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Immunomodulatory effects of nanoparticles on dendritic cells in a model of allergic contact dermatitis: importance of PD-L2 expression

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Nanoparticle (NP) skin exposure is linked to an increased prevalence of allergic contact dermatitis. In our prior studies using the mouse contact hypersensitivity (CHS) model, we reported that silica 20 nm (SiO_2) NPs suppressed the allergic response and titanium dioxide NPs doped with manganese ($mTiO_2$) exacerbated it. In this work, we conducted in vitro experiments using bone marrow-derived dendritic cells (BMDCs) to study the combinatorial effect of the potent 2,4-dinitrofluorobenzene (DNFB) hapten sensitizer with SiO_2 and $mTiO_2$ NPs on BMDC cytotoxicity, cytokine secretion and phenotype using the B7 family ligands. Results show that DNFB and $mTiO_2$ behave similarly and exhibit proinflammatory characteristics while SiO_2 promotes a naive phenotype. We observe that the B7-H3 (CD276) ligand is only expressed on CD80 + (B7-1) BMDCs. Results from adoptive transfer CHS studies, combined with BMDC phenotype analysis, point to the importance of PD-L2 expression in modulating the adaptive immune response. This work identifies metrics that can be used to predict the effects of NPs on contact allergy and to guide efforts to engineer cell-based therapies to induce hapten specific immune tolerance.

The prevalence of allergic skin disorders and adverse skin reactions are on the rise worldwide, contributing to severe morbidity, and having a significant impact on the patient quality of life[1,2]. It was estimated that the median prevalence of contact allergy is 21.2% (range 12.5–40.6%) in the general population of North America and Western Europe[3]. Irritant contact dermatitis results from an acute activation of the innate skin immune system following skin exposure to various types of chemicals (e.g. soaps, perfumes, solvents)[4]. Exposure to low molecular weight haptens can induce an adaptive immune response resulting in allergic contact dermatitis (ACD)[4–7]. In the sensitization phase of ACD, antigen-presenting cells in the skin get activated. They uptake haptenized proteins and migrate to the skin-draining lymph nodes where they present antigen to naïve T-cells that differentiate into CD8+ and/or CD4+ effector and memory T cells. Noncirculating tissue-resident memory (T_{rm}) cells seed the skin in 14–30 days and persist long term (> 1 year) in mouse models of ACD[8,9]. In the challenge phase, re-exposure to the hapten activates the antigen-specific memory T cells to induce an allergic response.

There are numerous chemicals in the home, workplace, and in the environment that can contribute to irritant and/or ACD. Recent studies suggest that environmental factors, including nanoparticles (NPs), can influence the prevalence and course of allergic disease, and NPs are suspected to be a contributing factor to the rise of ACD[10–12]. Sources of NPs are numerous. Natural sources include forest fires, soil erosion, dust storms, and volcanic activity, whereas anthropogenic sources include those produced in the laboratory for commercial use and those present in air pollution from factories, automobiles, and cigarette smoke[13–15]. Particulates in air pollution are known to induce oxidative stress and inflammation in the skin[16,17]. Studies suggest that oxidative stress in the skin is an initiating event that results in an immunosuppressive ACD response by activating the platelet-activating factor receptor (PAF-R) signaling pathway[18,19]. Prior studies in our lab reported that engineered NPs can modulate the adaptive immune response in a mouse model of allergic contact hypersensitivity (CHS)[20]. We observed that small (< 200 nm) negatively charged NPs, independent of composition, suppressed the allergic response in the

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challenge phase but similar sized positive NPs did not[21]. Moreover, certain NPs including highly carboxylated carbon nanotubes (CNT) and some TiO_2 NPs could exacerbate the allergic response[21–24]. The mechanisms of how NPs alter the adaptive immune response in the skin remain unclear, however, we did observe that NPs modulated early signaling events in the challenge phase (< 2 h)[21]. Since, NPs do not readily diffuse through the skin barrier as small molecular weight chemical haptens[20,25–29], it seems plausible that the NPs must alter epidermal derived signals that can affect mast cell (MC) and/or dendritic cell (DC) activation or function. These two cell types are critically important in transducing the CHS allergic response[30–32]. Mast cell deficiency in the skin dramatically reduces the allergic response due in part to impaired emigration of skin DCs to the lymph node in the sensitization phase[32–34]. Activated MCs are an important source of TNFα that promotes DCs to migrate to lymph nodes[34]. Furthermore, MCs and DCs interact to activate each other[7,32].

Recent studies in mice found that skin DCs (CD11c+, MHCII+), specifically epidermal Langerhans cells (CD103−, EpCAM+) and conventional cDC2 (CD103−, CD11c+, CD11b+), acquired OVA antigen in the skin and primed T cells in the lymph node but Langerhans cells were not required or sufficient to induce effector T cell response[35]. In fact, the function of epidermal Langerhans cells in the development of the CHS adaptive immune response has been debated and intensely studied for years using genetic ablation strategies[36–40]. It is now accepted that the intensity of the CHS response correlates directly with the efficiency of T cell priming, and that LCs are important only at low hapten exposure[38]. Dermal DCs subsets, specifically cDC2, are mainly responsible for the initiation and activation of the CHS response[31,38,41]. While, it is recognized that the physiochemical properties of NPs can alter DC function[42], how NPs may alter phenotypic changes in cDC2 to effect the efficiency of T cell priming in the context of skin allergy is unknown.

In this work, we focus on examining the effect that NPs have on the phenotype and activation of bone marrow-derived (CD11c+, MHCII+) dendritic cells (BMDC). Specifically, we studied ~ 20 nm negatively charged silica (SiO_2) NPs that suppressed the in vivo CHS response and < 100 nm negatively charged manganese-doped titanium dioxide NPs ($mTiO_2$) that exacerbated it[21]. Using flow cytometry, cytokine analysis, and the well-established dinitrofluorobenzene (DNFB) CHS mouse model, we characterized the BMDC phenotype focusing on several B7 family of co-stimulatory markers[43] including CD80 (B7-1), CD86 (B7-2), PD-L1 (B7-H1), PD-L2 (B7-DC) and CD276 (B7-H3). The rationale for selecting these markers is given below.

CD86 and CD80 are classic markers of DC activation and they have dual binding capacity to CD28, an activating T cell receptor that is constitutively expressed on naïve T cells and CTLA−4 a regulatory receptor which is upregulated upon T cell activation[44]. The dual binding capacity of CD86/CD80 to T cell receptors that enhance (CD28) or suppress (CTLA-4) proliferation has been the subject of much investigation[45–48]. CD80 and CD86 act cooperatively to modulate T-cell activation and tolerance induction[43,47]. It is believed that T cell fate is driven by the relative expression levels of the CD86/CD80 ligands on the DC and the CD28/CTLA-4 T cell receptors[46,49,50] as well as the fact that the binding affinity of CD86 and CD80 ligands to CTLA-4 is ~ 10 × stronger than to CD28 which leads to competitive binding between the activating and regulatory receptors[44,51,52].

Programmed death ligand 1 (PD-L1) and PD-L2 are ligands expressed on DCs that bind the PD-1 receptor on activated T-cells. Activation of the PD1 receptor inhibits T cell proliferation and proinflammatory cytokine production to suppress immune responses[53]. PD-L1 (B7-H1) is widely expressed on many cell types, including cancer cells, whereas PD-L2 (B7-DC) is only expressed on DCs[52]. It is reported that PD-L2 binds PD-1 with two to sixfold stronger affinity compared with PD-L1[54,55]. Others report that PD-L1 and PD-L2 bind PD-1 with comparable affinities but exhibit striking differences in their PD1 receptor association and dissociation characteristics[56]. Since PD-L1 and PD-L2 expression levels depend on distinct stimuli, it is suggested that they may have overlapping and differential roles in regulating T_H1 and T_H2 T cell responses[56,57]. Studies suggest PD-L2 positive DCs are needed to induce allergen tolerance by generating regulatory T cells (T regs)[58] and they contribute to generating potent LAP + Tregs[35].

CD276 (B7-H3) is a member of the B7 immune checkpoint family and is thought to promote an immunosuppressive response as it is highly expressed in many cancers that correlate with poor clinical outcomes[59]. Our interest in this marker stems from a study that showed activation of the arylhydrocarbon receptor in BMDCs generated Tregs cells that suppressed the allergic CHS response in mice[60]. Analysis of the DC markers (MHCII, CD86, PD-L1, B7-H3, B7-H4) following arylhydrocarbon receptor agonist exposure revealed a marked upregulation of B7-H3 which was associated with the immunosuppression[60].

In this study we identified key markers of BMDC activation by proinflammatory stimuli (lipopolysaccharide, DNFB, $mTiO_2$) including a upregulation of the CD86, CD80, and PD-L1 (B7-H1), as well as an upregulation of CD276 (B7-H3) and a negligible to slight downregulation of PD-L2 (B7-DC). Because of the dual binding affinity of CD80 and CD86 ligands to the CTL-4 (regulatory) and CD28 (activating) T-cell receptors, we further examined how the expression levels of PD-L1, PD-L2 and CD276 differed among the CD80/CD86 subpopulations compared to unstimulated immature BMDCs (imDCs). We observe that CD276 is only expressed on CD80+ cells and that both NPs modulate its expression. In single exposure studies, we find that DNFB and $mTiO_2$ behave similarly and exhibit proinflammatory characteristics. In contrast, SiO_2 is cytoprotective and promotes a naive imDC phenotype, particularly in DNFB co-culture studies. While PD-L1 is upregulated by proinflammatory stressors, PD-L2 is not and in fact, is down regulated by $mTiO_2$ exposure which correlates with an exacerbation of the allergic response in the in vivo adoptive transfer contact hypersensitivity (CHS) mouse model. These results show that NPs co-cultured with a potent sensitizer can alter the BMDC phenotype to effect the efficiency of T cell priming and the intensity of the CHS response in both the sensitization and challenge phases. This work points to metrics that can be used to predict the effects of NPs on contact allergy and to the novel use of NPs to engineer immunomodulatory responses in contact allergy.

Results

Effects of DNFB, SiO_2 and $mTiO_2$ on BMDC toxicity, cytokine secretion and co-stimulatory molecule expression

We performed cytotoxicity studies to establish concentration ranges to examine the effects of NPs and DNFB on BMDC phenotypes. We conducted single exposure studies to each stressor for 1 h to measure cytotoxicity as a function of concentration using the flow cytometry. Results (Fig. 1A) show a significant dose-dependent decrease in cell viability for DNFB and $mTiO_2$ but not for SiO_2. Exposing cells for 1 h to DNFB (0.1 mM) or $mTiO_2$ (0.05 mg/mL) caused a ~ 50% decrease in cell viability whereas exposing cells to SiO_2 was not cytotoxic. The effects of DNFB exposure correlated with notable changes in the BMDC morphology viewed in bright-field and in TEM images, with cells becoming round and losing dendrites (Figs. S1–S2). NP uptake in endosomal vesicles was also confirmed (Figs. S3–S4). Non-cytotoxic concentrations of each stressor were selected and the BMDCs were exposed for 5 h, 15 h, and 24 h (Fig. 1B). Results again show a significant decrease in cell viability over time for DNFB (0.001 mM) and $mTiO_2$ (0.005 mg/mL) but not for SiO_2 (0.01 mg/mL). The dose and time dependent cytotoxicity flow cytometry results were consistent with results using the PrestoBlue assay (Fig. S5).

Cytotoxicity studies suggest that DNFB and $mTiO_2$ behave similarly and are likely potent proinflammatory stressors compared to SiO_2. To further characterize interactions with BMDCs, we evaluated the key proinflammatory (IL-6, TNFα) and immunosuppressive (IL-10) cytokines secreted in the supernatant by ELISA. BMDCs were exposed to DNFB (0.001 mM), SiO_2 (0.01 mg/mL) and TiO_2 (0.005 mg/mL) as a function of time. Results showed that each compound increased IL-6 (Fig. 2A) and TNFα (Fig. 2B) secretion and exposure to $mTiO_2$ upregulated IL-10 (Fig. 2C). One-hour exposures as a function of concentration also showed that $mTiO_2$ upregulated IL-6,

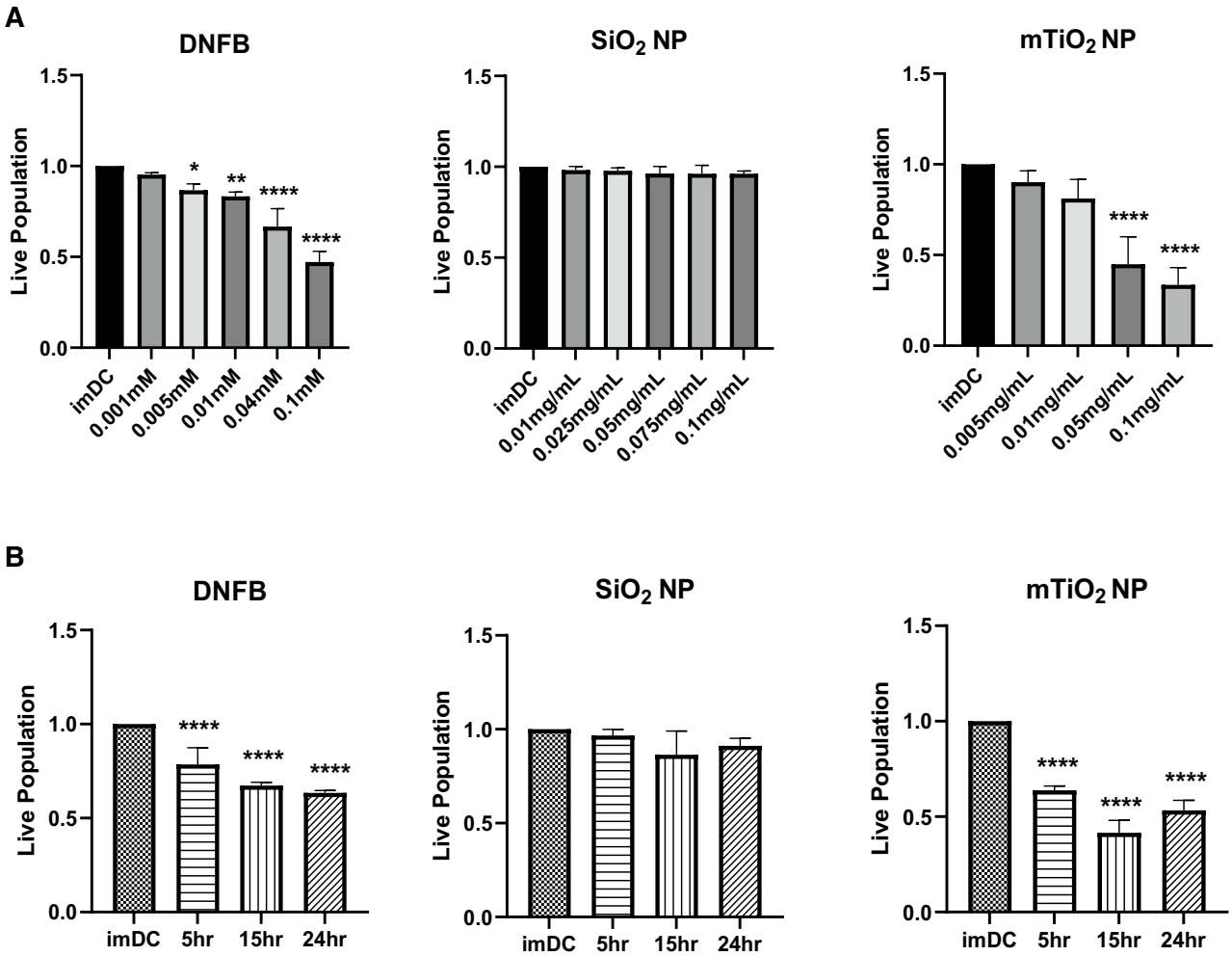

Figure 1. Effects of DNFB, SiO_2 and $mTiO_2$ exposure on BMDC cytotoxicity as a function of concentration and time. BMDCs were harvested on day 8 and treated with DNFB, SiO_2 and $mTiO_2$ to study cytotoxicity as a function of concentration and time by flow cytometry. (**A**) Cell viability as a function of concentration for a 1 h exposure. (**B**) Cell viability following exposure to the lowest non-cytotoxic concentrations from (**A**) and exposed over a period of 5 h, 15 h and 24 h. Results indicate that cytotoxicity of DNFB and $mTiO_2$ NPs on BMDCs was dose- and time- dependent. Live population was normalized the imDC no treatment control. Ordinary one-way ANOVA was performed and compared to imDC. N = 3–5. Mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

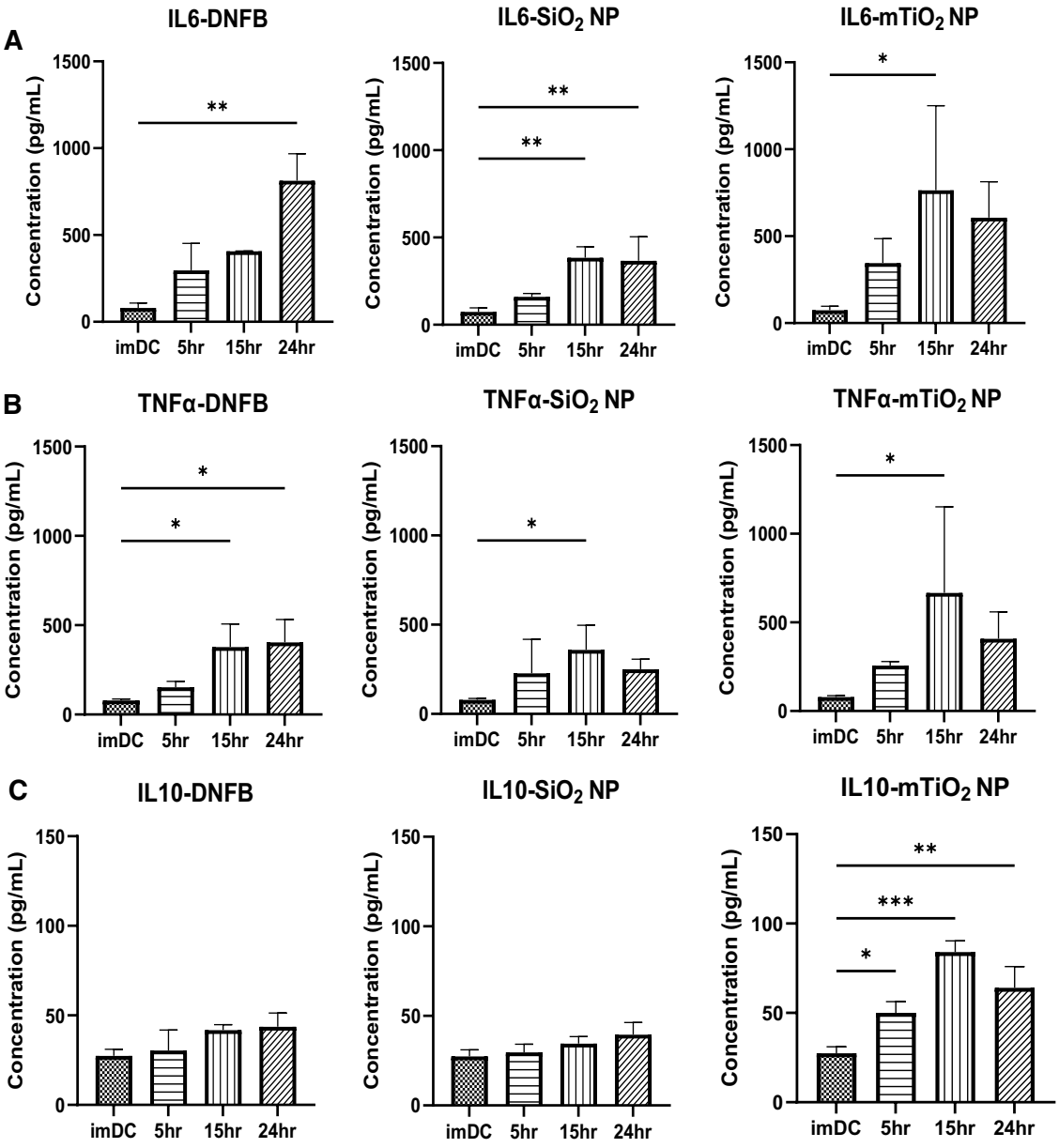

Figure 2. Effects of DNFB, SiO_2 and $mTiO_2$ exposure on BMDC cytokine secretion. BMDCs were exposed to a low non-cytotoxic concentrations of each stressor (0.001 mM DNFB, 0.01 mg/mL SiO_2 and 0.005 mg/mL $mTiO_2$) over a period of 5 h, 15 h and 24 h. ELISA was used to analyze cytokines in cell culture supernatants: (**A**) IL-6**,** (**B**) TNFα, (**C**) IL-10. All stressors tend to increase IL-6 and TNFα. Only $mTiO_2$ increased IL-10. Concentration was normalized against % of live cells. Ordinary one-way ANOVA was performed and compared to imDC. N = 3–5. Mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

TNFα, and IL-10 (Fig. S6). These results suggest that $mTiO_2$ is a potent proinflammatory stressor, behaving similarly to lipopolysaccharide (LPS) (50 ng/mL), that also upregulated IL-6, TNFα and IL-10 (Fig. S7). These observations were corroborated by intracellular flow cytometry staining for IL-10 and TNFα as a function of concentration and time (Figs. S8–S9) but in contrast to $mTiO_2$, we observe that SiO_2 tended to upregulate IL-10 without upregulating TNFα which, may suggest an immunosuppressive potential for SiO_2 exposure.

Next, we examined the effect of each stressor on the phenotype of the BMDCs by measuring the expression levels of the co-stimulatory molecules by flow cytometry. Single and double gating strategies, with FMO controls, were used (Fig. S10A–C). Single gating assesses the expression levels of each marker on the live cell population. Double gating analyzes the CD11c + MHCII+ subpopulation. To interpret results, we compared them to LPS exposure (50 ng/mL) over time which, for single gating (Fig. S11) and double gating (Fig. S12), induced the expression of CD86, CD80, and PD-L1. CD276 increased early (3–6 h) and then returned to baseline and no changes in PD-L2 expression were induced by LPS. For single gating, exposing BMDCs to DNFB (0.001 mM), SiO_2 (0.01 mg/mL), and $mTiO_2$ (0.005 mg/mL) caused differential changes in the CD11c + MHCII + phenotype as a function of time (Fig. 3) with DNFB producing effects most similar to LPS exposure. DNFB and $mTiO_2$

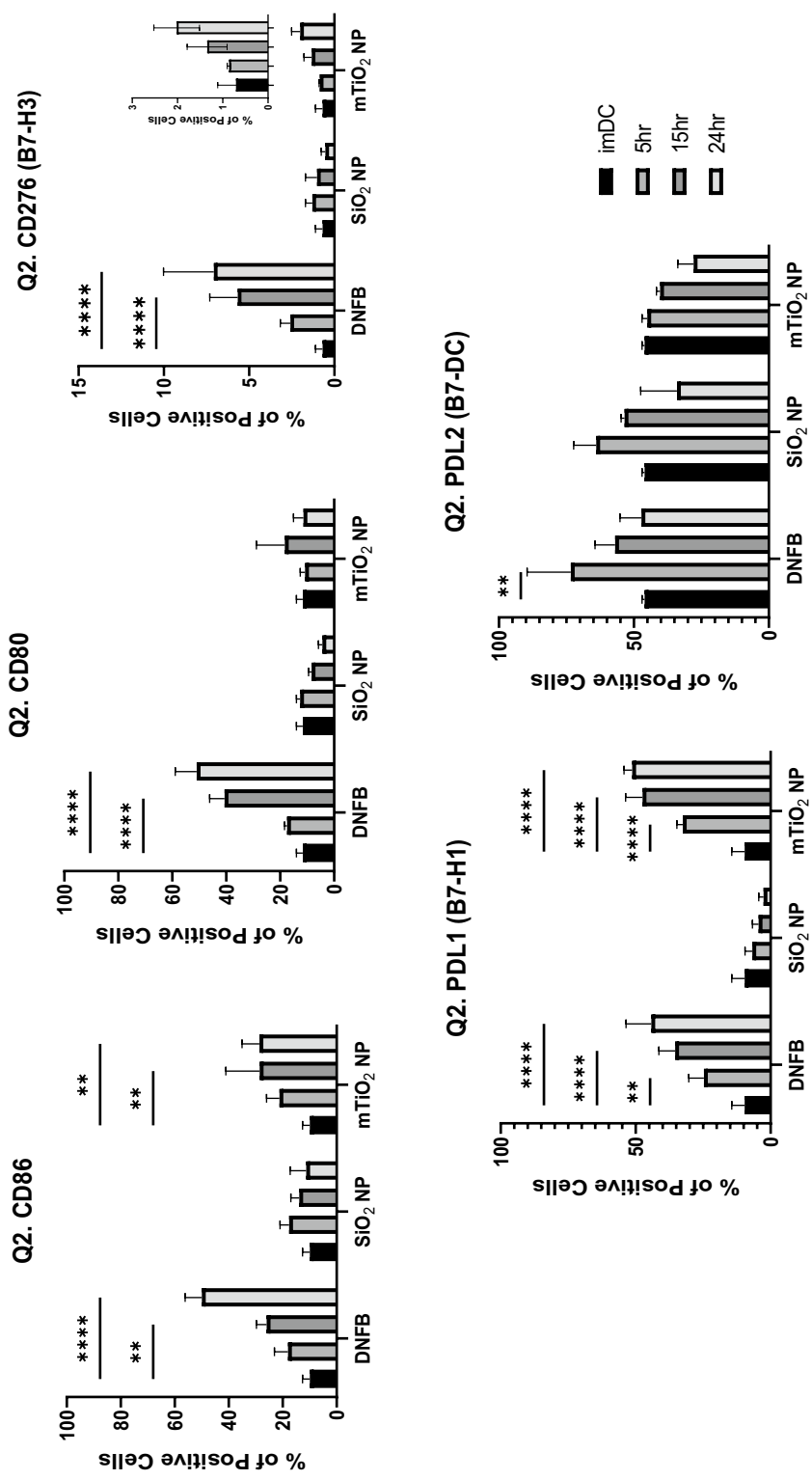

Figure 3. Co-stimulatory molecules from the B7 family quantified by flow cytometry gated on the CD11c+MHCII+subpopulation following BMDC exposure to each stressor over time. The phenotypic characteristics of BMDCs following exposure to a low concentration of each stressor (0.001 mM DNFB, 0.01 mg/mL SiO_2 and 0.005 mg/mL $mTiO_2$) was followed over time by flow cytometry gated under the CD11c+MHCII+(Q2.) subpopulation. DNFB and $mTiO_2$ behave more similar with upregulation of CD86, CD276, and PD-L1 over time. Only DNFB upregulated CD80. Exposure to SiO_2 did not alter these B7 ligands over time. Ordinary one-way ANOVA was performed and compared to imDC. N = 3–5. Mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

upregulated CD86 and PD-L1 expression whereas CD80 and CD276 were only upregulated by DNFB over time. PD-L2 was expressed on ~ 50% of imDC and exposure to each stressor did not alter its expression. Exposure to SiO_2 did not alter any co-stimulatory molecule suggesting BMDC maintained a naïve phenotype. Taken together, the results suggest that DNFB and $mTiO_2$ induce an activated BMDC phenotype and that $mTiO_2$ may be a more potent stressor as it failed to upregulate CD80, which is important for binding CTLA-4 to promote regulation.

The differential effects of each stressor on the expression of CD80 and CD86 led us to investigating the expression of PD-L1, PD-L2, and CD276 within the CD86/CD80 subpopulations over time (Fig. 4). Results showed that exposure to DNFB (0.001 mM) decreased the naïve CD80-CD86- double negative (DN) population and induced a steady significant rise in the activated $CD80^+CD86+$ double positive (DP) population which are trends expected for a proinflammatory stressor. Similarly, $mTiO_2$ (0.005 mg/mL) showed a trend toward upregulating the DP population, whereas SiO_2 (0.01 mg/mL) exposure over time did not. Both NPs tended to downregulate slightly the DN subpopulation up to 15 h but not as definitively as DNFB. This subtle decrease in the DN is due primarily to the upregulation of CD80+ single-positive cells (Fig. S13). The expression levels of CD276, PD-L1 and PD-L2 in the CD80/CD86 subpopulations also differed depending on the stressor, with DNFB and $mTiO_2$ exhibiting similar trends. Specifically, DNFB and $mTiO_2$ exposure up-regulated PD-L1 in the DN (Fig. 4) and the single positive subpopulations (Fig. S13) with no change in the DP subpopulations. In contrast, SiO_2 exposure down-regulated PD-L1 expression in the DP subpopulation over time with no change in DN subpopulation. No changes in PD-L2 expression in DP or DN subpopulations were induced by either stressor (Fig. 4) but decreases in PD-L2 expression in the CD86 and CD80 single positive cells were induced by SiO_2 only (Fig. S13). CD276 was only expressed CD80+ single (Fig. S13) and double positive cells (Fig. 4). Both NPs tended to increase CD276 on the activated DP cells at 24 h. In summary, this data shows that DNFB and $mTiO_2$ exposure promotes the upregulation of the activated DP phenotype, and increased PD-L1 expression in the naïve DN population over time. PD-L1 expression of an activated DP population was unchanged over time with exposure to DNFB and $mTiO_2$, whereas SiO_2 exposure tended to decrease PD-L1 expression pointing again to the similarities between DNFB and $mTiO_2$.

Effects of DNFB co-exposure with NPs on cytotoxicity, cytokine secretion and and co-stimulatory molecule expression

In prior studies, NPs co-exposed with DNFB modulated the in vivo CHS response in the challenge phase[21]. In this work, we were able to observe clear differences between SiO_2 and $mTiO_2$ in single exposure studies of cytotoxicity, cytokine production, and on phenotypic alterations of BMDC, with $mTiO_2$ behaving more similarly to DNFB. Here, we studied the effects of DNFB co-exposure with NPs on cytotoxicity and cytokine secretion compared to DNFB alone at 1 h (Fig. S14 and 24 h (Fig. 5). Consistent with earlier studies (Fig. 1), BMDC cultured with DNFB (0.001 mM) alone for 24 h was cytotoxic. Co-culture with SiO_2 (0.01 mg/mL) showed a protective effect (Fig. 5A). In contrast, co-culture with $mTiO_2$ (0.005 mg/mL) significantly exacerbated the toxic response (Fig. 5B). A cytoprotective effect of SiO_2 was also observed at 1 h exposure (Fig. S14) and is consistent with prior studies in keratinocytes[23] and fibroblasts[61]. We also tested the supernatant of each treatment group for IL-6, TNFα, and IL-10 by ELISA. Results show a downregulation of IL-6 with DNFB co-cultured with SiO_2 but not with $mTiO_2$ (Fig. 5C,D). Co-culture with either NP did not alter the levels of TNFα produced by DNFB exposure at 24 h (Fig. 5E,F) however, co-culture for 1 h with $mTiO_2$, but not SiO_2 NPs, showed elevated IL-6 levels (Fig. S14D) and both NPs elevated TNFα above that produced by DNFB at 1 h (Fig. S14E,F). Co-culture with $mTiO_2$, but not SiO_2 at 24 h (Fig. 5G,H), increased the secretion of IL-10, which is consistent with the $mTiO_2$ single exposure studies (Fig. 2). These co-culture studies demonstrate that SiO_2 exhibits a cytoprotective against effect DNFB exposure and exhibits an immunosuppressive effect as measured by a reduction in IL-6 secretion.

Next, the effects of DNFB co-exposure with NPs on the expression of the costimulatory molecules was investigated using flow cytometry (Fig. 6). Double gating on the CD11c+ MHCII+ subpopulation showed that co-exposing BMDC to DNFB (0.001 mM) with either SiO_2 (0.01 mg/mL) or $mTiO_2$ (0.005 mg/mL) similarly down-regulated the DNFB activation of CD86, CD80, CD276, and PD-L1. Co-exposure with $mTiO_2$ tended to downregulate PD-L2 expression to levels below the imDC levels.

Differentiating the effects of DNFB co-exposure with NPs on the expression levels of PD-L1, PD-L2 and CD276 in the BMDC CD80/CD86 subpopulations was less clear (Fig. 7). Consistent with single exposure studies at 24 h (Fig. 4), DNFB activates the BMDCs as evidenced by an increase in the C86+ CD80+ DP subpopulation and a decrease the naïve C86−CD80− DN subpopulation. Both NPs suppressed BMDC activation, maintaining the DP and DN subpopulations to imDC levels at 24 h. There were no statistically significant changes in CD276 expression. However, both NPs co-cultured with DNFB increased PD-L1 expression on the DP subpopulation at 24 h, which was also evident at 1 h for $mTiO_2$, but not for SiO_2, which exhibited a statistically significant decrease PD-L1 at 1 h relative to the imDC (Fig. S15). The most intriguing differential effect between the NPs co-cultured with DNFB was the observation that $mTiO_2$ induced a significant decrease in PD-L2 expression in the DP subpopulation at 24 h and SiO_2 did not (Fig. 7). This decrease was also evident after only 1 h of co-culture (Fig. S15). A second, potentially important difference between the NPs was observed at 1 h exposure in PD-L1 expression on the DP subpopulation where SiO_2 decreased expression whereas $mTiO_2$ upregulated it over DNFB levels (Fig. S15). However, at 24 h both NPs increased PD-L1 on the activated DP cells and decreased PD-L2 expression on the naive DN cells. Statistically significant changes in PD-L2 expression were not observed in the activated DP or the naive DN subpopulations in single-exposure studies (Fig. 4). The significant differences in PD-L1 and PD-L2 expression in the activated DP subpopulation caused by $mTiO_2$ co-cultured with DNFB and the opposing effects with SiO_2 co-culture maybe influential in the BMDC controlling the fate of the adaptive immune response.

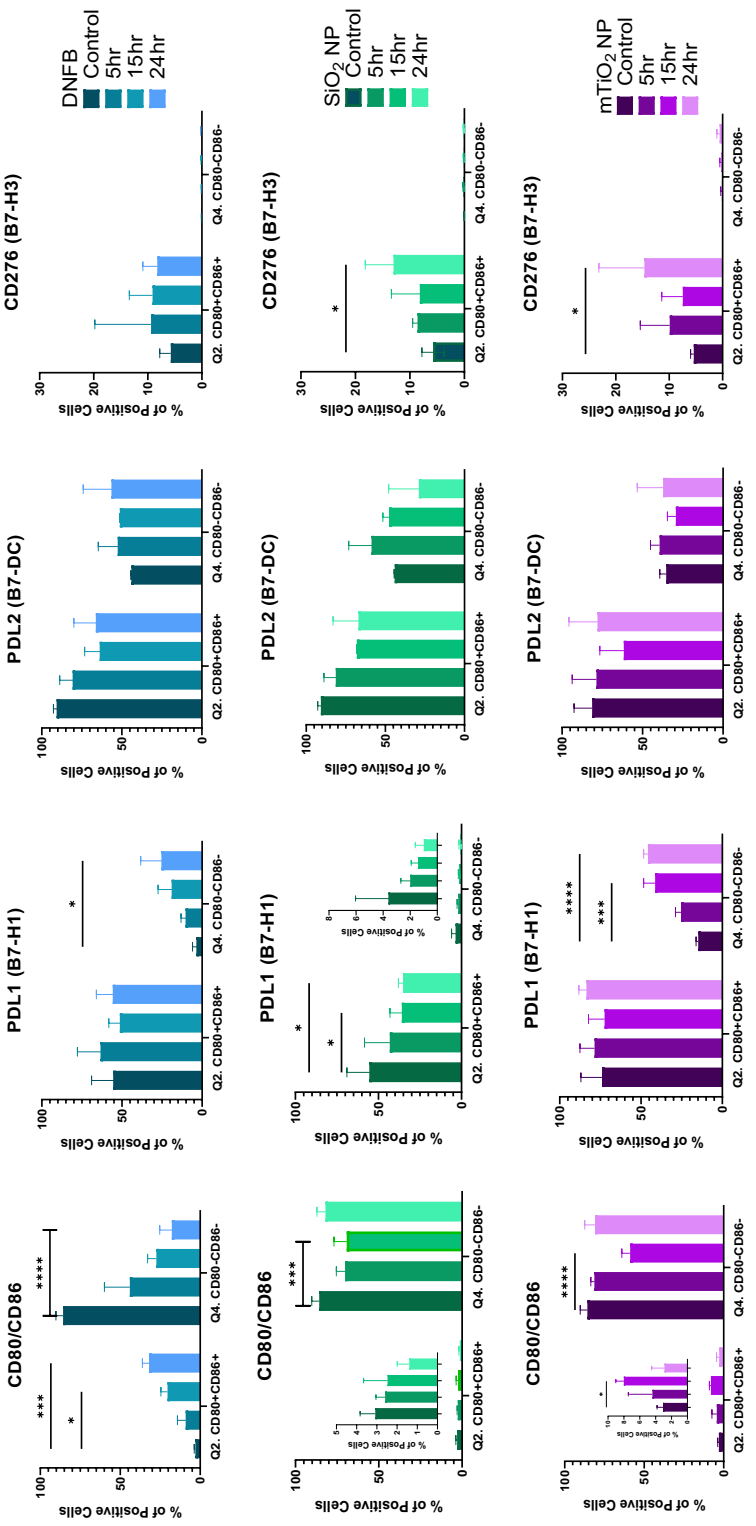

Figure 4. Co-stimulatory molecules from the B7 family quantified by flow cytometry gated on the CD11c+ MHCII+ and CD80/CD86 subpopulations following BMDC exposure to each stressor over time. Results for DNFB are colored in blue, SiO_2 in green and $mTiO_2$ in purple. The phenotypic characteristics of BMDCs following exposure to a low concentration of each stressor (0.001 mM DNFB, 0.01 mg/mL SiO_2 and 0.005 mg/mL $mTiO_2$) was followed over time, 5 h, 15 h, and 24 h, by flow cytometry gated under the CD11c+ MHCII + CD80/CD86 subpopulations. Presented here are changes the CD80 + CD86+ double positive (DP) (Q2.) and the CD80-CD86- double negative (DN) (Q4.) subpopulations including the expression levels of PD-L1 (B7-H1), PD-L2 (B7-DC) and CD276 (B7-H3) on the DP and DN subpopulations. For DNFB exposure, the activated DP population clearly increases as the naive DN decreases and PD-L1 expression increased on the DN at 24 h. $mTiO_2$ exposure over time parallels the effects of DNFB exposure. In contrast, SiO_2 exposure over time tended to decrease the DP subpopulation and it significantly decreased PD-L1 expression in the DP subpopulation. Both SiO_2 and $mTiO_2$ tended to increased CD276 in the DP at 24 h. The similarities between DNFB and $mTiO_2$ were evident in CD80/CD86 and PD-L1 expression and distinct from SiO_2. A two-way ANOVA was performed and compared to imDC. N = 3–5. Mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

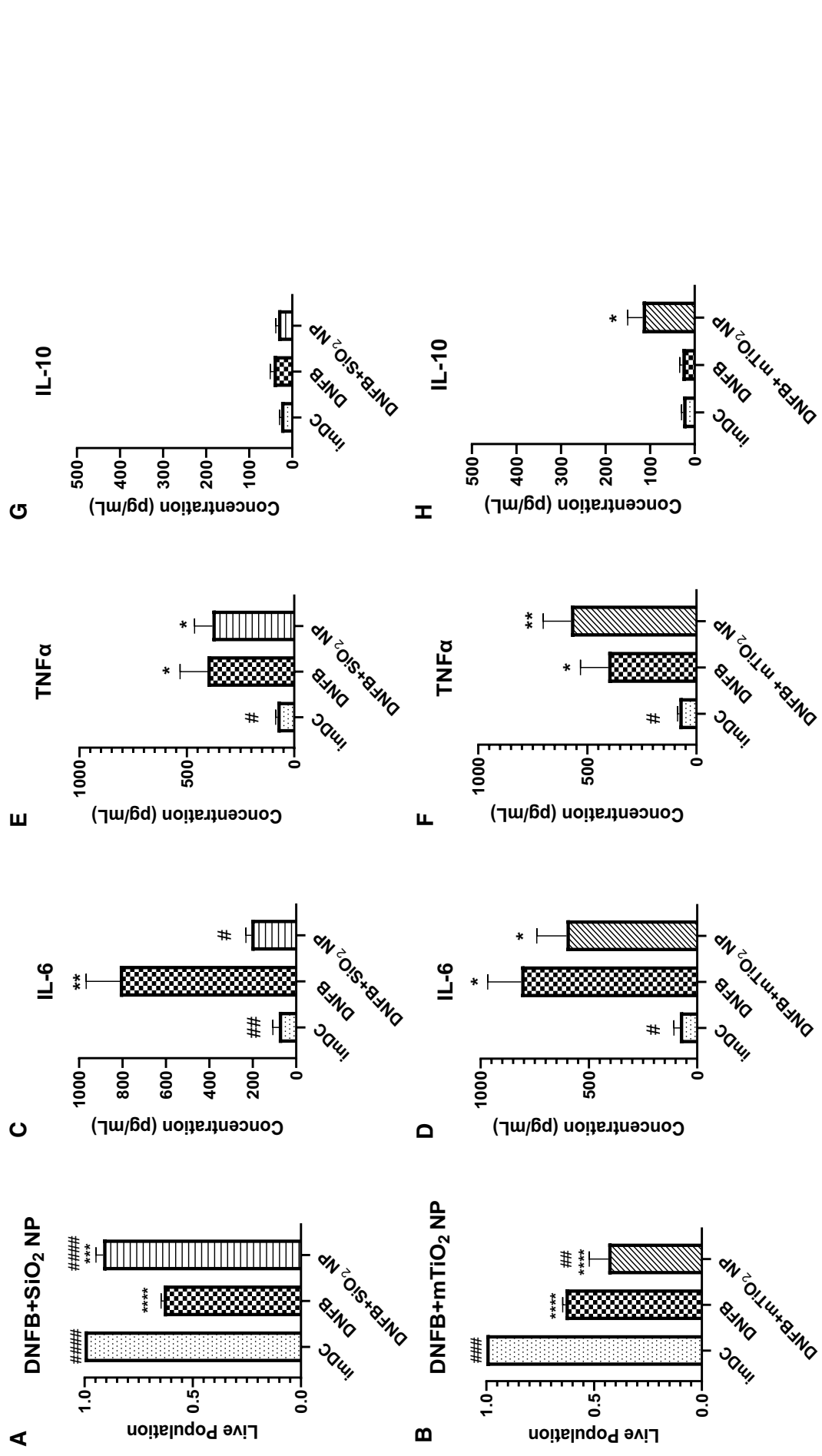

Figure 5. BMDC cytotoxicity and cytokine secretion following co-exposure to DNFB with SiO_2 or $mTiO_2$ NPs. BMDCs were co-exposed to DNFB plus SiO_2 or $mTiO_2$ nanoparticles for 24 h. Cell viability and secreted cytokines were measured and compared to DNFB treatment alone and to untreated imDC as a control. (**A**) SiO_2 NPs were shown to have cytoprotective effect in DNFB co-exposure whereas (**B**) $mTiO_2$ NPs induced higher cytotoxicity. Supernatants were collected to analyze cytokine production by ELISA for (**C**,**D**) IL-6 which showed co-culture with SiO_2 NPs decreased IL-6 production while co-culture with $mTiO_2$ did not. (**E**,**F**) addition of either NPs did not alter the levels of TNFα produced by DNFB alone and (**G**,**H**) the co-culture of DNFB with $mTiO_2$ NPs increased IL-10 production while SiO_2 NPs did not. Ordinary one-way ANOVA was performed and compared to imDC (*) and DNFB alone (#). Concentration was normalized against % of live cells. N = 3–5. Mean ± SD. $^{*/\#}p < 0.05$, $^{**/\#\#}p < 0.01$, $^{***/\#\#\#}p < 0.001$, $^{****/\#\#\#\#}p < 0.0001$.

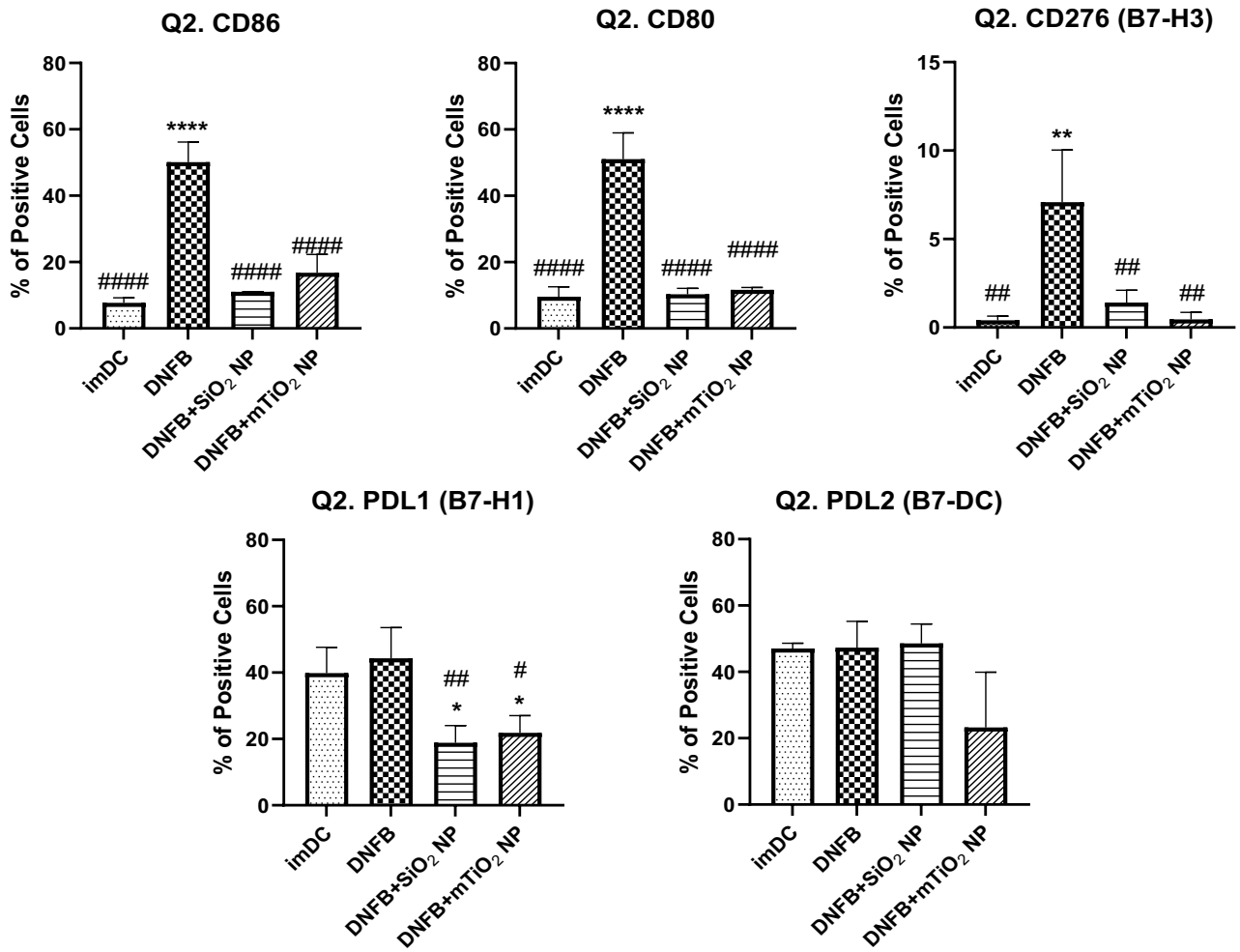

Figure 6. Co-stimulatory molecules from the B7 family quantified by flow cytometry gated on the CD11c + MHCII + subpopulation following co-exposure of DNFB with NPs. BMDCs were treated with DNFB (0.001 mM DNFB) and co-exposed with SiO_2 (0.01 mg/mL) or $mTiO_2$ (0.005 mg/mL) NPs for 24 h. Surprisingly, both NPs appeared to suppress the activation of all co-stimulatory molecules relative to DNFB only at 24 h, except for PD-L2, for which $mTiO_2$ significantly down regulated it. Ordinary one-way ANOVA was performed and compared to imDC (*) and DNFB (#). N = 3–5. Mean ± SD.*/$^{\#}p < 0.05$, **/$^{\#\#}p < 0.01$, ***/$^{\#\#\#}p < 0.001$, ****/$^{\#\#\#\#}p < 0.0001$.

Effects of NPs on the sensitization and challenge phases using the in vivo DNFB contact hypersensitivity model

Analysis of cytotoxicity, cytokine secretion and co-stimulatory markers taken together suggest that DNFB + SiO_2 co-exposure tends to promote a more naïve or regulatory BMDC phenotype, whereas DNFB + $mTiO_2$ promoted a proinflammatory BMDC phenotype. To test this, we used the in vivo CHS mouse model with adoptive transfer. We first tested the difference between topical and subcutaneous (S.C.) DNFB sensitization. BMDCs were treated with DNFB (0.01 mM) for 1 h. After two wash steps, the cells were resuspended in sterile saline and injected S.C. to sensitize the mice. A second group of mice was topically sensitized by applying 20 µL of 0.05% DNFB in acetone:olive oil vehicle (4:1)[7,21,60]. Five days later, ear thickness was measured, and the mice were challenged with 0.2% DNFB on one ear and with vehicle on the other. On day 6, ear thicknesses were remeasured. Results of ear swelling response showed no difference between the topical sensitization and S.C. sensitization (Fig. 8A).

In prior work we observed an immunomodulatory effect of NPs in the challenge phase with DNFB topical sensitization[21]. To test if these effects are similarly observed with S.C. sensitization, we S.C sensitized the mice with BMDC treated with 0.01 mM DNFB for 1 h. We challenged the mice on Day 5 with 0.2% DNFB alone, or mixed with NPs and measured the ear thickness on Day 6. Results show a similar result to our previous data[21] indicating that SiO_2 NPs suppressed and $mTiO_2$ NPs exacerbated the allergic response relative to challenge with DNFB alone (Fig. 8B). Next, we compared S.C. sensitization using BMDC treated with DNFB (0.01 mM) alone or BMDC co-cultured with SiO_2 (0.01 mg/mL) or $mTiO_2$ (0.005 mg/mL) for 1 h. After two wash steps, we S.C. injected the cells to sensitize the mice. Upon challenge, we treated one ear with 0.2% DNFB and the other with vehicle. Results show that mice sensitized with BMDC treated with DNFB + SiO_2 measured a decreased ear swelling relative to DNFB alone. In contrast, mice S.C. sensitized with BMDC treated with DNFB + $mTiO_2$ showed an

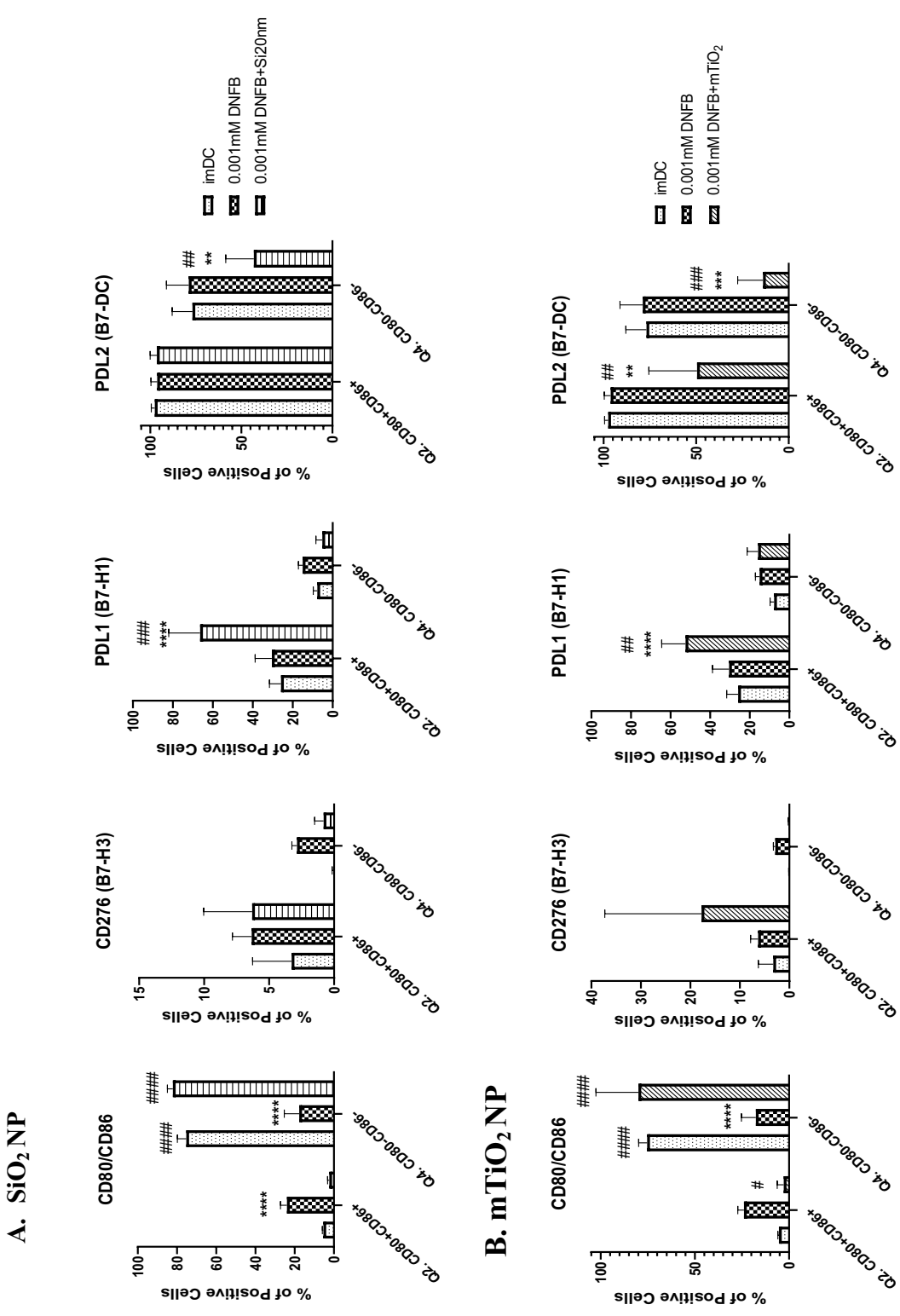

Figure 7. Co-stimulatory molecules from the B7 family quantified by flow cytometry gated on the CD11c+MHCII+CD80/CD86 subpopulations following 24 h DNFB alone or DNFB plus NP co-exposure. CD80/CD86 subpopulations were divided and CD276 (B7-H3), PD-L1 (B7-H1) and PD-L2 (B7-DC) were gated under, double positive (DP) population (Q2.) or double negative (DN) population (Q4.) to determine whether their expression varies under the different activation states of BMDCs by using the activation markers CD80/CD86 following non-cytotoxic concentrations of (**A**) 0.001 mM DNFB alone and plus 0.01 mg/mL SiO_2 co-exposure and (**B**) 0.001 mM DNFB alone and pls 0.005 mg/mL $mTiO_2$ co-exposure. NPs suppressed BMDCs activation, increased PD-L1 expression and $mTiO_2$ induced a significant decrease in PD-L2 expression in the DP subpopulation at 24 h while SiO_2 did not. A two-way ANOVA was performed and compared to imDC (*) and DNFB (#). N = 3–5. Mean ± SD. $^{**/\#\#}p < 0.01$, $^{***/\#\#\#}p < 0.001$, $^{****/\#\#\#\#}p < 0.0001$.

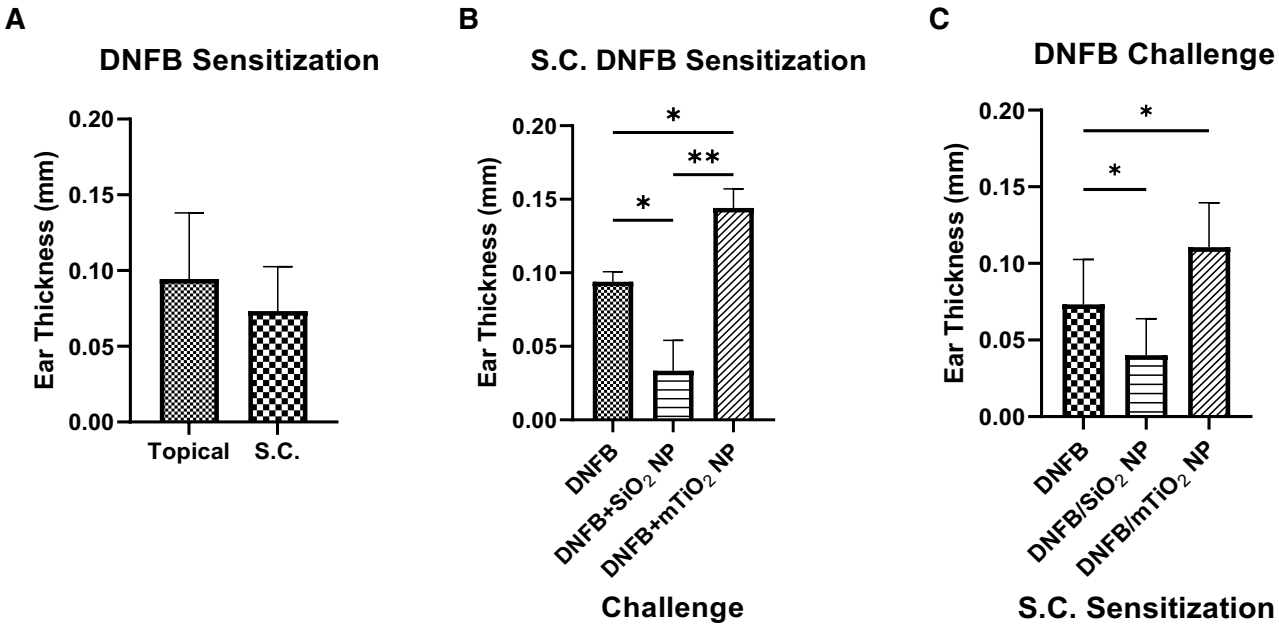

Figure 8. Comparison of CHS allergic response for different models of sensitization and challenge. (**A**) Comparison of ear swelling response for DNFB topical (0.05%) vs. S.C. (0.01 mM DNFB) sensitization with 0.2% DNFB challenge on one ear vs. vehicle on the other. No differences between these sensitization methods was observed. (**B**) Comparison of ear swelling response for S.C. sensitization with DNFB only and challenge with DNFB or DNFB co-exposure with NPs and vehicle on the other. Ear swelling was exacerbated with $mTiO_2$ NPs but decreased with SiO_2 NPs compared to DNFB alone. Ordinary one-way ANOVA was performed and compared to imDC (*). N = 3–12. Mean ± SD. *p < 0.05, **p < 0.01. (**C**) Comparison of ear swelling response for S.C. sensitization with BMDC treated 1 h with DNFB only or DNFB + SiO_2 or DNFB + $mTiO_2$ and challenge with 0.2% DNFB on one ear vs. vehicle on the other. Ear swelling was exacerbated with $mTiO_2$ but decreased with SiO_2 compared to DNFB alone.

increase in ear swelling relative to DNFB alone (Fig. 8C). These results suggest that BMDCs co-cultured with NPs and a potent sensitizer can alter the BMDC phenotype to effect the efficiency of T cell priming and the intensity of the CHS response. In prior work we did not measure an effect of NPs co-exposed topically with DNFB in the sensitization phase[21]. This is most likely due to the inability of the NPs to breach the skin barrier to an appreciable extent to interact sufficiently with skin dendritic cells to alter their phenotype.

Discussion

Engineered NPs have broad applications in many industries and are extensively under development for biomedical use[62–64]. For example, NPs are being engineered for use in vaccine development where they act as adjuvants and/or carriers to generate antigen-specific tolerogenic adaptive immunity[64]. This is a superior therapeutic strategy compared to suppressing the entire immune system which, can cause long-term damage[65]. Hence, at the forefront of the nanomedicine and nanotoxicology fields, is the need to understand and control how NPs interact with the immune system[66].

This work expands on our previous studies that showed NPs can modulate the adaptive immune response in a CHS mouse model[21]. We showed that SiO_2 NPs suppressed the allergic response in the challenge phase and $mTiO_2$ NPs exacerbated it[21–23]. The mechanism of how these NPs can alter adaptive immune responses remains unclear, which motivated this investigation to examine how these NPs could impact dendritic cell phenotype and function by quantifying BMDC cytotoxicity, cytokine production, the expression of the B7 family co-stimulatory ligands, and the in vivo adoptive transfer CHS model.

Results of this study show that BMDC treated with DNFB or $mTiO_2$, as a function of time and increasing concentration, are cytotoxic (Fig. 1) and they produce higher levels of proinflammatory cytokines (Fig. 2) compared to SiO_2 which exhibited a cytoprotective effect in DNFB co-culture studies whereas $mTiO_2$ exacerbated DNFB cytotoxicity (Fig. 5). TEM studies confirm that both NPs were taken up by the BMDCs and it showed that DNFB exposure caused BMDCs to lose dendrites and increase the presence of lysosomes (Fig. S2), which degrade exogenous materials[67]. $mTiO_2$ exposure induced a significant presence of lipid droplets whereas SiO_2 exposure showed only a small increase (Fig. S2). An increase in lipid droplets may result from oxidative stress[68], or it may indicate upregulation in metabolic activity through glycolysis which also drives the secretion of inflammatory cytokines[69].

Analysis of the B7 family of co-stimulatory ligands suggest that DNFB and $mTiO_2$ induce a proinflammatory BMDC (CD11c, MHCII+) phenotype by up-regulating CD86, CD80 and PD-L1 (Fig. 3) similar to LPS (Fig. S10) whereas, SiO_2 had little effect and in fact promoted a more naïve phenotype by inducing a decrease in the percent of CD86 + CD80+ cells over time (Fig. 4). Dendritic cells expressing low levels of CD80/CD86 present antigen

poorly and may induce tolerance[47,48]. We observed that potent proinflammatory stressors (LPS, DNFB, $mTiO_2$) upregulate the immunosuppressive PD-L1 ligand (Fig. 3, Fig. S12) which, is a mechanism by which the PD-1/PD-L1 pathway balances the pro-inflammatory effect by promoting the development of Foxp3 + Tregs to limit the immune responses[43,45,70,71].

The co-stimulatory molecule CD276 was found to be prominently expressed only on CD80+ cells (Fig. 4, Fig. S13) which, binds the inhibitory CTLA-4 receptor with high affinity[49,50]. Despite its link to promoting an immunosuppressive BMDC phenotype following activation of the arylhydrocarbon receptor[60], CD276 expression was down regulated by both NPs in our DNFB co-culture studies (Fig. 6), suggesting that CD276 plays a minimal role driving the adaptive immune response in this CHS adoptive transfer model. Both NPs induced similar effects in modulating the expression levels of CD86, CD80, PD-L1 and CD276 induced by DNFB with the key exception of PD-L2, where $mTiO_2$ downregulated PD-L2 on the activated CD86+ CD80+ subpopulation and SiO_2 did not (Figs. 6 and 7). This suggests an important role of PD-L2 in directing the efficiency T cell priming in the DNFB-CHS model where SiO_2 treated BMDC suppresses the allergic response in both the sensitization and challenge phases whereas, $mTiO_2$ exacerbated it (Fig. 8). This finding is consistent with studies of allergic asthma, that showed PD-L2 expression in the lung was protective against the initiation and progression of airway inflammation[72–75]. Our results point to the importance of PD-L2 expression in the sensitization phase with DNFB, a T_H1 skewing hapten[76,77]. Studies suggest that PD-L1 and PD-L2 participate in the differential regulation of T_H1 and T_H2 cells[78]. The PD-L1/PD-1 interaction causes a T_H2 response and an increased IL-4 secretion while the PD-L2/PD-1 interaction causes a T_H1 response and an increase in INF-γ secretion[73]. In future studies it would be important to analyze full cytokine panels that contain T cell polarizing signals as well as chemokines important for lymph node trafficking. Quantifying the expression of the chemokine receptor CCR7 on the engineered BMDC is important as upregulation directs their migration to T-cell zones in lymph nodes[79]. The percent of the S.C. injected BMDC that traffic to the lymph node could be determined using fluorescently labeled BMDC and correlated to CCR7 expression. Quantifying these metrics is important for engineering tolerogenic dendritic cell therapies for treating autoimmune and severe allergic disorders[65,80].

While our studies point to differences in PD-L2 expression as a potential mechanism for the differential effects of SiO_2 and $mTiO_2$ on DNFB sensitization (Fig. 8), it is important to note that the adoptive transfer of ex vivo engineered BMDC preparations contains a heterogeneous mix of B7 phenotypes. Additional studies would be informative to examine the relative importance of each BMDC subset more fully in driving the allergic response. Specifically, the different CD86/CD80 subpopulations (DP, DN, single positive) could be sorted to test which phenotype induces potent allergic responses. Studies show that dendritic cells expressing high levels of CD80 but not CD86 are protective and can induce immune tolerance via promoting CD25+ regulatory T cells[81]. It is also important to investigate the effect of protein coronas that form on NPs exposed to biological fluids and cell culture media[82,83]. Differences in the corona protein composition or abundance could alter the NPs interaction with the BMDCs. Corona composition is highly dependent on surface charge[84]. SiO_2 and $mTiO_2$ are both negatively charged so we anticipate that similar compositional coronas would form but this should be confirmed in proteomics studies. It is also possible that culturing BMDC with DNFB may haptenize the cells making them directly antigenic. Injection of haptenized BMDC could activate endogenous antigen presenting cells (APCs) in the skin or in the lymph node. Sensitization via this mechanism could be confirmed using transgenic mouse models with deleted endogenous APCs, however, the striking differential effects of the NPs observed in sensitization phase suggest a role for the ex vivo engineered BMDC in directing the observed adaptive immune responses.

This study corroborated our earlier studies using topical DNFB sensitization that showed (Fig. 8B) these NPs affected the allergic response in the challenge phase with $mTiO_2$ exacerbating the ear swelling and SiO_2 suppressing it[21]. While the mechanism in the challenge phase remains unclear, it seems plausible that the NPs could modulate epidermal-derived signals that affect MC and/or DC activation. These signals could be alarmins produced by keratinocytes or the NPs could modulate of the endogenous expression of CD80 or CD86 on keratinocytes. Studies using transgenic mouse models that over expressed CD80 or CD86 on basal keratinocytes showed a differential ability induce a chronic inflammatory response in the DNFB CHS model[85]. The immunomodulation IL-10 cytokine was also persistently increased in the mouse ear skin in the CD80 transgenic mouse which is consistent with a potent proinflammatory response observed with LPS and $mTiO_2$ treatment in this study.

In summary, this work points to metrics that can be used to predict the effects of NPs on contact allergy and points to the novel use of NPs to engineer immunomodulatory responses in contact allergy. Given that skin contact allergy is on the rise[1,2], as is the creation of novel engineered nanomaterials for industrial, biomedical and consumer use[62–64,86], there is a need for assays that can predict the impact that NPs may have on the immune response in the context of skin allergic disease. Further, immunoengineering is an important growing field for developing cell-based therapies to induce antigen specific immune tolerance[42,64,87,88].

Materials and methods

Animals

Hairless SKH mice back-crossed 6 generations with C57BL/6 mice were used in this study and all previous work[21–24]. All animal experimental protocols were reviewed and approved by the University of Rochester Committee on Animal Resources (UCAR #2010-24E). Experiments involving animals and reporting of data were carried out in compliance with the ARRIVE guidelines and all methods were carried out in accordance with relevant guidelines and regulations.

Cell culture

BMDCs were harvested and cultured from tibiae and femurs of 8–12 weeks mice using a standard protocols[89]. Bone marrow cells were suspended in: RMPI 1640 (Gibco Cat# 11875-093) containing 10% FBS (Gibco Cat#

10082147), 1% pen strep (Gibco Cat# 15140-122), 2 mM Glutamax (Gibco Cat# 35050-061), 1 mM sodium pyruvate (Gibco Cat# 11360-070), 50 µM β-ME (Gibco Cat# 31350-010), and 20 ng/mL GM-CSF (Gibco Cat# PMC2011). A flow cytometry panel was defined (Table S1) to characterize the development of BMDCs over time. While GM-CSF-derived BMDC cultures are heterogeneous, studies confirm they are comprised conventional DCs[90] and BMDCs are extensively used in both fundamental research and in clinical protocols[91]. After 8 days of culture the majority of cells were CD11c+ (86.1%), CD11b+ (98.1%) and MHCII+ (67.6%) with no detection of T or B cells (Fig. S16). On average 10% of cells stain positive for F4/80 a macrophage marker and 71.3% of the cells were CD11c+, CD11b+ and MHCII+ triple positive. Eight-day old BMDCs were used for initiating all experiments.

SiO_2 and $mTiO_2$ NP characterization

Using dynamic light scattering and zeta potential measurements the SiO_2 NPs (nanoComposix Cat# SISN20-25M) exhibited a hydrodynamic diameter of 33.5 nm (± 3.3 nm), zeta potential of – 21.9 mV (± 10.1 mV) and a PDI of 0.236 in ultrapure water (pH 6.5)[23]. The $mTiO_2$ NPs (Sigma-Aldrich Cat# 677469, < 100 nm) exhibited a hydrodynamic diameter of 556.4 nm (± 34.4 nm), zeta potential of –9.05 mV (± 1.3 mV) and a PDI of 0.296 in ultrapure water (pH 6.5)[22]. The lower surface charge on the $mTiO_2$ NPs suggest a greater tendency to agglomerate as is evidenced by the hydrodynamic diameter being larger than the vendor reported primary size particle size < 100 nm. Transmission electron microscope (TEM, Hitachi 7650) was also used to measure free NPs and NPs inside the cells. The average size of the NPs inside the cells, which for $mTiO_2$ NPs was found to be 51.6 ± 12.5 nm and SiO_2 NPs were found to be 20.6 ± 3.5 nm (Figs. S3, S4).

Generation of BMDCs exposed to DNFB, SiO_2, and $mTiO_2$ NPs

The BMDCs were exposed to DNFB, SiO_2, or $mTiO_2$ at different concentrations for 1 h and non-cytotoxic concentrations (0.001 mM DNFB, 0.01 mg/mL Si20 nm and 0.005 mg/mL $mTiO_2$) were chosen for the 24 h time studies and subsequent single exposure experiments. The co-exposure of DNFB with SiO_2 NPs did not give any discernible toxicity in vitro, for which, the concentration of DNFB was increased ten-fold (0.01 mM) for some experiments. The concentrations for the co-exposure of DNFB with $mTiO_2$ NPs remained at 0.001 mM and 0.005 mg/mL, respectively.

Flow cytometry

For cell staining and analysis of co-stimulatory molecule expression, the antibodies and the concentrations used per 1 M cells are summarized in Table S1. We used flow cytometry (Cytek Aurora, Cytek Biosciences) and FlowJo (v10.7.2) to analyze cells. The gating strategy is shown in Fig. S10A. For each experiment FMO controls with BMDC are used. Examples of the FMO gating are illustrated in Fig. S10B,C.

Cytotoxicity and cytokine analysis

Cytotoxicity was measured by flow cytometry using the eFluor™ 780 viability dye that labels dead cells. We normalized the BMDCs live population against imDC control cells that were not treated with any stressor. In addition, we used the PrestoBlue cytotoxicity assay (Invitrogen, P50200) according to manufacture protocol, to confirm the trends observed using flow cytometry. The cell culture supernatant was collected and analyzed for the pro-inflammatory cytokines IL-6, TNFα and the immunosuppressive cytokine IL-10 by ELISA (Invitrogen Cat# 88-7064, 88-7324 and 88-7105, respectively) following manufacture instructions. These cytokines were selected as they represent important innate proinflammatory and immunosuppressive makers.

Contact hypersensitivity (CHS) mouse model

As in our prior work[21], mice were sensitized topically on the back by applying 20 µL DNFB (0.05%) diluted in an acetone and olive oil vehicle in a 4:1 ratio. After 5 days, we performed challenge with 20 µL of 0.2% DNFB on one ear and vehicle (4:1 acetone and olive oil ratio) on the other ear. Alternatively, we sensitized the mice subcutaneously (S.C) by injecting BMDCs (2×10^6) treated for 1 h with DNFB (0.01 mM) only or DNFB (0.01 mM) plus NPs; SiO_2 (0.01 mg/mL) or $mTiO_2$ (0.005 mg/mL). After 5 days, we performed challenge with 20 µL of 0.2% DNFB on one ear, and the other ear treated with vehicle (4:1 acetone and olive oil ratio) or 0.2% DNFB plus SiO_2 or $mTiO_2$ NPs using the same doses and protocols in prior work[22,23]. The magnitude of the allergic response post challenge was quantified by measuring ear thickness using a digital caliper (Mitutoyo Cat# 209-931) with a resolution of 0.005 mm on day 5, before the application of the challenge dose (pre-challenge). On day 6, both ears were remeasured (post-challenge). Ear swelling (mm) was measured as: (post-challenge) – (pre-challenge).

Statistics

We used GraphPad Prism 9 to analyze all statistical analyses. Ordinary one-way or two-way analysis of variance (ANOVA) was used to compare expression levels of co-stimulatory molecules to imDC (*) and DNFB (#). imDC were not treated with any stressor and served as control. Two-tailed, unpaired with unequal variances, student's t-test was used to compare the ear thickness between two different sensitizing treatments in the CHS in vivo study. p-values < 0.05 were considered significant. All data are presented with standard deviation. The experiments were replicated at least three times.

Data availability

All data are available in the main text or the supplementary materials. Raw data will be made available upon request. Contact: Lisa DeLouise: lisa_delouise@urmc.rochester.edu.

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Author contributions

L.A.D. contributed to the conceptualization of the work. J.P.P. helped generate the CHS mouse data. A.W.L. generated all of the flow data, figures and statistical analysis. L.A.D., A.W.L. contributed to the study design, discussion of the results, and writing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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