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Immunomodulatory Synthetic Glycocluster Molecule Prevents Melanoma Growth *In Vivo*

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Triacedimannose (**TADM**) is a synthetic trivalent acetylated glycocluster and a transmembrane macrophage activator independent of the mannose receptor. **TADM** induces Th1-type immune responses and suppresses Th2-type cytokines in acute and chronic allergic inflammation models in vivo. We, therefore, wanted to test whether **TADM** could also facilitate anti-tumour tissue responses similar to what has been observed for the immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4. A syngeneic mouse melanoma model was selected since metastatic melanoma has been successfully targeted by checkpoint inhibitors in the clinic. **TADM** inhibited the growth of B16 mouse melanoma tumours at levels comparable to an anti-PD-1 antibody. **TADM**-treated tumours encompassed significantly more apoptotic cells as measured by TUNEL staining, and interferon-gamma (IFN-γ) expression was increased in the spleens of **TADM**-treated mice compared to untreated controls. **TADM**-treated mice also demonstrated increased Ly6 C low monocytes and neutrophils in the spleens. However, **TADM**-treated tumours showed no discernible differences in infiltrating immune cells. **TADM** can alone suppress the growth of melanoma tumours. **TADM** likely activates M1 type macrophages, type N1 neutrophils, and CD8+ and Th1 T cells, suppressing the type 2 immune response milieu of melanoma tumour with a strong type 1 immune response.

Introduction

Immunoadjuvants are compounds designed to supplement therapies to stimulate immune responses. These have been extensively investigated for their potential to enhance treatments targeting various immunological disorders, notably cancer. Currently studied adjuvants in cancer immunotherapy include Monophosphoryl lipid A (MPLA) and CpG-ODN.[1] Both are microbial particles shown to function via Toll-like Receptors (TLR) to generate robust anti-tumour immune responses by induction of both innate and adaptive immune cells.[2] *Candida albicans* mannan containing β-1,2-mannoside structures induce IFN-ɣ responses and lymphoproliferation in practically all human beings induced not only by intact mannan but also by small hydrolysed β-1,2-manno-oligosaccharide fragments.[3,4] These fragments also induce TNF responses in macrophages.[5] The fungal cell wall mannans are known to induce immune reactions in a melanoma model.[6]

In previous work, we synthesised oligosaccharide constructs mimicking the natural β-1,2-mannoside structures and enhanced their immunostimulatory properties by creating oligovalent glycocluster molecules. Triacedimannose (TADM) is a synthetic trivalent acetylated glycocluster consisting of β-1,2–linked dimannoses, identified as a potential lead compound in

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in vitro studies out of a library of a total of 30 candidates, ranging from mono- to pentavalent β-1,2–dimannoside assemblies.[7] Later work demonstrated similar properties for two of its close structural analogues, **TADM-EL** and **TADM-LL**.[8] In human in vitro peripheral blood mononuclear cell models and in vivo murine models, **TADM** induces both IFN-ɣ and TNF.[7–9] The induction of TNF implies that macrophages are activated, and induction of IFN-ɣ indicates that $CD4^+$ Th1 type T cells (Th1) and $CD8^+$ cytotoxic T cells (CTL) are activated. As **TADM** has been shown to induce Th1-type immune responses and suppress Th2-type cytokines in ovalbumin-induced acute and timothy-induced chronic allergic inflammation models *in vivo*, we here tested if **TADM** and other trivalent oligosaccharide derivatives could also function to enhance the tumour-associated antigen-induced immune reaction and suppress the growth of melanoma tumours in a mouse model.[9,10]

The prognosis of metastatic melanoma has improved during the last decade after the approval of immunotherapeutic agents.[11,12] Metastatic melanoma tumours are associated with extensive somatic genetic alterations and the capability to suppress the function of the immune system. Antibodies against the immune checkpoint proteins PD-1 and CTLA-4 are currently used as monotherapy and in combinations in the treatment of metastatic melanoma.[13] Both treatments target tumour cells' mechanisms to inhibit immune reactions, especially T cell activation.[14]

In this work, we show that **TADM** inhibited the growth of B16 mouse melanoma tumours at levels comparable to an anti-PD-1 antibody. **TADM**-treated tumours encompassed significantly more apoptotic cells as measured by TUNEL staining, and interferon-gamma (IFN-γ) expression was increased in the spleens of **TADM**-treated mice compared to untreated controls. **TADM**-treated mice also demonstrated increased numbers of Ly6 C low monocytes and neutrophils in the spleens. However, **TADM**-treated tumours showed no discernible differences in infiltrating immune cells.

Results

Effect of the Compounds on *In Vivo* Melanoma Tumor Growth and Apoptosis

We have recently shown that a trivalent mannobiose **TADM** can modulate induced allergic inflammation by balancing the Th2-biased immunity towards Th1 and Treg responses.[9] In order to address the potential of synthetic glycocluster molecules for melanoma immunotherapy, we tested the capability of four trivalent compounds, **TADM**, **TADM-EL**, **TADM-LL**, and **TDM** (Figure 1), for their potential to suppress the growth of melanoma cells in a syngeneic mouse model. Compound TDM, included as a reference in the present study, is structurally identical to TADM but lacks acetylation of its carbohydrate moieties. In our earlier study, TDM did not display in vitro activity characteristics for TADM and its two acetylated analogues.[7]

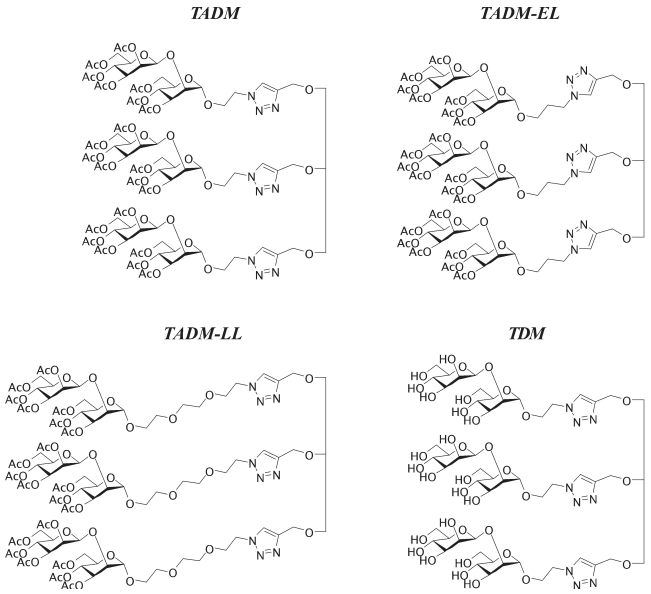

Figure 1. Structures of the glycocluster compounds.

B16 mouse melanoma cells were seeded subcutaneously to female black C57BL/6 mice (10/treatment group). The animals were treated once every two days (q2d) with the PBS buffer alone or 200 μg of the tested compounds (Figure 2**A**). Of the tested compounds, **TADM** most efficiently reduced the tumour growth ($P = 0.0057$, compared to the vehicle alone) (Figure 2B). **TADM-LL**, **TADM-EL** and **TDM** did not significantly reduce tumour growth ($P = 0.66$, $P = 0.070$ and $P = 0.16$, respectively) (Figure 2B).

Since **TADM** was the most efficient of the tested mannobioses for inhibiting tumour growth, we compared its efficacy to anti-PD-1 treatment as a single treatment. As expected, **TADM** significantly reduced the tumour growth rate (Figure 2C) compared to the untreated control ($P = 0.0011$). Anti-PD-1 also reduced tumour growth ($P = 0.0030$), similar to **TADM** (Figure 2C). Together, these findings indicate that the compound **TADM** effectively inhibits melanoma tumour growth *in vivo* with an activity comparable to anti-PD-1.

TUNEL-staining revealed significantly more apoptotic TUNEL-positive cells in both **TADM** and anti-PD-1-treated samples compared to control tumours ($P < 0.0001$ for TADM; $P = 0.013$ for anti-PD-1) (Figure 2D and E) at the day 12 of the experiment, i.e. 48 hours after the last drug injections. Moreover, **TADM** was more efficient in inducing apoptosis than anti-PD-1 ($P = 0.014$).

Effect of Glycoclusters on Melanoma Cell Growth *In Vitro*

To address any direct effects of the compounds on the cancer cell growth, B16 melanoma cells were treated with 0.05 mg/ml **TADM** or **TADM-LL** for 24, 48, or 72 hours, followed by quantification of cell viability by an MTS c assay. **TADM** or **TADM-LL** did not affect cell growth during the 3-day experiment with the concentrations studied (Figure 3A), indicating an indirect mechanism for the *in vivo* anti-tumour activity.

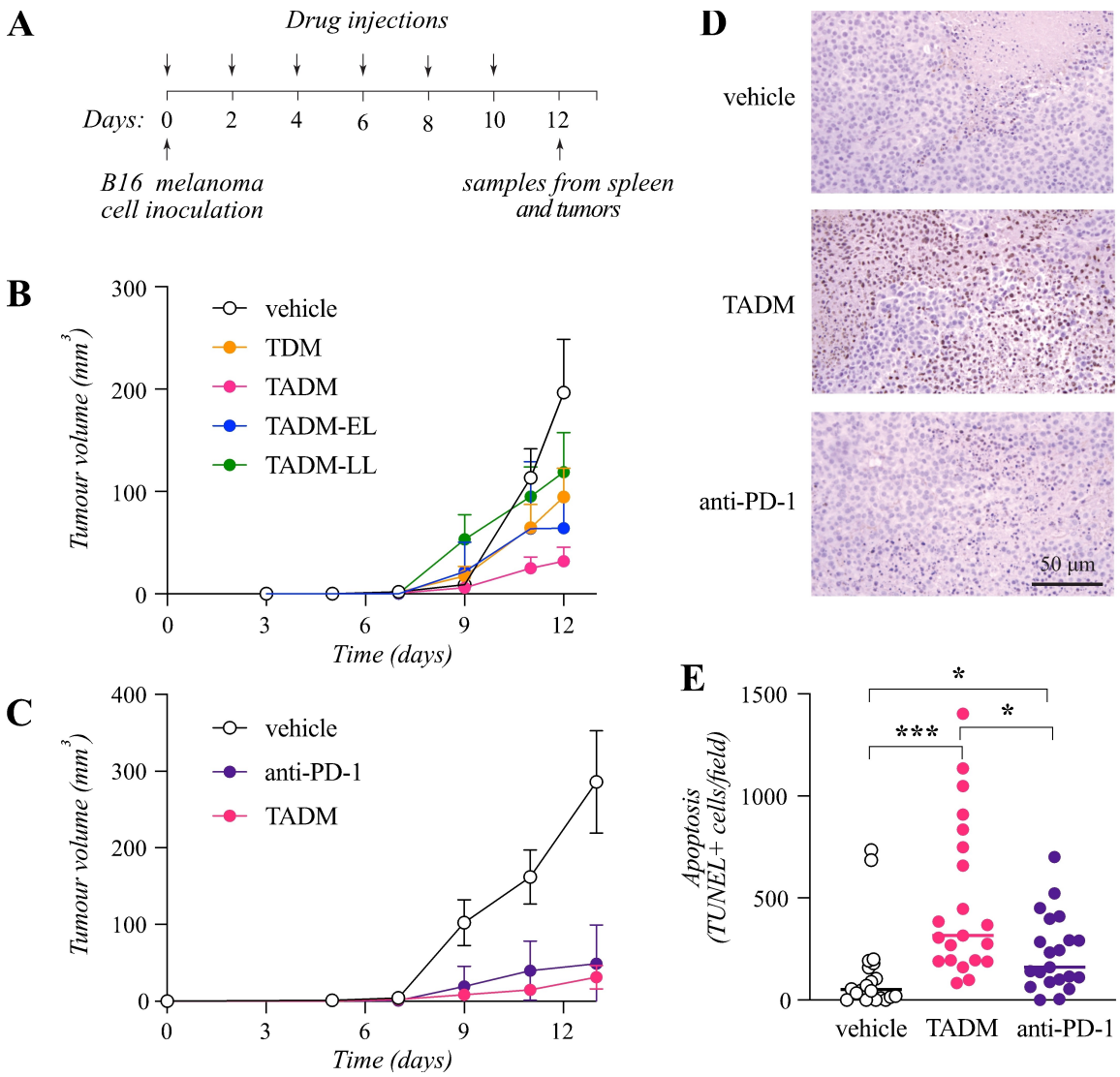

Figure 2. Effect of glycocluster compounds on melanoma tumours *in vivo*. A: Schematic representation of the experimental design used in the *in vivo* melanoma tumour growth assay. B: Growth of subcutaneous B16 melanoma tumours in C57BL/6 mice (n = 9–10, per treatment group) treated q2d with PBS buffer (vehicle), **TADM** (200 µg/injection), **TADM-EL** (200 µg/injection), **TADM-LL** (200 µg/injection), **TDM** (200 µg/injection), or anti-PD-1 antibody (250 µg/injection). n = 10; median +/−SEM is shown. C: Growth of subcutaneous B16 melanoma tumours in C57BL/6 mice treated q2d by PBS buffer (vehicle), anti-PD-1 antibody (150 µg/injection), or **TADM** (200 µg/injection). n = 10; median +/− SEM is shown. D–E: TUNEL staining analysis of apoptosis of tumours treated as in panel C. D) Representative images showing apoptotic TUNEL-positive cells as brown. E) Quantification of TUNEL-positive cells from three images/tumour (n = 5–7/treatment group). The median for each treatment group is indicated with a line, Mann-Whitney test. *, $P < 0.05$; ***, $P < 0.001$.

Effect of TADM on THP-1 Macrophage Polarisation In Vitro

To elucidate whether the immunostimulatory effect of **TADM** could be mediated by polarizing macrophages towards an immunostimulatory M1-like phenotype upon **TADM** treatment, THP-1 monocytes were treated with 50 µg/ml **TADM** for up to six days. The effect of **TADM** on gene expression was assessed with RT-qPCR. **TADM** significantly induced the expression of TNFA after 48 hours ($P < 0{,}01$) and six days ($P < 0{,}001$), a gene encoding for Tumour necrosis factor-α, an important immunostimulatory cytokine secreted by proinflammatory M1-like macrophages.[15] **TADM** did not affect the expression of two M2-like immunosuppressive macrophage markers, macrophage mannose receptor MCR1/CD206 or CD163 (Figure 3B).[16,17]

Inflammatory Cells in the Spleen

To explore the systemic effects of the compounds in an *in vivo* model, C57BL/6 mice were inoculated with B16 melanoma cells and treated with either **TADM** or anti-PD-1. Mice were sacrificed on day 12 of the experiment (Figure 2C). Single-cell suspensions of spleen samples were analysed by flow cytometry to characterise any changes in inflammatory cell numbers (Figure 4A). The number of B cells, CD4 or CD8 T cells, or natural killer cells in the spleen were not significantly altered in any of the treatment groups, consistent with a previous report indicating no significant effect of anti-PD-1 in the same syngeneic subcutaneous melanoma model.[18]

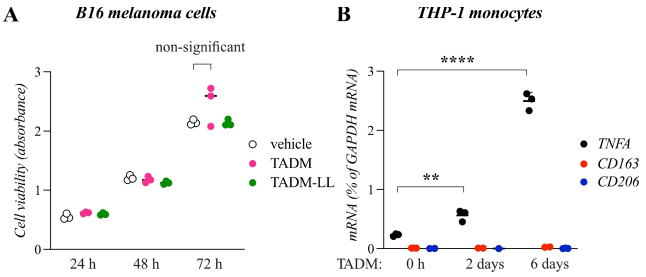

Figure 3. Effect of the glycocluster compounds *in vitro*. A: The indicated compounds (0.05 mg/ml) were tested for their effect on B16 melanoma cell growth using MTS cell viability assays (n = 2) and one-way ANOVA. B: **TADM** (0.05 mg/ml) was tested for its effect on polarisation of THP-1 monocytes to express proinflammatory M1-type (TNFA) or anti-inflammatory M2-type (CD163 and CD206) macrophage markers by real-time RT-PCR (n = 3). The median for each group is indicated with a line, one-way ANOVA. **, $P < 0.01$, ****, $P < 0.0001$.

The number of Ly6 C-high monocytes or macrophages derived from these progenitors was not changed following the tested treatments. Instead, Ly6 C low monocytes were moderately increased following **TADM** and PD-1 treatment ($P =$ **0.040 and** 0.001, respectively). The Ly6 C low monocytes patrol the endothelial surface in the blood vessel and recruit neutrophils as required.[19] Also, the number of neutrophils was significantly increased following both tested treatments ($P =$ 0.012 for **TADM** and $P < 0.0001$ for anti-PD-1 compared to untreated control cells).

Gene Expression in the Spleen

To further understand the systemic inflammatory effects of the treatments, the spleen samples isolated from the **TADM**- and anti-PD-1-treated mouse melanoma models were also analysed by real-time RT-PCR (Figure 4B). Expression of the gene encoding the proinflammatory cytokine IFN- ɣ was found to be significantly increased following treatment with both anti-PD-1 ($P = 0.0008$) and TADM ($P = 0.026$). However, no significant differences were observed in the expression of T-bet (Tbx21), a transcription factor crucial in the lineage development of CD4 helper T cells into Th1 cells, as well as in the differentiation of other T cell subtypes.[20] Notably, mirroring the impact of **TADM** on spleen neutrophil counts, administration of **TADM** also led to an increase in the expression of the gene responsible for encoding myeloperoxidase (MPO) ($P = 0.021$) (Figure 4B), an enzyme highly expressed in neutrophils and an integral microbicidal protein involved in neutrophil-driven inflammation.[21] MPO expression displayed a trend towards elevation in animals treated with anti-PD-1; however, this increase did not achieve statistical significance ($P = 0.14$) (Figure 4B).

Immunohistochemical Analysis of Tumour Sections

In an effort to elucidate the treatments' tumour growth-preventing effects, immunohistochemical staining of paraffin-embedded tumours was carried out. However, no significant difference was observed in the number of CD3-positive lymphocytes or the blood vasculature in any treated tumours compared to control animals (Figure 5A).

To investigate the immune cell infiltration into the tumour microenvironment more comprehensively, immunohistochemical staining for primary macrophage and T cell subtypes was carried out and quantified as positive cells/mm2 using QuPath software 0.4.4.[22] (Figure 5B). No discernible differences were observed in the number of F4/80+ macrophages across all treatment groups. Furthermore, neither treatment induced the polarisation of macrophages to skew either to M2-like immunosuppressive (CD206) or M1-like proinflammatory (CD86) phenotypes. Likewise, no statistically significant differences were detected between the two treatment groups and the control group in terms of cytotoxic CD8 T cell infiltration in the tumours. Furthermore, the analysis of neither CD4 helper T cells nor FoxP3+ regulatory T cells showed differences between treatment groups.

A comprehensive description of all methodologies employed is available in the supplementary information (**S1**).

Discussion

We show here that a synthetic trivalent mannobiose **TADM** is alone sufficient to reduce the growth rate of melanoma tumours and stimulate apoptosis in a syngeneic B16 melanoma model. The growth inhibition was associated with increased IFN-ɣ expression and the number of neutrophils in the spleen, indicating a systemic immune reaction was elicited in response to treatment with **TADM**. **TADM** did not directly affect the viability of the B16 cells *in vitro*, further supporting an indirect systemic or stromal effect. However, when tumour tissues were analysed at the end of the treatment,

Cancer tumours induce a weak immune response due to poor antigens and immunosuppressive mechanisms, such as the type 2 immune response milieu tumours utilise. The enhancement of immune response against cancer cells has recently been the hotspot of cancer research. It includes the disruption of the immunosuppressive pathways, for example, by blocking the PD-1 or CTLA-4 related pathways on T cells. In addition, the presence of microbial particles enhancing the innate immune reaction via TLRs is shown to produce antitumor activity. In addition to innate immune reaction, the bacterial TLR ligands can enhance T cell activity, putatively through APC activation, and induce IFN-ɣ production.[23]

TADM is a synthetic trivalent acetylated glycocluster of β-1,2-linked dimannoses and a transmembrane macrophage activator independent of the mannose receptor.[24] **TADM** has been shown to induce Th1 type immune responses and suppress Th2 type cytokines in ovalbumin-induced acute and timothy-induced chronic allergic inflammation models in vivo.[9,10] These observations encouraged us to test whether **TADM** could also facilitate anti-tumour immune responses similar to what has been observed with the immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4. A syngeneic

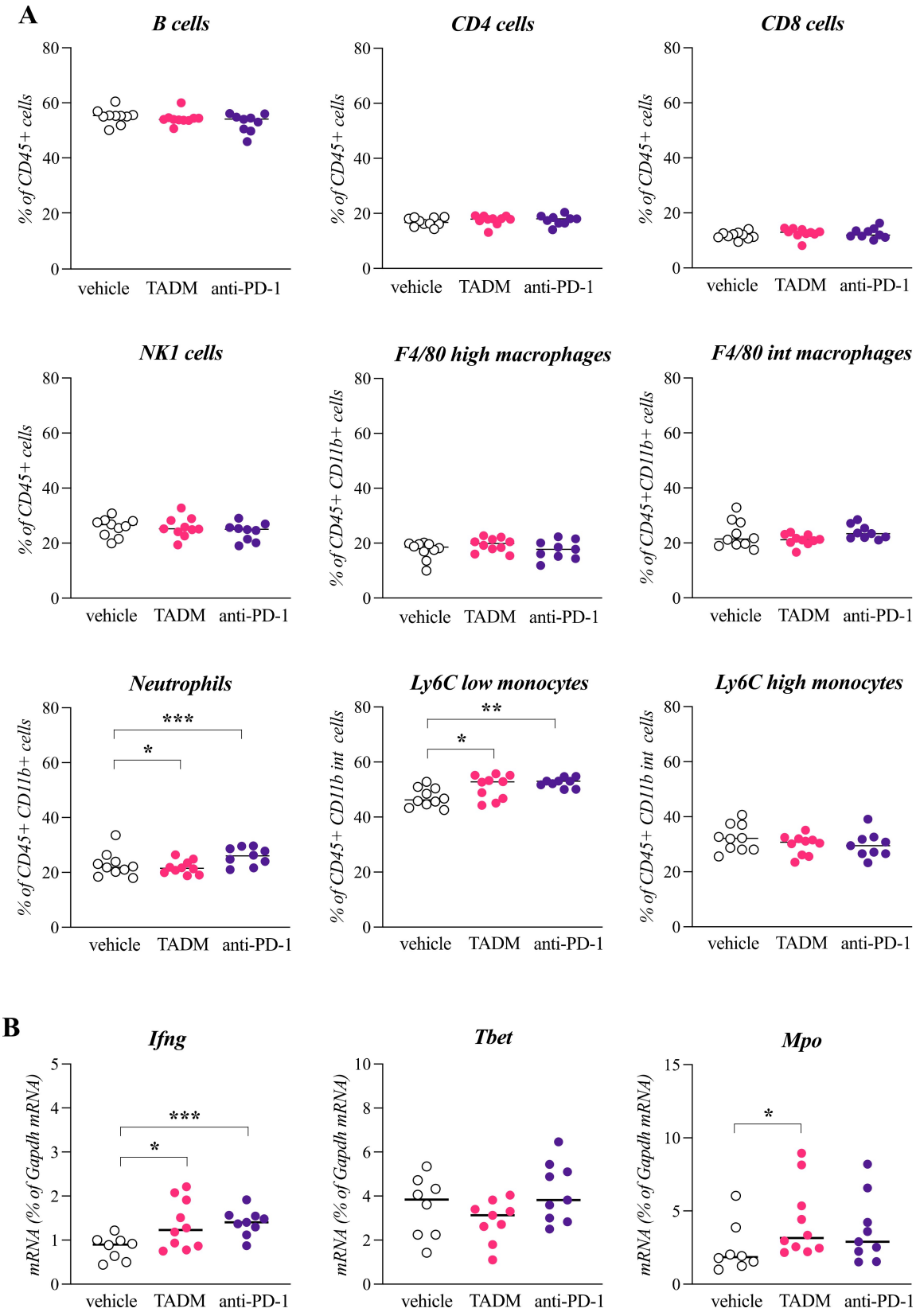

Figure 4. Inflammatory cells and cytokine mRNA expression in the spleens of TADM- and anti-PD-1 treated mice carrying B16 melanoma tumours. A: Flow cytometry analysis of inflammatory cells in the spleen (n = 9–10 per treatment group). One-way ANOVA and Kruskal-Wallis test for normally and non-normally distributed data, respectively. Shapiro-Wilk test was used to test for the normality of data. B: Gene expression in spleen samples analysed by real-time RT-PCR. Mice (n = 9–10 per treatment group) were treated as shown in Figure 2A and sacrificed on day 12 of the experiment. The median for each group is indicated with a line, unpaired t-test. *, $P < 0.05$: **, $P < 0.01$; ***, $P < 0.001$.

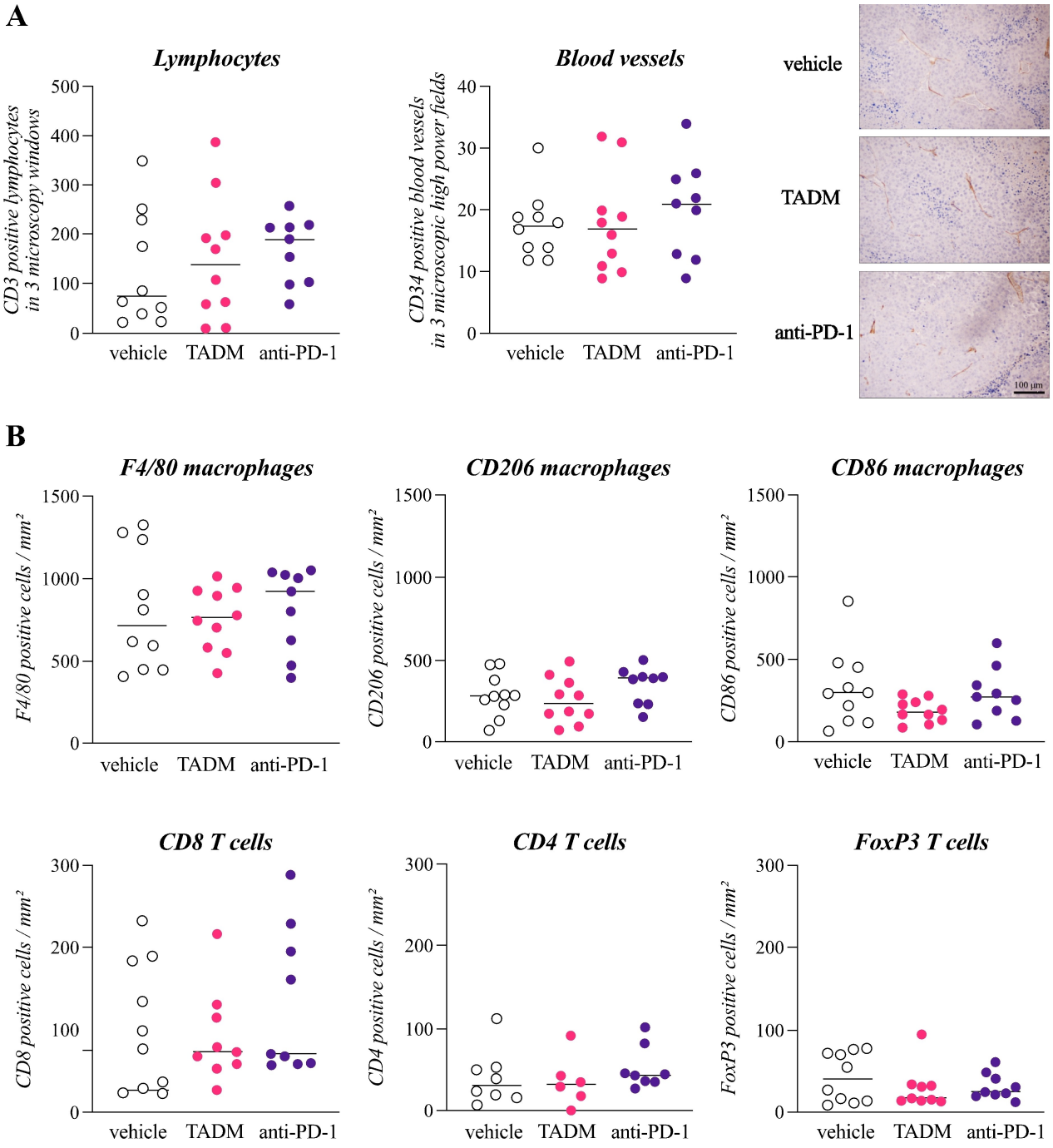

Figure 5. Infiltrating immune cells and blood vasculature in the B16 melanoma tumour microenvironment of TADM- and anti-PD-1-treated mice. A: Manual quantification of CD3-positive lymphocytes (left) and CD34-positive blood vessels (right) in tumours in three microscopy fields (n = 9–10 per treatment group). Kruskal-Wallis test. B: Immune cell infiltration in tumour microenvironment quantified from immunohistochemical stainings with QuPath software 0.4.4. Mice (n = 9–10 per treatment group) were treated as shown in Figure 2A and sacrificed on day 12 of the experiment. The median for each group is indicated with a line. One-way ANOVA and Kruskal-Wallis test for normally and non-normally distributed data, respectively. The Shapiro-Wilk test was used to test for normality of data.

mouse melanoma model was selected since metastatic melanoma has been successfully targeted by checkpoint inhibitors in the clinic.

When the systemic effects were analysed using samples obtained from the C57BL/6 mouse spleens at the end of the treatment regimen, both **TADM** and anti-PD-1 were observed to increase IFN-γ expression moderately. IFN-γ is expressed and secreted primarily by activated CD4+ Th1 cells, CD8+ cytotoxic T cells, γδ T cells, and natural killer cells.[25] The anti-human PD-1 antibody nivolumab has consistently been found to increase IFN-γ expression in CD4 T cells.[26] As there was no increase in the number of any of the lymphoid lineages tested following **TADM** or anti-PD-1 treatments in the spleens in our syngeneic model, one could hypothesise that the cells present were more

active in producing IFN-ɣ. In any case, the induction of IFN-ɣ expression in the spleens supported the activity of **TADM** in favouring a Th1-biased immune phenotype.

Notably, as assessed by flow cytometry, **TADM** and anti-PD-1 also moderately but significantly increased the number of Ly6 C low monocytes in the spleens. Ly6 C low monocytes have been documented to patrol the blood vessel lumens and recruit neutrophils.[27,28] Consistently, the number of neutrophils also increased in the spleens of **TADM**- and anti-PD-1-treated mice. Neutrophils have been found to exist in two phenotypes: N1 and N2. N2 neutrophils promote tumour growth.[29] MPO is highly expressed in N1 neutrophils. MPO expression was consistently increased in **TADM**- and anti-PD-1-treated groups.

Macrophages are highly plastic cells of the innate immune system and take cues from their surrounding microenvironment. Depending on the surrounding immunological environment, they exist on a polarisation spectrum from proinflammatory (M1-like) to immunosuppressive (M2-like). M1-like macrophages are the classical macrophages that secrete TNF and phagocyte and kill, among others, tumour cells, while M2-like macrophages, albeit important in tissue homeostasis, are also tolerogenic, secreting IL-4 and IL-10.[30] There is a strong opinion that the tumour-associated macrophages that promote tumour growth and metastasis skew towards an immunosuppressive, M2-like phenotype. **TADM** has been shown to be able to enter macrophages in vitro directly through the cell membrane without binding to the mannose receptor, and the acetyl groups are at least partially cleaved inside the cell.[24] The activated M1-like macrophages can enhance the adaptive immune response against melanoma cells by activating both Th1 and CD8 cytotoxic lymphocytes.

Immunohistochemical staining of the tumour microenvironment revealed no distinct differences in the abundance of immunosuppressive or proinflammatory macrophages and cytotoxic, helper, or regulatory T cells between the treatment groups and the control cohort. Although reported to be moderately responsive to anti-PD-1 treatment as a monotherapy.[31,32] The B16 melanoma model utilised in this study is recognised for its immunologically 'cold' phenotype, characterised by limited immune cell infiltration and activity within the tumour microenvironment.[18,33–35] The pronounced anti-tumour effects observed in the **TADM** treatment cohort, despite the immunologically inert nature of the model, highlight the potential translational impact. Significantly, also anti-PD-1 did not induce significant changes in the measured immune cell population in this model while promoting a similar reduction in tumour growth to **TADM**. Thus, further investigation is warranted in an alternative, more immunogenic model to elucidate the mechanisms underlying the observed therapeutic efficacy.

Conclusions

In summary, our findings show the potential of synthetic beta-1,2-linked manno-oligosaccharide adjuvants, mimicking fungal mannans, as potent suppressors of melanoma tumour growth. **TADM** likely activates M1-like macrophages, type N1 neutrophils, and CD8+ and Th1 T cells, suppressing the type 2 immune response milieu of melanoma tumours with a robust type 1 immune response. While these results shed light on a mechanism of action for **TADM** that involves other cell types than the cancer cells themselves, the molecular and cellular details remain to be elucidated. Moreover, exploring the synergistic potential of **TADM** with cytotoxic or targeted cancer therapies indicates intriguing avenues for future research, warranting thorough examination of its role as an immunoadjuvant.

Supporting Information Summary

Please see supporting information for experimental methods and flow cytometry analysis workflow. All trivalent mannobioses tested in this work were from earlier batches, prepared as described previously.[7,8] Complete characterization data for these compounds, including ^{1}H and ^{13}C NMR, MALDI-TOF or HRMS, and optical rotation, is available in these original articles and/or their corresponding supporting information.

Acknowledgements

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Adjuvant · Cancer · Glycocluster · Melanoma · Immunotherapy

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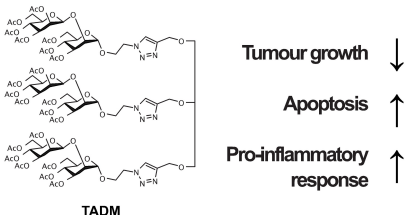
RESEARCH ARTICLE

Triacedimannose (TADM) a novel synthetic glycocluster molecule and an activator of macrophages, is able to induce pro-inflammatory responses in vivo. Here, we show that TADM demonstrates efficacy in a murine melanoma model in hindering tumour growth, inducing apoptosis, and enhancing anti-tumour immune response comparable to established anti-PD1 checkpoint inhibitor treatment.

Synthetic oligosaccharide as immunoadjuvant for cancer therapy



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Immunomodulatory Synthetic Glycocluster Molecule Prevents Melanoma Growth *In Vivo*