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CpG-C immunotherapeutic efficacy is jeopardized by ongoing exposure to stress: Potential implications for clinical use

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Abstract

Bi-directional influences between stress hormones and immune responses have been repeatedly documented, however, in the clinical setting they are rarely considered when immunotherapeutic approaches are used or studied in patients. As some immunotherapeutic treatments have shown great potential in animal models but have had limited success in patients, we hypothesize that on-going psychological and physiological stress responses in patients, which do not characterize the setting of animal studies, contribute to this discrepancy. In the current study we examined the interaction between on-going water stress and CpG-C immunotherapy to determine whether stress that precedes immunotherapy can modulate the efficacy of CpG-C immunostimulation. C57BL/6 mice were exposed to water stress or served as controls. Two hrs following the commencement of the stress protocol animals were injected with CpG-C, non-CpG, or PBS, and sacrificed 1, 4 or 12hrs thereafter. We found that in CpG-C-treated animals stress eliminated the elevation of plasma IL-12, and synergistically elevated corticosterone levels. Furthermore, stress markedly reduced the total number of myeloid ($33D1^+$), plasmacytoid ($mPDCA\text{-}1^+$) and plasmacytoid-derived ($33D1^+mPDCA\text{-}1^+$) dendritic cells in CpG-C-treated animals, as well as the numbers of these cell sub-types expressing CD11b, CD80 and CD69. These changes were more dramatic in the blood than in the spleen. Overall, these findings indicate that under no-stress conditions CpG-C induces a robust immune response, which is significantly diminished when immunostimulation is attempted during on-going stress. If these findings hold in humans, potential prophylactic treatments should be found to limit the deleterious effects of on-going stress on the efficacy of immunotherapy.

Keywords

CpG-C; stress; dendritic cells; IL-12; corticosterone

Introduction

The relationship between stress responses and the immune system is bi-directional, as both systems profoundly modulate each other. Certain cytokines have been shown to signal the

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brain regarding on-going peripheral immune responses, and consequently bring about the activation of neuroendocrine stress responses (Hickey, 2001; Kusnecov and Goldfarb, 2005; Rossi-George et al., 2005). Furthermore, activated leukocytes can themselves secrete stress hormones, such as adrenocorticotropic hormone (ACTH) (Blalock et al., 1985) and catecholamines (CAs) (Josefsson et al., 1996), potentially activating peripheral endocrine responses and directly impacting other cells. Most importantly, activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis, and the release of their respective stress hormones (e.g., catecholamines and corticosteroids) following stress conditions were shown to modulate immune activity and resistance to cancer progression (Ben-Eliyahu, 2003; Glasner et al., 2010; Shakhar and Blumenfeld, 2003). Mechanisms mediating such stress-induced immune regulation include the direct activation of functional receptors for stress hormones expressed by most lymphocytes (Smith and Blalock, 1988), and the direct sympathetic innervation of primary and secondary lymphoid organs (Elenkov et al., 2000; Sredni-Kenigsbuch, 2002).

Although this stress-immune relationship has been thoroughly documented, in the clinical setting it is rarely considered when immunotherapeutic approaches are used or studied in patients. The use of immunotherapy in cancer patients has gained momentum in the past decade, as it is now recognized that the patient's immune system has a critical role in shaping malignant development and controlling residual disease (Dunn et al., 2004; Neeson and Paterson, 2006). However, although pre-clinical studies in animal models showed great potential (Boggio et al., 2000; Brunda et al., 1993; Kobayashi et al., 2002; Zou et al., 1995), their applications in cancer patients have been less successful (Atkins et al., 1997; Colombo and Trinchieri, 2002; Hurteau et al., 2001; Parris, 2007; Portielje et al., 1999). Given that immunostimulators act through activating leukocytes, it is feasible that stress hormones would impact leukocytes' responsiveness to immunostimulators. Therefore, it is probable that psychological or physiological stress experienced by patients receiving immunostimulation for a pre-diagnosed disease will impact the efficacy of immunostimulation. Such potential limiting processes are less likely to occur in animal pre-clinical studies, as animals are usually not exposed to stressors during administration of immunostimulators (Goldfarb et al., 2009; Rosenne et al., 2007; Schwartz et al., 2008).

One such immunostimulatory agent is CpG-C, which activates a robust proinflammatory immune response by binding to the intracellular receptor Toll-like receptor 9 (TLR9). TLR9 has been shown to be differentially expressed in humans and rodents (Krieg, 2002), and is located predominantly within plasmacytoid dendritic cells (pDCs) and B cells in humans, while in mice it is also abundant within monocytes, macrophages and myeloid dendritic cells (mDCs). In both species its activation is MyD88-dependent, which downstream recruits IRAK4 that in turn associates with TRAF6, ultimately leading to the activation of two distinct signaling pathways, and finally to the activation of IRF7 and NFκB (Guiducci et al., 2009). IRF7 activation results in the secretion of type I interferons, while the activation of NFκB brings about proinflammatory cytokine production, dendritic cell maturation and survival.

We have recently shown that when CpG-C was administered a day prior to surgical stress in rats, it nearly nullified the deleterious effects of stress and laparotomy on the host's ability to clear tumor cells from the lungs (Goldfarb et al., 2009). However, the context of this CpG-C treatment is far removed from the clinical situation, where patients receive immunotherapy while experiencing and coping with the psychological and physiological stressors that precede treatment. As various immunostimulators, including CpG, are under investigation for the treatment of a wide array of cancers and other maladies, it is crucial to understand these processes so that clinically applicable interventions can be developed to maximize efficacy of biological response modifiers (BRMs).

Therefore, in the current study we examined whether on-going stress that precedes immunotherapy can modulate the efficacy of CpG-C immunostimulation in two immune compartments, the peripheral blood and the spleen. As dendritic cells are one of the major cell populations activated by CpG-C (Marshall et al., 2003), we examined three different dendritic cell populations and their unique response to CpG-C treatment following stress. Furthermore, as CpG-C has been shown to induce the secretion of both pro-inflammatory (Marshall et al., 2005) and anti-inflammatory (Duramad et al., 2003) cytokines, the secretion of both IL-12 and IL-10 was evaluated to examine the change in their balance following both immunostimulation and stress.

Methods

Animals and counterbalancing

One hundred and sixty two male C57BL/6 mice were purchased at the age of 4-6 weeks (Harlan Laboratories, Jerusalem, Israel), and housed 4 per cage in our vivarium with ad-lib access to food and water on a 12:12 light:dark cycle at 22±1°C. All mice were allowed at least 4 weeks to acclimate to the vivarium and were 10 weeks old on the day of experimentation. The order of injections and blood withdrawal were counterbalanced across all experimental groups. The experiment was run in 2 replicates in which all groups were represented (approximately 9 animals per group). The study was approved by the Institutional Animal Care and Use Committee of Tel Aviv University.

Oligodeoxynucleotides (ODN) and their administration

CpG-C (ODN 2395: 5′-TCGTCGTTTTCGGCGCGCGCCG-3′) with a phosphorothioate backbone and non-CpG ODN (ODN 2137: 5′-TGCTGCTTTTGTGCTTTTGTGCTT -3′) were purchased from Coley Pharmaceuticals Canada (Ottawa, Canada), and contained undetectable levels of endotoxin as measured by the limulus amebocyte lysate assay. Two different controls were used: Phosphate buffered saline (PBS), and non-CpG, which lacks C-G motifs. Both CpG-C and non-CpG were diluted in PBS, and were administered i.p. at a dose of 50μg/mouse.

Experimental procedure

Mice were divided into 3 treatment groups (CpG-C, non-CpG and PBS) that were further subdivided to undergo water stress or to serve as no-stress controls. Each of these 6 groups was then further subdivided into 3 different treatment/stress exposure times before blood draw (1, 4 and 12hrs following the injection of CpG-C/non-CpG/PBS), forming the 18 experimental groups. The animals undergoing stress were placed in opaque cages with 0.5cm of room-temperature water with free access to food and water. Two hrs following the commencement of the stress protocol, mice were injected with CpG-C, non-CpG or PBS, and the timing of the different stress groups was set such that all protocols ended at the same time of day, and all animals were sacrificed simultaneously (maintaining group counterbalancing) (Figure 1). All animals were sacrificed by an overdose of isoflurane (Nicholas Pirani India Limited, Andhra Pradesh, India) and 0.5ml of blood was withdrawn by cardiac puncture into syringes containing EDTA (1.8mg/ml blood, Sigma, Rehovot, Israel). At this time, spleens were harvested and halved. One half was flash frozen in 2-methylbutane (Sigma, Rehovot, Israel) and kept on dry ice until storage at -80°C. These half spleens were later used for the assessment of mRNA levels by real-time RT-PCR (see below). The other half of the spleen was placed into a tissue grinder with 2.5ml of PBS and was ground and filtered to create single-cell suspensions. Fifty μl of the suspension were then used for FACS analysis (see below). The blood harvested was centrifuged at 2000rpm for 20mins and plasma was collected and stored at -80°C for further analysis. The exact volume of plasma removed from each sample was replaced with PBS to return samples to

their original volume and 50ul of the reconstituted blood were immediately taken for FACS analysis.

Fluorescence activated cell scanner (FACS)

FACS analysis was used to assess the number of dendritic cell sub-populations (e.g., $33D1^+$ dendritic cells, plasmacytoid dendritic cells (pDCs)), as well as total lymphocytes in the blood and spleen. Standard procedures were used to prepare cells for FACS analysis (Melamed et al., 2005). Dendritic cells were identified within the lymphocyte cell population. Plasmacytoid dendritic cells were identified using FITC-conjugated anti-mouse mPDCA-1 (Miltenyi Biotec, Germany) (Krug et al., 2004), while 33D1 dendritic cells by PE-conjugated anti-mouse 33D1 (eBioscience, San Diego, CA). Other monoclonal antibodies (mAb) used were PE-Cy5-conjugated anti-mouse CD69 (Biolegend, San Diego, CA), APC-conjugated anti-mouse CD80 (eBioscience, San Diego, CA), and APC-Alexa Fluor 750-conjugated anti-mouse CD11b (eBioscience, San Diego, CA). Flow cytometry analysis was conducted using a FACScan (Becton Dickinson). Lymphocytes were identified based on forward and side scatters. To assess the total number of cells or cell sub-populations per μl blood or single cell spleen suspension, 300 polystyrene microbeads per μl of sample (20μm diameter, Duke Scientific, Palo Alto, CA) were added to each sample. Following cytometry, the formula: (# events per cell population/# microbead events) × 300 was used to calculate the number of cells per μl sample. The coefficient of variation for this method in our laboratory was found to be 6% or less for identical samples.

Measurement of plasma corticosterone, IL-12p70, and IL-10

Plasma was separated as detailed above and was used for the assessment of IL-12p70 and IL-10 by quantitative enzyme-linked immunosorbant assay (ELISA) (Mouse ELISA Ready-SET-Go!, eBioscience, San Diego, CA), and of corticosterone by radioimmunoassay (RIA) (ImmuChem double antibody corticosterone ^{125}I RIA kit, MP Biomedicals, Orangeburg, NY), as per the manufacturers' instructions.

RNA extraction, reverse transcription, and real-time RT-PCR for splenic IL-12p40 and TLR9

Total RNA was isolated from halved mouse spleens using the MasterPure RNA Purification kit (Epicentre Biotechnologies, Madison, WI) as per manufacturer's instructions. RNA quantification and purity were assessed using NanoDrop 2000 spectroscopy (Thermo Scientific, Wilmington, DE), and 1μg of RNA was converted to cDNA using the Verso cDNA kit (Thermo Scientific, Waltham, MA) as per the manufacturer's instructions. Real-time PCR was conducted using TaqMan probes (Applied Biosystems, Foster City, CA) for IL-12p40 (Mm00434174_m1), the CpG receptor, TLR9, (Mm00446193_m1), and the mouse housekeeping gene β-actin (Mm00607939_s1) was used as an endogenous control to normalize each sample and gene. PCR reactions were performed in a 10μl volume using 0.5μl TaqMan probe, 100ng cDNA template, 5μl TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), and 2.5μl DNase/RNase molecular grade water (Ambion, Austin, TX). The conditions of PCR amplification were: an initial denaturation at 95°C for 15mins followed by 40 cycles of denaturing at 95°C for 10sec, and annealing at 60°C for 1min. Reactions were run in duplicate. Real-time PCR was conducted using an Applied Biosystems 7300 Real-Time PCR system.

Statistical Analysis

Two-way or three-way factorial analysis of variance (ANOVA) with a pre-determined significance level of 0.05 was conducted. Provided significant group differences were found, Fisher's protected least significant differences (Fisher's PLSD) contrasts were performed to compare specific pairs of groups, based on *a priori* hypotheses. When appropriate

(comparing only two groups), an unpaired, two-tailed student's t-tests was used to determine statistically significant differences with a pre-determined significance level of 0.05. All statistical analyses were conducted using StatView software (SAS Institute, San Francisco, CA).

Results

CpG-C treatment did not affect plasma corticosterone levels in unstressed animals, but synergistically elevated corticosterone levels in stressed animals

Plasma corticosterone levels were elevated following water stress [$F_{(1,141)}$=363.213, p<0.0001], indicating that the stress paradigm used herein indeed provoked a physiological stress response (Figure 2). Interestingly, while CpG-C did not elevate plasma corticosterone levels in non-stressed animals, it elevated corticosterone levels in animals exposed to 12hrs of water stress (water stress × treatment interaction [$F_{(2,141)}$=3.593, p=0.0301], water stress × time-interval interaction [$F_{(2,141)}$=30.39, p<0.0001], and Fisher's PLSD p< 0.05 for the comparison between the PBS and the CpG-C stress groups).

Water stress interrupted the CpG-C-induced rise in IL-12 plasma protein and splenic mRNA levels

CpG-C raised IL-12p70 plasma levels in non-stressed animals, but had no effect in animals subjected to water stress, as indicated by a significant interaction between water stress and treatment [$F_{(2,13}$4)=9.698, p=0.0001] (Figure 3a), and by direct Fisher's PLSD contrasts between CpG-C and either PBS or non-CpG (p<0.0001).

We examined relative expression of splenic IL-12p40 mRNA only in the CpG-C-treated animals and the PBS control groups at the 4hr time-interval, based on the above findings and as no increases in plasma protein levels were evident in either of the control groups (PBS or non-CpG). Only half of the samples taken at the 4hr time-interval were above detection level (equally distributed across all groups). Only these samples were used in the analysis. A two-way ANOVA indicated a significant main effect of CpG-C, elevating IL-12p40 mRNA levels [$F_{(1,13)}$=9.32, p=0.0092] (Figure 3b), and this was in line with the elevation in IL-12p70 plasma levels. Although the interaction between water stress and treatment did not reach statistical significance, an unpaired t-test revealed a significant difference between CpG-C and PBS treatment in non-stressed animals [$t_{(5)}$=3.937, p=0.011], but not in the animals exposed to water stress, suggesting that the rise in IL-12p40 mRNA following CpG-C treatment was interupted.

Levels of splenic TLR9 mRNA were not affected by water stress or CpG-C treatment at either the 1 or 4hr time points tested (data not shown).

CpG-C immunochallenge elevated plasma IL-10 levels, but water stress inhibited this elevation

Data were taken only from the second replication, due to a defective ELISA plate used for analysis of the plasma from the first replication, and thus only 3-4 and 2-5 animals remained in each of the control groups (PBS and non-CpG, respectively) across the different time-intervals. Statistical analysis was conducted following the unification of the two control groups, as they did not differ from each other and in order to achieve sufficient statistical power. Plasma IL-10 levels showed significant water stress by treatment [$F_{(1,63)}$=4.848, p=0.0313], as well as water stress by time-interval interactions [$F_{(2,63)}$=4.432, p=0.0158] (Table 1), indicating that water stress interrupted not only the rise in plasma IL-12p70, but also the rise in IL-10 at the same 4 and 12hr time-points following CpG-C administration.

No effects of CpG-C on lymphocyte concentrations but a significant effect for stress

When changes in cell populations were examined by FACS analysis, water stress caused a 2-fold decrease in lymphocyte numbers/μl blood [$F_{(1,142)}$=114.984, p<0.0001], without interaction (Figure 4). No effects of CpG-C treatment or of the different time points were evident. In the spleen, a water stress by time-interval interaction was apparent such that the reduction in total lymphocyte numbers was larger as the length of stress was prolonged [$F_{(2,139)}$=4.731, p=0.0103] (data not shown).

Stress and CpG-C treatment affected the number, activation, and maturation of 33D1$^+$ dendritic cells, plasmacytoid dendritic cells, and plasmacytoid-derived (33D1$^+$mPDCA-1$^+$) dendritic cells in the blood

CpG-C treatment significantly elevated the number of all circulating dendritic cell populations (33D1$^+$ DCs (Figure 5), pDCs (Figure 6) and plasmacytoid-derived (PD) DCs expressing both 33D1 and mPDCA-1 (Figure 7) markers) (see Table 2 for all statistics), and did so mostly 4hrs following immunochallenge. PD DCs also showed an increase in number 12hrs following CpG-C treatment. However, the numbers of all DCs subtypes were significantly diminished in the blood of animals exposed to water stress. Nevertheless, the effect of CpG-C was still evident at the 4h time-interval in stressed animals, but was absent at the 12hr time-interval, even in the PD DC population. When the numbers of specific sub-populations of DCs were examined, the same pattern of results emerged for populations expressing the adhesion molecule CD11b, the co-stimulatory marker CD80, and the activation marker CD69. CD11b was also examined on pDCs, and in accordance with the literature (Asselin-Paturel et al., 2003) none of the pDCs were found to express this marker. Expression levels were also examined and significant main effects were detected for CD69 on circulating dendritic cells expressing mPDCA-1 (**pDCs:** treatment [$F_{(2,139)}$=14.964, p<0.0001], time-interval [$F_{(2,139)}$=9.186, p=0.0002], water stress [$F_{(1,139)}$=4.836, p=0.0295], Figure 6d. **PD DCs:** treatment [$F_{(2,140)}$=6.567, p=0.0019], time-interval [$F_{(2,140)}$=3.065, p=0.0498], water stress [$F_{(1,140)}$=12.813, p=0.0005], data not shown). However, no significant interactions between the effects of CpG-C treatment and water stress were evident.

Stress did not affect splenic 33D1$^+$ dendritic cells numbers but had a small effect on the number of splenic dendritic cells expressing mPDCA-1

In contrast to the dramatic effect of water stress on the number of different DC populations in the blood, the same populations in the spleen were either unaffected (33D1$^+$ DCs) or showed a reduction that was significantly smaller in magnitude (Table 2). Furthermore, effects of CpG-C treatment and of the different time intervals examined were not found in most cases, including the different cellular markers studied (CD11b, CD80, CD69), indicating a qualitative difference in the effect of our stress and immunostimulation protocols between the two immune compartments inspected. Expression levels were also examined and significant main effects were detected for CD69 on splenic 33D1$^+$ dendritic cells (treatment [$F_{(2,144)}$=6.962, p=0.0013], time-interval [$F_{(2,144)}$=7.763, p=0.0006]), and for CD11b on splenic PD DCs (water stress [$F_{(2,143)}$=12.942, p=0.0004], time-interval [$F_{(2,143)}$=5.442, p=0.0053]).

Discussion

CpG immunotherapy is presently being investigated in clinical trials for an array of different conditions including various cancers, and as an adjuvant for vaccines developed for both healthy and diseased populations (Vollmer and Krieg, 2009). As stress has been extensively documented to modulate immune responses, including the suppression of some immune indices (Elenkov et al., 2000), it is essential to understand its impacts on the efficacy of CpG

immunotherapy *in vivo*. In the current study we demonstrated that on-going water stress, which comprises both psychological and physiological characteristics, can markedly impair the immune response of C57BL\6 mice to CpG-C immunostimulation. It should be noted that in the stress paradigm used mice were not immersed in water, but rather stood in 0.5cm of room temperature water, and their corticosterone levels increased to 180-550ng/ml, which is characteristic of most non-severe stress paradigms in C57BL\6 mice (Bailey et al., 2004; Coutellier et al., 2009; Spyrka and Hess, 2010). Interestingly, CpG-C alone did not affect corticosterone levels in unstressed animals, as seen following challenge with other BRMs (Jiang et al., 2004) and in contrary to previous reports (Jiang et al., 2004; Tran et al., 2007). Nevertheless, CpG-C did elevate corticosterone levels in stressed mice following 12, but not 1, or 4hrs of ongoing stress.

A dramatic demonstration of the deleterious effects of on-going stress on the immune response to CpG-C seen herein is the alteration in T helper 1 (Th1) and Th2 cytokine secretion. CpG-C has been shown to induce versatile cytokine secretion including both the pro-inflammatory IL-12 (Marshall et al., 2005) and the anti-inflammatory IL-10 (Duramad et al., 2003) following challenge. Here we found that while CpG-C significantly elevated IL-12p70 protein levels in the plasma of unstressed mice, this effect was completely nullified in animals exposed to a few hours of water stress. We also found an interruption in the elevation of splenic IL-12p40 mRNA levels in animals treated with CpG-C and exposed to stress *in vivo*. These findings differ from previous studies reporting that following dexamethasone administration splenic IL-12p70 levels were unchanged in pDCs stimulated with CpG-B *in vitro* compared to PBS-treated controls (Abe and Thomson, 2006), although the experimental approach of the two studies clearly differ. As the secretion of IL-12 by DCs is important for subsequent secretion of IFNγ (Sabatte et al., 2007), it is pivotal to maintain IL-12 levels during stress to gain the beneficial effects of CpG-C immunostimulation. In this regard, the anti-inflammatory cytokine IL-10 has been shown to reduce the secretion of IFNγ by PBMCs through the inhibition of IL-12 following stimulation with CpG-C (Duramad et al., 2003). Therefore, the reduction seen in IL-10 levels following water stress in the current study could not explain the inhibition seen in IL-12 levels following stress. Thus, future studies should focus on identifying endocrine mediating mechanisms and developing prophylactic regimens that will prevent the abolishment of the IL-12 response following CpG-C- in the context of stress.

As dendritic cells constitute the major cell population responsible for the elevation in IL-12 levels along with other effects of CpG-C, our study focused on their assessment following *in vivo* CpG-C immuochallenge and pre-exposure to stress. We evaluated pDCs (mPDCA-1^+), known for their robust type I interferon secretion following CpG-C stimulation, mDCs ($33D1^+$), known for their antigen-presenting capacities, and plasmacytoid-derived DCs (mPDCA-1^+33D1^+) that share similar characteristics with mDCs (Soumelis and Liu, 2006). Examination of pDCs and mDCs revealed that in the circulation CpG-C elevated the number, the activation and the maturation profiles of these cells. However, water stress markedly reduced their numbers without abolishing the CpG-C effect. Thus, although CpG-C still had an impact under stress conditions, the DC numbers found in stressed animals were significantly lower, most likely affecting the magnitude of the immune response to CpG-C. In this respect, it has also been shown that DCs are susceptible to the effects of stress, such that exposure to exogenous corticosterone results in an inability to mature following stimulation, impaired secretion of pro-inflammatory cytokines, and failure to prime naïve $CD8^+$ T cells (Elftman et al., 2007).

The changes seen in the numbers of DCs following stress seem unique, as much smaller effects were evident when the entire lymphocytic population was examined. Additionally, no changes in lymphocyte numbers were demonstrated following CpG-C immunochallenge,

suggesting that the robust interaction between CpG-C treatment and stress is most likely unique to specific cell populations. Overall, these findings employing a behavioral stress paradigm and *in vivo* administration of CpG-C, are in line with previous reports showing that exposure to dexamothasone reduced the number of both splenic pDCs and mDCs (Abe and Thomson, 2006).

Within the DC population, the most interesting findings are those regarding plasmacytiod-derived (PD) DCs. Here, following CpG-C immunostimulation we found that all of the PD DCs cells express the mDC marker 33D1 and the adhesion molecule CD11b, which is not expressed on pDCs, and is usually restricted to cells of myeloid lineage. The difference in function is also conveyed by a larger percentage of these cells (~25-30%) that express the co-stimulatory molecule CD80 within the CpG-C treated group. In contrast, CD80 is hardly expressed on pDCs (~10%), as their main function is secretion of type I IFNs and not antigen-presentation to T cells (Soumelis and Liu, 2006). Furthermore, the rise in PD DC number following CpG-C treatment was dramatic compared to that seen in both the mDC or pDC populations. Within this unique cell population we found that stress not only decreased the number of cells in the circulation in animals treated with CpG-C, but also changed the kinetics of this response, as elevated PD DC numbers were evident only 4hrs following immunochallenge but not at 12hrs, as seen in the unstressed animals treated with CpG-C.

Comparison of the effects of CpG-C and stress on the different DC subsets in the two immune compartments examined (the circulation and the spleen) revealed a clear difference between the two. Whereas CpG-C had a robust effect on these cell subtypes in the blood, no such effect was seen in the spleen, and while water stress dramatically affected all DC cell subtypes in the circulation, in the spleen it had almost no impact on mDCs and only a small impact on pDCs. Only the splenic PD DCs showed a response to stress comparable to that seen in the blood. It therefore seems as though cells resident to the spleen are less susceptible to external stimuli compared to the same cell populations in the blood. O'keeffe et al. (2002) (O'Keeffe et al., 2002) found that pDCs in the blood have a different, and less differentiated, phenotype than pDCs found in mouse lymphoid organs, including the spleen. The differences between the cells in the two immune compartments indicate a compartment-specific susceptibility pattern and differentiation capacity, which needs to be further examined following *in vivo* exposure to stress and CpG-C immunostimulation.

Looking for potential mechanisms mediating the immune perturbations described above we examined transcript levels of TLR9, as the activity of CpG ODNs depends on their activation of this receptor (Hemmi et al., 2000). We did not detect changes in TLR9 mRNA levels in the spleen following CpG-C treatment or behavioral stress. This is in accordance with findings that reported no changes in splenic TLR9 mRNA following CpG-A immunochallenge (Cao et al., 2008), as well as no changes in adrenal and pulmonary TLR9 mRNA and protein levels following CpG-B treatment (Knuefermann et al., 2007; Tran et al., 2007). Furthermore, no changes in TLR9 protein levels within murine splenic pDCs were found by others following *in vivo* administration of the glucocorticoid dexamothasone (Abe and Thomson, 2006). It seems, therefore, that the combination of behavioral stress and CpG-C treatment does not affect transcript levels of the evolutionarily preserved TLR9. The dampened immune response to CpG-C seen following stress may be due to unchanged antigen intake and environment sampling by dendritic cells (Elftman et al., 2007). Specificaly, following the encounter of a mitogen, dendritic cells briefly increase their endocytotic activity, then antigen uptake is drastically down-regulated (West et al., 2004). Corticosterone has been shown to prevent this down-regulation, thus interfering with dendritic cell maturation and function (Elftman et al., 2007) and perhaps IL-12 secretion. Beyond this hypothesized mechanism and to the best of our knowledge, the molecular

mechanism underlying the interaction between stress and the efficacy of CpG immunotherapy have yet to be elucidated.

In the current study we have shown that while CpG-C immunostimulation in unstressed mice activated an immune response that included cytokine upregulation and augmentation of relevant DC subpopulation numbers, the effect of the same treatment on mice experiencing on-going stress was severely diminished. Clearly, patients treated with biological response modifiers for the potential resolution of various medical conditions cope with a range of stressors; however, animal studies do not adequately simulate this on-going stress response. The limited number of studies that addressed this potential interaction in animals has provided initial indications that stress can indeed modulate the beneficial effects of immunostimulation (Ghanta et al., 1985; Li et al., 1997). The current study clearly supports this notion, employing the immunostimulator CpG-C. As DCs play a crucial role in both innate immunity and the activation of adaptive immune responses, it is likely that a dampened DC response following stress, such as the one described in the current study, may substantially contribute to the disparity between some pre-clinical findings and clinical trials employing biological response modifiers. Independently of the effects of stress, TLR9 has been shown to be differentially expressed in humans and rodents (Krieg, 2002). This discrepancy should also be taken into account when advancing from pre-clinical to clinical trials and studying the effects of stress. Therefore, future studies should examine neuroendocrine mediating mechanisms and potential prophylactic interventions, both psychological and pharmacological, to reduce the harmful effects of stress on the immune response to CpG-C and other biological response modifiers.

Research Highlights

- Stress interrupts CpG-C-induced production and secretion of IL-12
- Stress reduces dendritic cell subtype numbers in CpG-C-treated animals
- Stress reduces activation and maturation of dendritic cell subtypes
- Stress effects on dendritic cells are more dramatic in the blood than in the spleen

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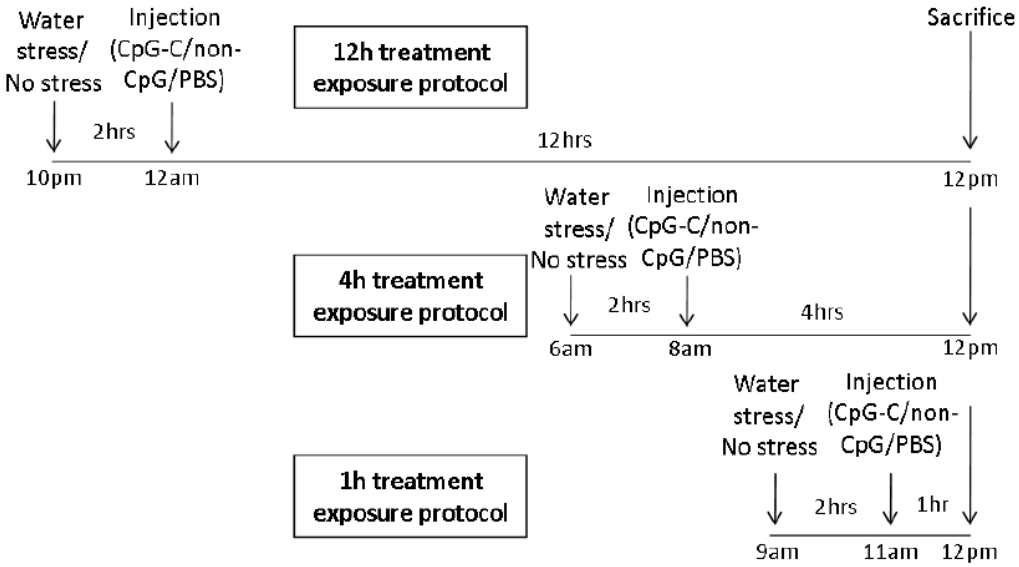

Figure 1. Experimental design and schedules of the different stress and CpG-C treatment protocols

Mice were divided to undergo water stress or to serve as no-stress controls. The animals undergoing stress were placed in opaque cages with 0.5cm of room-temperature water with free access to food and water, while the no-stress groups were left in their home cages. Two hrs following the commencement of the stress protocol, mice were injected with CpG-C, non-CpG, or PBS. Each of these 6 groups was then further subdivided into 3 different stress exposure times before blood draw (1, 4 and 12hrs following the injection of CpG-C/non-CpG/PBS), forming 18 experimental groups. The timing of the different stress protocols was set such that all protocols ended at the same time of the day, and all animals were sacrificed simultaneously.

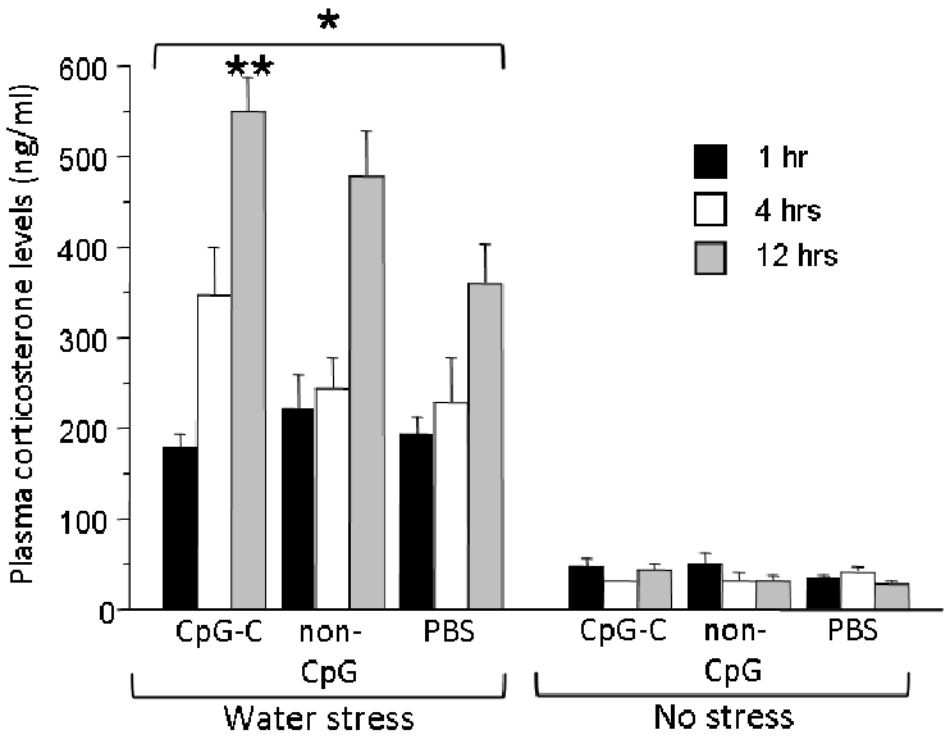

Figure 2. CpG-C treatment did not affect plasma corticosterone levels in unstressed animals, but elevated corticosterone levels in animals subjected to 12hr of water stress

Mice were exposed to water stress or served as home cage controls. Two hrs following the commencement of the stress protocol animals were injected with CpG-C, non-CpG or PBS, and sacrificed 1, 4 or 12hrs thereafter. Plasma corticosterone levels were elevated following water stress (*, $p<0.05$). CpG-C did not elevate plasma corticosterone levels in non-stressed animals, but elevated corticosterone levels in animals exposed to 12hrs of water stress compared to the PBS-water stress group (**, $p<0.05$). Data are presented as mean + SEM.

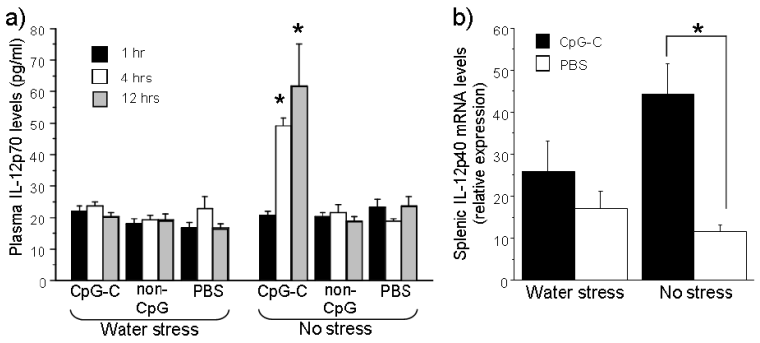

Figure 3. Water stress interrupted the CpG-C-induced rise in IL-12 plasma protein and in splenic mRNA levels

Mice were exposed to water stress or served as home cage controls. Two hrs following the commencement of the stress protocol animals were injected with CpG-C, non-CpG or PBS, and sacrificed 1, 4 or 12hrs thereafter. **(a)** CpG-C treatment significantly raised IL-12p70 plasma levels in non-stressed animals (*, $p<0.05$), however, no such effect was seen in animals subjected to water stress, yielding a significant interaction between water stress and CpG-C treatment ($p<0.05$). **(b)** Splenic IL-12p40 mRNA was studied only in CpG-C-treated animals and the PBS control groups at the 4hr time-interval. A significant difference between CpG-C and PBS treatment was found in non-stressed animals (*, $p<0.05$) but not in the animals exposed to water stress, suggesting that the rise in IL-12p40 mRNA following CpG-C treatment was interupted. Data are presented as mean + SEM.

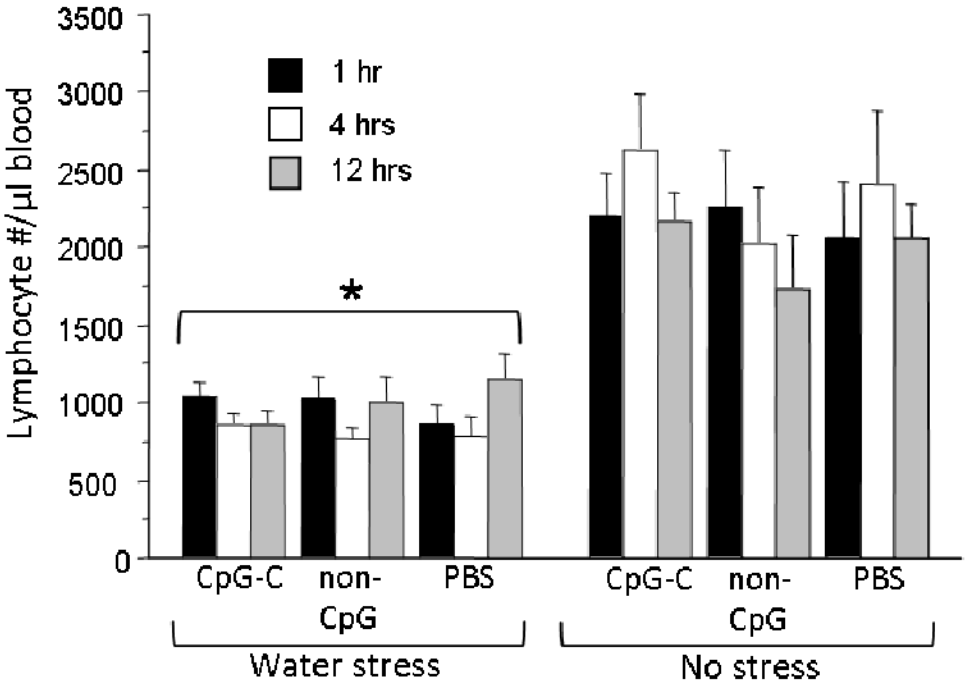

Figure 4. Stress, but not CpG-C affected lymphocyte concentrations in the blood

Mice were exposed to water stress or served as home cage controls. Two hrs following the commencement of the stress protocol animals were injected with CpG-C, non-CpG or PBS, and sacrificed 1, 4 or 12hrs thereafter. FACS analysis found that water stress caused a 2-fold decrease in lymphocyte numbers/µl blood (*, $p<0.05$), however, no effects of CpG-C treatment were detected. Data are presented as mean + SEM.

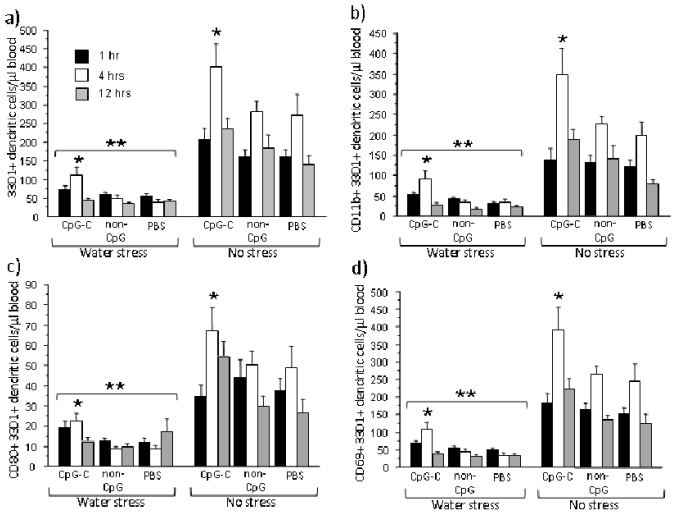

Figure 5. Stress and CpG-C treatment affected the number, activation, and maturation of blood 33D1$^+$ dendritic cells and their subpopulations

Mice were exposed to water stress or served as home cage controls. Two hrs following the commencement of the stress protocol animals were injected with CpG-C, non-CpG or PBS, and sacrificed 1, 4 or 12hrs thereafter. FACS analysis found that CpG-C treatment significantly elevated the total number of circulating 33D1$^+$ dendritic cells (DCs) and did so mostly 4hrs following immunochallenge (*, $p<0.05$), however, the number of 33D1$^+$ DCs was significantly diminished in the blood of animals exposed to water stress (**, $p<0.05$) **(a)**. When the numbers of specific sub-populations of 33D1$^+$ DCs were examined, the same pattern of results emerged for populations expressing the adhesion molecule CD11b **(b)**, the co-stimulatory marker CD80 **(c)**, and the activation marker CD69 **(d)**. Data are presented as mean + SEM.

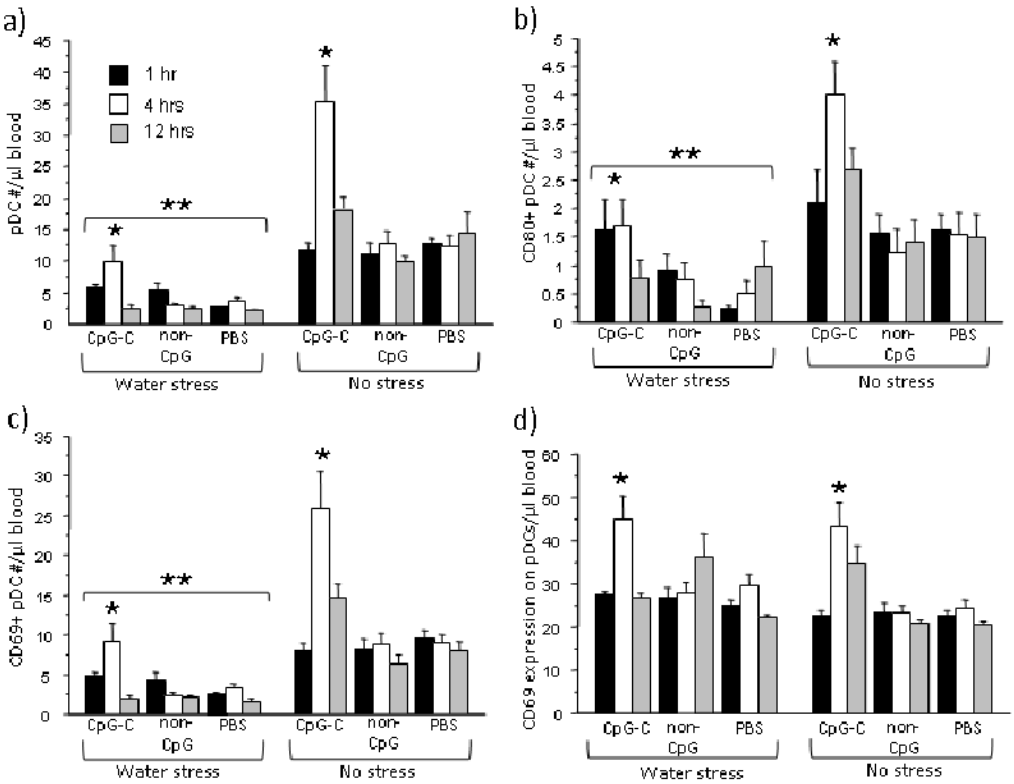

Figure 6. Stress and CpG-C treatment affected the number, activation, and maturation of blood plasmacytoid dendritic cells and their subpopulations

Mice were exposed to water stress or served as home cage controls. Two hrs following the commencement of the stress protocol animals were injected with CpG-C, non-CpG or PBS, and sacrificed 1, 4 or 12hrs thereafter. FACS analysis found that CpG-C treatment significantly elevated the total number of circulating plasmacytoid dendritic cells (pDCs) and did so mostly 4hrs following immunochallenge (*, $p<0.05$), however, the number of pDCs was significantly diminished in the blood of animals exposed to water stress (**, $p<0.05$) **(a)**. When the numbers of specific sub-populations of $33D1^+$ DCs were examined, the same pattern of results emerged for populations expressing the co-stimulatory marker CD80 **(b)**, and the activation marker CD69 **(c)**. The expression levels of CD69 were also elevated 4hrs following CpG-C treatment, irrespective of exposure to stress (*, $p<0.05$) **(d)**. Data are presented as mean + SEM.

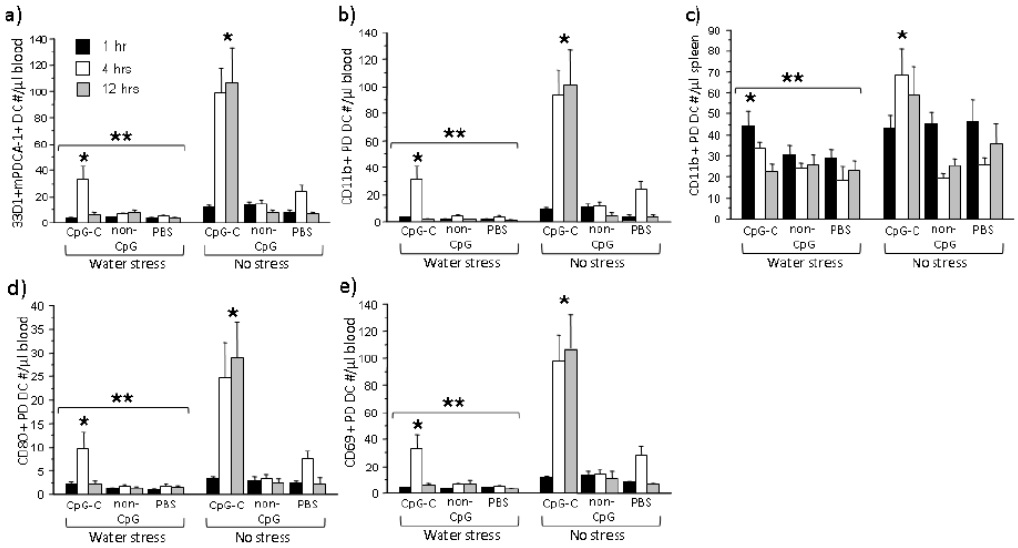

Figure 7. Stress detrimentally affected the impact of CpG-C treatment on the number, activation, and maturation of plasmacytoid-derived dendritic cells

Mice were exposed to water stress or served as home cage controls. Two hrs following the commencement of the stress protocol animals were injected with CpG-C, non-CpG or PBS, and sacrificed 1, 4 or 12hrs thereafter. FACS analysis found that CpG-C treatment significantly elevated the total number of circulating plasmacytoid-derived dendritic cells (PD DCs) and did so at 4 and 12hrs following immunochallenge (*, $p<0.05$). Stress significantly reduced the number of this population across all conditions (**, $p<0.05$), and significantly reduced the impact of CpG-C treatment, completely abolishing it at the 12hr time point, yielding a significant interaction between stress and CpG-C treatment ($p<0.05$) **(a)**. When the numbers of specific sub-populations of PD DCs were examined, the same pattern of results emerged for populations expressing the adhesion molecule CD11b in the blood **(b)**, and the spleen **(c)**, the co-stimulatory marker CD80 in the blood **(d)**, and the activation marker CD69 in the blood **(e)**. Data are presented as mean + SEM.

Table 1

Plasma IL-10 levels (pg/ml)

Stress	Time-interval	CpG-C	Control
	1 hr	183.4 ± 14.1	133.4 ± 9.0
Water stress	**4 hrs**	151.3 ± 7.1	121.0 ± 3.3
	12 hrs	166.7 ± 24.3	147.2 ± 19.4
No stress	**1 hr**	159.3 ± 15.2	134.5 ± 5.6
	4 hrs	231.9 ± 26.4 *	136.7 ± 11.9
	12 hrs	218.4 ± 22.2 *	128.4 ± 10.1

* Significantly different from the control (non-CpG and PBS), $p<0.05$.

Table 2

The effect of water stress and CpG-C treatment at differing time intervals on the numbers of dendrtic cell populations in the blood and the spleen

Cell population	Blood – *F* values for:				Spleen - *F* values for:			
	Water stress ↓ (WS)	**CpG-C ↑ (treatment)**	**Time-interval (TI)**	**Interactions**	**Water stress ↓ (WS)**	**CpG-C (treatment)**	**Time-interval (TI)**	**Interactions**
33D1$^+$ dendritic cells								
Total #	$F(1,134)=149.377^{**}$	$F(2,134)=8.536^{**}$	$F(2,134)=13.859^{**}$	WS × TI $F(1,134)=9.11^{**}$	NS	NS	NS	Triple $F(4,136)=4.717^{**}$
CD11b+	$F(1,133)=130.172^{**}$	$F(2,133)=10.426^{**}$	$F(2,133)=17.389^{**}$	WS × TI $F(1,133)=9.028^{**}$	$F(1,140)=5.751^{*}$	NS	$F(1,140)=3.467^{*}$	WS × TI $F(2,140)=6.267^{**}$
CD80+	$F(1,136)=98.811^{**}$	$F(2,136)=5.132^{**}$	$F(2,136)=3.743^{*}$	WS × TI $F(1,136)=4.114^{*}$	NS	NS	NS	NS
CD69+	$F(1,132)=137.414^{**}$	$F(2,132)=9.667^{**}$	$F(2,132)=15.116^{**}$	WS × TI $F(1,132)=9.386^{**}$	NS	NS	NS	Triple $F(4,137)=4.488^{**}$
Plasmacytoid dendritic cells (mPDCA-1$^+$)								
Total #	$F(1,136)=94.936^{**}$	$F(2,136)=15.25^{**}$	$F(2,136)=7.843^{**}$	All second degree $F(2,136)=3.56\text{-}7.644^{*,**}$ and triple $F(4,136)=3.163^{*}$	$F(1,131)=4.583^{*}$	NS	NS	WS × treatment $F(2,131)=3.076^{*}$ WS × TI $F(2,131)=4.88^{**}$
CD11b+	NA	NA	NA	NA	NA	NA	NA	NA
CD80+	$F(1,140)=26.784^{**}$	$F(2,140)=13.713^{**}$	NS	NS	$F(1,139)=4.737^{*}$	NS	NS	WS × TI $F(2,139)=4.495^{*}$
CD69+	$F(1,136)=72.741^{**}$	$F(2,136)=18.483^{**}$	$F(2,136)=9.218^{**}$	WS × treatment $F(2,136)=4.902^{**}$ Treatment × TI $F(4,136)=8.244^{**}$ Triple $F(4,136)=2.808^{*}$	$F(1,131)=3.968^{*}$	NS	NS	WS × TI $F(2,131)=3.479^{*}$
Plasmacytoid-derived dendritic cells(33D1$^+$ mPDCA-1$^+$)								
Total #	$F(1,136)=18.795^{**}$	$F(2,136)=20.919^{**}$	$F(2,136)=6.489^{**}$	WS × treatment $F(2,136)=10.896^{**}$ Treatment × TI $F(4,136)=5.096^{**}$ Triple $F(4,136)=3.364^{*}$	$F(1,138)=11.072^{**}$	NS	NS	NS
CD11b+	$F(1,135)=18.738^{**}$	$F(2,135)=20.198^{**}$	$F(2,135)=6.632^{**}$	WS × treatment $F(2,135)= 10.423^{**}$ Treatment × TI $F(4,135)=4.852^{**}$ Triple $F(4,135)=3.371^{*}$	$F(1,125)=11.094^{**}$	$F(2,125)=8.783^{**}$	NS	NS
CD80+	$F(1,141)=13.58^{**}$	$F(2,141)=16.31^{**}$	$F(2,141)=4.872^{**}$	WS × treatment $F(2,141)=6.619^{**}$ Treatment × TI $F(4,141)=3.429^{*}$ Triple $F(4,141)=2.641^{*}$	$F(1,140)=8.062^{**}$	NS	NS	NS
CD69+	$F(1,139)=21.029^{**}$	$F(2,139)=20.59^{**}$	$F(2,139)=7.117^{**}$	WS × treatment $F(2,139)=10.452^{**}$ Treatment × TI $F(4,139)=4.981^{**}$	$F(2,138)=10.223^{**}$	NS	NS	NS

* $p<0.05$,

** $p<0.01$,

NA – not applicable, NS – non-significant