.W. Baldwin, J. Polonsky, and E. Lederer. 1979. Immunotherapy of an ascitic rat hepatoma with cord factor (trehalose-6,6′-dimycolate) and synthetic analogues. *International Journal of Cancer* 24: 780–785.
22. Nishikawa, Y., T. Katori, K. Kukita, and T. Ikekawa. 1982. Synthesis and anti-tumour effects of 6,6′-di-*O*-acyl-α,α'-trehaloses. *Nippon Kagaku Kaishi* 10: 1661–1666.
23. Kodar, K., J.L. Harper, M.J. McConnell, M.S.M. Timmer, and B.L. Stocker. 2017. The Mincle ligand trehalose dibehenate differentially modulates M1-like and M2-like macrophage phenotype and function via Syk signaling. *Immunity, Inflammation and Disease* 5: 503–514.
24. Werninghaus, K., A. Babiak, O. Groß, C. Hölscher, H. Dietrich, E.M. Agger, J. Mages, A. Mocsai, H. Schoenen, K. Finger, F. Nimmerjahn, G.D. Brown, C. Kirschning, A. Heit, P. Andersen, H. Wagner, J. Ruland, and R. Lang. 2009. Adjuvanticity of a synthetic cord factor analogue for subunit Mycobacterium tuberculosis vaccination requires FcRγ–Syk–Card9–dependent innate immune activation. *The Journal of Experimental Medicine* 206: 89–97.
25. Schweneker, K., O. Gorka, M. Schweneker, H. Poeck, J. Tschopp, C. Peschel, J. Ruland, and O. Groß. 2013. The mycobacterial cord factor adjuvant analogue trehalose-6,6′-dibehenate (TDB) activates the Nlrp3 inflammasome. *Immunobiology* 218: 664–673.
26. Giamarellos-Bourboulis, E.J., M. Mouktaroudi, E. Bodar, J. Van Der Ven, B.J. Kullberg, M.G. Netea, et al. 2009. Crystals of monosodium urate monohydrate enhance lipopolysaccharide-induced release of interleukin 1 βby mononuclear cells through a caspase 1-mediated process. *Annals of the Rheumatic Diseases* 68: 273–278.
27. Chen, C.J., Y. Shi, A. Hearn, K. Fitzgerald, D. Golenbock, G. Reed, S. Akira, and K.L. Rock. 2006. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *The Journal of Clinical Investigation* 116: 2262–2271.
28. Taus, F., M.B. Santucci, E. Greco, M. Morandi, I. Palucci, S. Mariotti, et al. 2015. Monosodium urate crystals promote innate anti-mycobacterial immunity and improve BCG efficacy as a vaccine against tuberculosis. *PLoS One* 10: 1–16.
29. Kuhn, S., E.J. Hyde, J. Yang, F.J. Rich, J.L. Harper, J.R. Kirman, and F. Ronchese. 2013. Increased numbers of monocyte-derived dendritic cells during successful tumor immunotherapy with immune-activating agents. *Journal of Immunology* 191: 1984–1992.
30. Shi, Y., J.E. Evans, and K.L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425: 516–521.
31. Hu, D.-E., A.M. Moore, L.L. Thomsen, and K.M. Brindle. 2004. Uric acid promotes tumor immune rejection. *Cancer Research* 64: 5059–5062.
32. Dziaman, T., Z. Banaszkiewicz, K. Roszkowski, D. Gackowski, E. Wisniewska, R. Rozalski, M. Foksinski, A. Siomek, E. Speina, A. Winczura, A. Marszalek, B. Tudek, and R. Olinski. 2014. 8-Oxo-7,8-dihydroguanine and uric acid as efficient predictors of survival in colon cancer patients. *International Journal of Cancer* 134: 376–383.
33. Slobodnick, A., S. Krasnokutsky, R.A. Lehmann, R.T. Keenan, J. Quach, F. Francois, and M.H. Pillinger. 2018. Colorectal Cancer among gout patients undergoing colonoscopy. *Journal of Clinical Rheumatology*: 1. https://doi.org/10.1097/RHU.0000000000000893.
34. Fleetwood, A.J., T. Lawrence, J.A. Hamilton, and A.D. Cook. 2007. Granulocyte-macrophage Colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: Implications for CSF blockade in inflammation. *Journal of Immunology* 178: 5245–5252.

35. Hamilton, T.A., C. Zhao, P.G. Pavicic, and S. Datta. 2014. Myeloid colony-stimulating factors as regulators of macrophage polarization. *Frontiers in Immunology* 5: 1–6.
36. Khan, A.A., S.H. Chee, R.J. McLaughlin, J.L. Harper, F. Kamena, M.S. Timmer, and B.L. Stocker. 2011. Long-chain lipids are required for the innate immune recognition of trehalose diesters by macrophages. *Chembiochem* 12: 2572–2576.
37. Martin, W.J., M. Walton, and J.L. Harper. 2009. Resident macrophages initiating and driving inflammation in a monosodium urate monohydrate crystal-induced murine peritoneal model of acute gout. *Arthritis and Rheumatism* 60: 281–289.
38. Haabeth, O.A.W., K.B. Lorvik, H. Yagita, B. Bogen, and A. Corthay. 2016. Interleukin-1 is required for cancer eradication mediated by tumor-specific Th1 cells. *Oncoimmunology* 5: e1039763.
39. He, Y., H. Hara, and G. Núñez. 2016. Mechanism and regulation of NLRP3 Inflammasome activation. *Trends in Biochemical Sciences* 41: 1012–1021.
40. Helft, J., J. Böttcher, P. Chakravarty, S. Zelenay, J. Huotari, B.U. Schraml, D. Goubau, and C. Reise Sousa. 2015. GM-CSF mouse bone marrow cultures comprise a heterogeneous population of CD11c(+)MHCII(+) macrophages and dendritic cells. *Immunity* 42: 1197–1211.
41. Na, Y.R., D. Jung, G.J. Gu, and S.H. Seok. 2016. GM-CSF grown bone marrow derived cells are composed of phenotypically different dendritic cells and macrophages. *Molecules and Cells* 39: 734–741.
42. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Théry, and S. Amigorena. 2002. Antigen presentaion and T cell stimulation by dendritic cells. *Annual Review of Immunology* 20: 621–667.
43. Wang, C., X. Yu, Q. Cao, Y. Wang, G. Zheng, T.K. Tan, et al. 2013. Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC Immunology* 14: 1–10.