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Helper T cells down-regulate CD4 expression upon chronic stimulation giving rise to double-negative T cells

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Abstract

Double-negative T (DNT) cells are $\text{TCR}^+\text{CD3}^+\text{CD4}^-\text{CD8}^-\text{NK1.1}^-$ cells that constitute a small but significant proportion of the TCR^+ T cells. Their developmental pathway and pathological significance remain unclear. In the present study, we utilized chronic *in vitro* stimulation of CD4^+ T cells to mimic immune hyper-activation of autoimmune lymphoproliferative syndrome and systemic lupus erythematosus, conditions characterized by DNT cells accumulation. After approximately 4–5 rounds of stimulation, the $\text{CD3}^+\text{CD4}^-$ population became apparent. These cells did not express CD8, NK1.1, TCR , or B220, exhibited a highly proliferative effector phenotype, and were dependent on T cell receptor (TCR) stimulation for survival. Moreover, $\text{CD3}^+\text{CD4}^-$ cells expressed MHC class II-restricted TCR , indicative of their origin from a CD4^+ T cell population. The results presented herein illustrate a novel method of DNT cell generation *in vitro* and suggest that immune hyper-activation could also be implicated in the genesis of the disease-associated DNT cells *in vivo*.

Keywords

Double-negative T cells; CD4 co-receptor; helper T cells; Kv1.3

1. Introduction

$\text{CD3}^+\text{CD4}^-\text{CD8}^-\text{NK1.1}^-$ T cells (double-negative T cells, DNTs) constitute approximately 1% of T cells expressing α and β chains of the T cell receptor (TCR) in the peripheral lymphoid organs of normal mice and humans [1, 2]. Although DNT cells were first reported over 20 years ago [3, 4], their origin and significance remain controversial. Thymic and extrathymic origins of the DNT cells have been proposed in the literature, but neither has been definitively proven. Thymic origin has been speculated to occur via escape of negative selection, followed by activation in the periphery [5, 6]. Alternatively, because DNT cells are defined as $\text{CD3}^+\text{CD4}^-\text{CD8}^-$ cells, it has been hypothesized that they could arise in the periphery from CD4^+ or CD8^+ precursors [7, 8].

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Autoimmune lymphoproliferative syndrome (ALPS) results from defective lymphocyte apoptosis and is characterized by accumulation of DNT cells in the peripheral circulation and lymphoid tissues, as well as lymphadenopathy, splenomegaly, and autoimmunity [9]. The dramatic increase in the percentage of DNT cells from the physiologic 1% to 40% in patients with ALPS is useful diagnostically [10]; however, their origin and function in ALPS pathogenesis remain unclear. Fas/CD95-deficient (*lpr*) mice have been widely used to model ALPS and to study DNT cell genesis. Defective apoptosis in these mice prevents deletion of autoreactive T cells and, during immune stimulation, leads to DNT cell accumulation in the lymph nodes, indicating potential peripheral origin of these cells [11]. In addition, an expanded population of DNT cells has been observed in peripheral blood of patients with systemic lupus erythematosus (SLE) and other immune-mediated diseases [12]. The role of DNT cells in the pathogenesis of autoimmune and inflammatory conditions remains unclear, and DNT cells with both immunosuppressive and pathogenic phenotypes have been described [13]. However, it is presently unknown whether the two phenotypes develop along mutually exclusive pathways, whether they arise from the same precursor cell, or whether certain environmental cues could lead to conversion of a regulatory DNT cell into a pro-inflammatory cell.

Because DNT cells accumulate in mice and humans with inflammatory conditions characterized by immune hyper-activation such as ALPS and SLE, we hypothesized that DNT cells could arise from hyper-stimulated pathogenic CD4⁺ T lymphocytes. Herein, we demonstrate that chronic *in vitro* stimulation of CD4⁺ helper T (T_H) cells gives rise to DNT cells, suggesting that DNT cells could be arising from over-stimulated T_H cells during the course of ALPS and SLE as well. Moreover, the DNT cells generated in our studies exhibit a hyper-activated phenotype, which favors a pathogenic, rather than an immunosuppressive, function of these cells in autoimmune diseases and inflammation.

2. Materials and Methods

2.1. Mice

C57BL/6 mice and mice with a transgenic T cell receptor (TCR) specific for MOG 35-55 (2D2 mice) were purchased from The Jackson Laboratories (Bar Harbor, ME). Kv1.3 KO mice on the C57BL/6 background were a kind gift from Dr. Leonard Kaczmarek (Yale University, New Haven, CT) and were bred and maintained in our animal facility. All mice were maintained in a federally approved animal facility at The Johns Hopkins University (Baltimore, MD) in accordance with the Institutional Animal Care and Use Committee. Mice of 8 to 12 weeks of age were used in all of the experiments. Age- and sex-matched mice were used in all experiments.

2.2. Cell isolation and culture

Spleens were isolated from naïve mice, and single-cell suspensions were made by passing through a 70-µm nylon cell strainer. T_H cells were isolated from splenocytes by negative selection using EasySep Mouse CD4⁺ T cell Enrichment Kit (StemCell Technologies, Vancouver, British Columbia, Canada), following manufacturer's protocol. Cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% v/v FBS (Life Technologies), 100 µg/ml penicillin and streptomycin (Quality Biological, Gaithersburg, MD), 0.5 µM 2-mercaptoethanol (Life Technologies), 10 mM HEPES buffer (Quality Biological), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), and MEM NEAA (Sigma-Aldrich). The cells were stimulated with Dynal anti-CD3/CD28-coated beads (Invitrogen) for multiple rounds. For each round of stimulation, the cells were cultured in the presence of anti-CD3/CD28 beads for 4 days, followed by 3 days of rest in fresh medium in the absence of anti-CD3/CD28 beads. After 3 days of rest, the cells were

ficolled to remove dead cells and replated in fresh medium with anti-CD3/CD28 beads for the next round of stimulation.

2.3. Flow cytometry

For intracellular cytokine staining, cells were stimulated with Cell Stimulation Cocktail plus protein transport inhibitors (eBioscience, San Diego, CA) for 6 hours. Surface antigens were stained with the following antibodies: anti-CD4 (BD Biosciences, San Jose, CA, clone RM4-5), anti-CD3 (eBioscience, 145-2C11), anti-CD8 (BD Biosciences, 53-6.7), anti-NK1.1 (BD Biosciences, PK136), anti-B220 (BD Biosciences, RA3-6B2), anti-TCR (eBioscience, eBioGL3), anti-TCR (BD Biosciences, H57-597 to TCR α -chain), anti-V β 3.2 (BD Biosciences, RR3-16), anti-V β 11 (BD Biosciences, RR3-15), anti-CD44 (BD Biosciences, IM7), and anti-CD25 (BD Biosciences, 7D4). For intracellular staining, cells were fixed and permeabilized with the Foxp3 staining buffer kit (eBioscience) and stained for intracellular cytokines and proteins with anti-IFN γ (BD Biosciences, XMG1.2), anti-IL-17 (BioLegend, TC11-18H10.1), and anti-CLTA4 (eBioscience, UC10-4B9). For analysis of apoptosis, the cells were washed in Annexin V Binding Buffer (BD Biosciences) according to manufacturer's instructions and stained with Annexin V-APC (BD Biosciences) and 7-AAD (BD Biosciences). Flow cytometric analyses were performed on a FACSCalibur instrument (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

2.4. Proliferation Assay

Cultured cells were labeled with CFSE (Life Technologies) immediately prior to the 5th restimulation. The cultures were stimulated with anti-CD3/CD28-coated beads for 72 hours or not stimulated, and CFSE dilution was determined by flow cytometric analysis to evaluate the proliferation of CD4⁺ and CD4⁻ cells.

2.5. Statistical analysis

Statistical analysis was conducted using the GraphPad Prism software (GraphPad, San Diego, CA). Two-tailed Student *t*-test was used to analyze normally distributed data. Results were considered significant if the *p* value was < 0.05: * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.

3. Results

3.1. Chronic stimulation leads to a reduction in the proportion of CD4⁺ cells in pure T_H cell cultures

T_H cells were isolated from the spleens of C57BL/6 mice by negative selection, which routinely results in over 97% pure cultures. The cells were cultured in complete RPMI 1640 medium and stimulated with anti-CD3/CD28-coated beads for multiple rounds, with each round consisting of 4 days of stimulation, followed by 3 days of rest in the absence of anti-CD3/CD28 beads. Following 5 rounds of stimulation, the cultures were composed of approximately 80% CD3⁺CD4⁺ cells and 20% CD3⁺CD4⁻ cells (Fig. 1a). Since the negative selection method used for isolation of the CD4⁺ T cell population does not result in 100% pure cultures, we wanted to evaluate whether the changes observed in our cultures were due to a contaminating cell population. Flow cytometric examination of CD8, NK1.1, TCR, and B220 receptor expression revealed that the CD3⁺CD4⁻ cell population did not express significant levels of these receptors, demonstrating that the CD3⁺CD4⁻ cells did not represent a common contaminating lymphocytic lineage (Fig. 1b).

3.2. DNT cells arise from CD4⁺ T cells

Since the CD3⁺CD4⁻ cells did not express markers of the typical contaminating populations, we next sought to investigate their origin. First, endocytosis of the CD4 receptor was evaluated as a potential cause of reduction in the purity of the CD4⁺ T cell culture. To this end, the cultured cells were stained for both surface and intracellular CD4, which revealed that the CD3⁺CD4⁻ cells did not express intracellular CD4 and, hence, likely did not endocytose the CD4 co-receptor (Fig. 2a). Next, evaluation of TCR expression demonstrated that over 97% of both CD4⁺ and CD4⁻ cells express TCR (Fig. 2b), indicating that the CD3⁺CD4⁻ cells are in fact TCR⁺CD3⁺CD4⁻CD8⁻NK1.1⁻ T cells, i.e. DNT cells.

To determine whether the DNT population could be arising from CD4⁺ T cells, we utilized CD4⁺ T cells from 2D2 TCR-transgenic mice, which express MHC class II-restricted TCR specific for myelin oligodendrocyte glycoprotein (MOG) peptide (V 3.2/V 11 TCR) and produce few CD8⁺ T cells. 2D2 T_H cells were isolated and cultured as described above for WT cells. Directly *ex vivo*, over 90% of CD4⁺ T cells expressed the 2D2 TCR (Fig. 2c, left). Presence of the DNT population and its expression of the 2D2 TCR were examined during the 5th round of stimulation. Interestingly, the proportion of CD4⁺ T cells expressing V 3.2/V 11 TCR was dramatically reduced after 5 weeks of culture (Fig. 2c, right), indicating that chronic stimulation may alter TCR expression on TCR-transgenic CD4⁺ T cells. Following 5 rounds of stimulation, DNT cells constituted approximately 10% of the culture (data not shown). The majority of these cells expressed the 2D2-TCR, similar to the CD4⁺ T cells (Fig. 2c, right). These findings indicate that TCR-transgenic CD4⁺ T cells can potentially give rise to DNT cells, which maintain the TCR specificity.

3.3. DNT cells exhibit faster proliferation and less apoptosis than CD4⁺ T cells

After establishing that the DNT cells express MHC class II-restricted TCR and thus appear to arise from a CD4⁺ T cell population, we sought to evaluate their proliferative capacity as compared to that of the cells that maintained CD4 expression. CD4⁺ T cells depend on anti-CD3/CD28 stimulation for proliferation and survival. Similarly, when cultured in the absence of anti-CD3/CD28-coated beads for longer than a typical 3-day rest, the DNT cells failed to proliferate or survive (data not shown). Evaluation of CFSE dilution revealed that the DNT cells proliferated dramatically faster than their CD4⁺ neighbors in response to anti-CD3/CD28 stimulation (Fig. 3a). Furthermore, increased proliferative capacity was associated with reduced apoptosis, as evaluated by 7-AAD and annexin V staining (Fig. 3b). Thus, although both DNT and CD4⁺ T cells were dependent on TCR stimulation, DNT cells exhibited greater proliferative capacity and were more resistant to apoptosis than the CD4⁺ T cells. Alternatively, it is possible that the more rapidly proliferating CD4⁺ T cells are more resistant to apoptosis and preferentially lose CD4 expression.

3.4. DNT cells exhibit an effector phenotype and do not express CTLA4

To further characterize their phenotype, we examined expression of CD44, CD25, and CTLA4 (Fig. 4a-c) on DNT and CD4⁺ T cells during the 5th round of stimulation. While CD44 expression was not significantly different between the two populations, CD25 expression was significantly increased in the DNT cells as compared to the CD4⁺ cells, indicating an effector phenotype with an increased propensity for heightened activation. DNT cell CTLA4 expression was significantly lower than that of CD4⁺ T cells (Fig. 4c). Furthermore, both populations secreted equivalent amounts of the pro-inflammatory cytokines IFN γ and IL-17 (Fig. 4d). Overall, these data indicate that the DNT cells exhibit a phenotype resembling that of the effector CD4⁺ T cells, characterized by high CD25 and CD44 expression, as well as by production of pro-inflammatory cytokines.

3.5. DNT cell genesis is increased in the absence of Kv1.3 channel

The Kv1.3 channel is an outward rectifying potassium channel that maintains membrane depolarization and allows intracellular calcium accumulation during T cell activation. Kv1.3 has been shown to play a role in cell cycle progression, apoptosis, and activation status of CD4⁺ T cells [14-16]. Hence, we sought to examine whether the absence of Kv1.3 affects the genesis of DNT cells from pure T_H cell cultures. To this end, we cultured both WT and Kv1.3 KO CD4⁺ T cells as described above for WT cells. Remarkably, DNT cells appeared sooner and were more abundant in the Kv1.3 KO CD4⁺ T cell cultures than in WT cultures (Fig. 5), suggesting that DNT cell development from CD4⁺ T cells could be increased by anti-apoptotic signals as in the case of Kv1.3 deletion and ALPS.

4. Discussion

Herein, we report a novel observation that chronic *in vitro* stimulation leads to downregulation of CD4 expression on murine T_H cells, giving rise to the DNT cell population. The DNT cells in our cultures did not express CD8, NK1.1, B220, or TCR receptors, but were TCR⁺ and MHC class II-restricted. The DNT cells exhibited an effector phenotype characterized by high CD44 and CD25 expression and IFN- γ and IL-17 production, high proliferative capacity that was dependent on TCR stimulation, and reduced apoptosis. The apparent downregulation of CD4 co-receptor by T_H cells during chronic stimulation demonstrated in the present study could provide insight into the genesis of DNT cells in ALPS, SLE, and other inflammatory disorders. Moreover, the hyper-activated effector phenotype of DNT cells favors a pathogenic role of these cells in autoimmunity and inflammation.

In our study, chronic *in vitro* stimulation of pure T_H cell cultures led to a decrease in the proportion of CD4⁺ cells with a concomitant increase in CD3⁺CD4⁻ cells, suggesting that the DNT cells arose from activated CD4⁺ T cells as a result of downregulation of CD4 co-receptor expression. The CD4⁻ cells first appeared during the 4th round of stimulation, after which the proportion of CD4⁻ cells continued to increase. These findings are in agreement with a previous study demonstrating that CD4⁻ cells can be converted from proliferating CD4⁺ T cells [17]. However, in this study, Zhang *et al.* demonstrated a dramatic accumulation of CD4⁻ cells as early as 4 days following stimulation of CD4⁺ T cells with allogeneic dendritic cells and recombinant IL-2 or IL-15. In our study, the cells were stimulated with anti-CD3/CD28-coated beads that mimic TCR stimulation and without addition of exogenous cytokines, which could potentially explain the difference in the timing of CD4⁻ T cell appearance between our study and the study by Zhang and colleagues.

In our study, the CD4⁻ cells did not expand during the first 3 weeks of stimulation, arguing against their origin from a contaminating population. Although it is possible that the increase in the number of DNT cells could be due to expansion of a pre-existing population of DNT cells, we would expect this expansion to not be delayed for over 3 rounds of stimulation. Of note, Vogtenhuber *et al.* have shown that a small fraction of CD4^{low/neg}CD25⁺ T cells pre-existing in purified CD4⁺CD25⁺ Tregs can outgrow the Tregs [18]. Culture of CD4⁺CD25⁺ Tregs with anti-CD3 mAb for 3 days and an additional 5-8 days with IL-2 alone led to CD4^{low/neg}CD25⁺ cells constituting approximately 50% of the total culture. Very pure Treg cultures established after FACS sorting did not give rise to the DNT cells, suggesting that CD4^{low/neg}CD25⁺ T cells do not arise from CD4⁺ precursors. However, in this study, the CD4^{low/neg}CD25⁺ cells consisted of several distinct populations, including TCR⁺, CD8⁺, and NKT cells, all of which had similar functional phenotypes. DNT cells are defined by the lack of CD8, NK1.1, and TCR receptor expression; hence, the CD4^{low/neg}CD25⁺ T cells accumulated in these studies were not DNT cells by definition.

An accumulation of DNT cells is characteristic of several murine and human autoimmune and inflammatory conditions [19]. Although much progress has been made in understanding the function of DNT cells, their origin under both physiological conditions and during immune hyper-activation, as in the case of ALPS and in *lpr* mice, remains unclear. DNT cells have been hypothesized to accumulate in *lpr* mice in a failed attempt to control the systemic autoimmune disease mediated by self-reactive T cells, in addition to their increased survival propagated by defective Fas-mediated apoptosis [20, 21]. Moreover, it has been proposed that DNT cells have a regulatory function in *lpr* mice, and potentially in patients in ALPS, but cannot kill the autoreactive CD4⁺ or CD8⁺ T cells via Fas-FasL pathway due to the Fas mutation existing in these conditions [1, 5], thus contributing to the development of autoimmune disease [21]. Alternatively, defective apoptosis in *lpr* mice and in patients with ALPS could allow CD4⁺ T cells to avoid activation-induced cell death (AICD) and to continue proliferation, thus potentially giving rise to DNT cells. Activation of CD4⁺ T cells via TCR normally leads to rapid proliferation and ultimately to AICD [22]. *In vivo*, such contraction of the immune response following an immune challenge helps reduce the number of CD4⁺ T cells back to homeostatic levels, thus limiting lymphocyte accumulation and reactions against self. Interestingly, examination of apoptosis in our cultures revealed that significantly fewer DNT cells were undergoing apoptosis compared to CD4⁺ T cells, suggesting that DNT cells were more resistant to AICD. These findings are in agreement with previous observations demonstrating that DNT cell viability is minimally affected by TCR cross-linking as compared to CD8⁺ T cells [23]. In this study, Khan *et al.* demonstrate that DNT cell expression of Fas receptor was decreased with a concomitant increase of pro-survival Bcl-xL and Bcl-2, thus accounting for their decreased apoptosis.

To further explore the role of apoptosis and cell cycle progression in the development of DNT cells, we cultured CD4⁺ T cells lacking expression of the Kv1.3, a voltage-gated potassium channel that has been shown to be involved in the regulation of apoptosis and cell cycle in CD4⁺ T cells [15]. Interestingly, following chronic stimulation of Kv1.3-deficient CD4⁺ T cells, the DNT cell population appeared earlier and accumulated to a greater extent than in the WT cultures. Although other mechanisms may be contributing to the observed phenotype, it is likely that the reduced rate of apoptosis that occurs in the absence of Kv1.3 channel could allow DNT cells to differentiate from CD4⁺ precursors more rapidly. While the findings presented in this study contribute to the understanding of DNT cell origin and their developmental pathway, further studies are required to elucidate the mechanisms underlying the genesis of this population and their functional relevance. Nonetheless, our data reveal an easy manner in which these cells can be generated for future analysis and argues for their pathogenic role in autoimmunity and inflammation.

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Highlights

- Chronic *in vitro* stimulation of CD4⁺ T cells gave rise to CD3⁺CD4⁻ DNT cells
- DNT cells generated herein exhibited a highly proliferative effector phenotype
- DNT cells were dependent on T cell receptor (TCR) stimulation for survival
- DNT cells expressed MHC class II-restricted TCR
- DNT cells appear to originate from a CD4⁺ T cell population

Fig. 1. Chronic stimulation leads to a reduction in the proportion of CD4⁺ cells in pure T_H cell cultures

CD4⁺ T cells isolated by negative selection from C57BL/6 splenocytes were stimulated with anti-CD3/CD28-coated Dynal beads for 4 days, followed by 3 days of rest without the beads, for a total of 5 rounds of stimulation. **(a)** Expression of CD4 and CD3 was examined by flow cytometric analysis 72 hours following 1st, 3rd, 4th, and 5th restimulation (stim 1, 3, 4, and 5). The CD3⁺CD4⁻ population first became apparent during the 4th round of stimulation and increased in proportion during subsequent weeks of activation. **(b)** During the 5th round of stimulation, the CD3⁺CD4⁻ population was further examined for the expression of CD8, NK1.1, TCR, and B220. The flow plots shown are gated on the CD3⁺CD4⁻ population. All data are representative of at least 3 independent experiments.

Fig. 2. DNT cells arise from CD4⁺T cells

CD4⁺ T cells were isolated and cultured as in Figure 1. **(a)** To examine potential internalization of CD4 receptor, cultured cells were subjected to flow cytometric analysis during the 4th round of stimulation. The cells were surface-stained for CD3 and labeled with the same clone of anti-CD4 antibody (RM4-5) prior to and following fixation/permeabilization. **(b)** Expression of $\alpha\beta$ TCR on CD4⁺ and CD4⁻ cells was examined during the 5th round of stimulation. The gate was set on CD3⁺CD4⁺ (left) or CD3⁺CD4⁻ (right) population. **(c)** CD4⁺ T cells were isolated from 2D2-TCR transgenic animals and cultured as described in Figure 1. Expression of 2D2-TCR (V α 3.2/V β 11) was examined by flow cytometry directly ex vivo (gated on CD4⁺ cells, left) or following 5 rounds of stimulation (gated on CD3⁺CD4⁺ and CD3⁺CD4⁻ cells, right). All data are representative of at least 3 independent experiments

Fig. 3. DNT cells exhibit faster proliferation and less apoptosis than CD4⁺ T cells

CD4⁺ T cells were isolated and cultured as in Figure 1. **(a)** Following 4 rounds of stimulation, the cultures were labeled with CFSE and restimulated (stim) or not (no stim) for the 5th round of stimulation. CFSE dilution was examined on CD3⁺CD4⁺ (CD4⁺) and CD3⁺CD4⁻ (CD4⁻) cells 72 hours later. **(b)** 72 hours following 1st (left, gated on CD3⁺CD4⁺ cells) and 5th (right, gated on CD3⁺CD4⁺ or CD3⁺CD4⁻ cells) restimulation, apoptosis was examined by 7-AAD and annexin V staining. The proportion of 7-AAD⁺Annexin V⁺ cells is presented in the bar graph. All data are representative of at least 3 independent experiments

Fig. 4. DNT cells exhibit effector memory phenotype and do not express CTLA4

CD4⁺ T cells were isolated and cultured as in Figure 1. 72 hours following 1st stimulation (stim 1) or 5th restimulation (stim 5), the CD3⁺CD4⁺ (CD4⁺) and CD3⁺CD4⁻ (CD4⁻) cells were examined for expression of CD44 (a), CD25 (b), and CTLA4 (c) by flow cytometric analysis. (d) IFN γ and IL-17 production was also examined 72 hours following the 5th restimulation (gated on CD3⁺CD4⁺ or CD3⁺CD4⁻ cells). The percentage IFN γ ⁺ and IL-17⁺ producing cells is graphed on the right and is representative of at least 2 independent experiments

Fig. 5. DNT cell genesis is increased in the absence of Kv1.3 channel

Splenic CD4⁺ T cells were isolated from C57BL/6 mice (WT) or Kv1.3 KO mice (KO) and cultured as in Figure 1. **(a)** The proportions of CD3⁺CD4⁺ and CD3⁺CD4⁻ cells were quantified by flow cytometric analysis of CD4 and CD3 expression. Flow plots for cultures from the 5th stimulation are shown. **(b)** The proportion of CD3⁺CD4⁺ cells as percentage of total CD3⁺ cells is graphed for each round of stimulation for WT and KO cultures, * $p < 0.05$. All data are representative of at least 3 independent experiments