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Review

The Y_1 receptor for NPY: A key modulator of the adaptive immune system

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ABSTRACT

Growing evidence suggests that the neuropeptide Y (NPY) system plays an important role in the immune system. Yet, little is known about the expression of NPY and receptors in the immune system. Moreover, original contradicting results have confused the picture and hampered a clear understanding of its role in the immune system. The use of Y_1 receptor-deficient mice, combined with advanced methods to investigate immune functions, have provided the solution to the problem raised by previous disparities. From results obtained using Y_1-deficient mice ($Y_1^{-/-}$), we uncovered a bimodal role for Y_1 on immune cells. Y_1 expression on antigen-presenting cells (APC) is essential for their function as T cell priming elements. Conversely, Y_1 signaling in T cells plays a regulatory role without which T cells are hyper-responsive. The opposite role of Y_1 on APC and T cells has reconciled previous disparities by showing that signaling via Y_1 protects against inflammation by inhibiting T cell responses, whereas $Y_1^{-/-}$ mice are protected in the same inflammatory models due to defective APCs.

Contents

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Abbreviations: NPY, neuropeptide Y; APC, antigen-presenting cell; DC, dendritic cell; Th, T helper; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; DSS, dextran sodium salt; DTH, delayed type hypersensitivity; MLR, mixed lymphocyte reaction; TLR, Toll-like receptor

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1. Introduction

The induction of an adaptive immune response is a complex process involving the coordination of a number cell types and molecules. Antigen-presenting cells (APCs) trap, process and present antigens to naïve T lymphocytes which then differentiate into antigen-specific effector cells. More specifically, macrophages and dendritic cells (DCs) act as APCs, taking up antigen and presenting it to $CD8^+$ and $CD4^+$ T cells as peptide fragments bound to MHC class I or class II molecules, respectively. The interaction between T cells and APCs is a fundamental step in the induction of an adaptive immune response, which sets the stage for the proper activation of both T and B cell antigen-specific effector functions. Newly activated T cells can differentiate into T helper (Th) 1 or 2 cells, depending on the cytokine environment. Appropriate activation of APCs is a pivotal event in determining the polarization of T cell responses, and, for instance, production of IL-12 by APCs, is key to promote Th1 T cell differentiation. IL-12 acts directly on T cells to induce proliferation and IFNγ production and in combination with IFNγ to promote isotype switching to IgG2a in B cells [9]. Several autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis are caused by a sustained and inappropriate Th1-dominant autoreactive T cell responses.

In recent years the NPY system has emerged as a set of molecules potentially playing an important role in the induction of a number of immune responses by acting on a variety of immune cells. Previously described roles for the NPY system include immune cell distribution, production of cytokine by T helper cells and inflammatory mediator release from macrophages [3]. Further evidence supporting a role for NPY in regulating immune functions comes from the observation that high numbers of NPY containing nerve fibers are present in lymphoid organs, also importantly, interacting with leukocytes [8]. Moreover, the Y_1 receptor for NPY is widely expressed on leukocytes, including T, B cells and APCs such as DCs and macrophages [10].

Two recent studies have described a novel role for NPY via its Y_1 receptor in regulating the induction Th1 responses. In the first study, treatment of mice with NPY or an agonist to the Y_1 receptor suppressed experimental autoimmune encephalomyelitis (EAE), a Th1 T cell-driven autoimmunity model [2]. Conversely, blocking Y_1 receptor signaling using an antagonist to Y_1 resulted in an earlier onset of disease [2]. This data suggested a suppressive role for NPY, via signaling through its Y_1 receptor on T cells. However, a conflicting picture arose from the second study using another Th1-mediated model of inflammatory colitis, the dextran sodium salt (DSS)-induced colitis. In this model, Y_1-deficient mice ($Y_1^{-/-}$) or those treated with a Y_1 receptor antagonist were protected against weight loss and disease activity induced by DSS [5], thus suggesting this time that an absence of Y_1 receptor signaling has a protective effect in this model. The challenge at this stage is to explain why Y_1 signaling protected in some Th1-mediated settings and yet participated in inflammation in some others.

2. Immunological phenotype of $Y_1^{-/-}$ mice and new clues

To further dissect the role of the Y_1 receptor in regulating immune responses, we examined the immunological phenotype of $Y_1^{-/-}$ mice [10]. Mice lacking the Y_1 receptor had smaller spleens than WT controls, and this correlated with a global reduction in B cell numbers in all secondary lymphoid organs [10]. Reduced B cell numbers had little effect on total serum Ig levels; however, a specific loss of IgG2a production was observed, and this was not corrected upon antigen challenge [10]. In addition, initial evidence for an impairment of T cell activation in $Y_1^{-/-}$ mice arose from the reduced ratio of effector to naïve T cells observed in the lymph nodes of these mice, despite normal T cell development in the thymus [10]. Using a

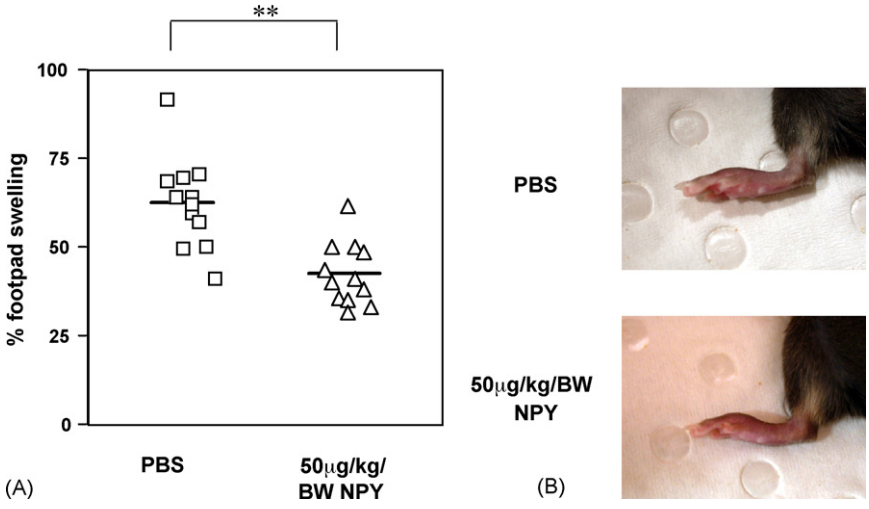

Fig. 1 – NPY inhibits footpad swelling elicited in a DTH response. (A) Footpad swelling in WT mice 24 h after secondary challenge with mBSA (n = 10–12 mice per group). Mean values represented by black bars. Each symbol represents an individual animal. Squares: i.p. PBS injection daily. Diamonds: 50 μg/ml NPY injected daily i.p. $^{**}P < 0.005$ as determined by t-test. Footpad swelling was measured using a caliper and calculated as described previously [4]. (B) Pictures of an inflamed paw of a mouse injected with PBS (top panel) or NPY (bottom panel) i.p. daily, representative of (A).

classical Th1 T cell-mediated inflammatory model, delayed type hypersensitivity (DTH), we tested the *in vivo* effect of NPY on inflammation. In this model, antigen challenge in the footpad results in antigen presentation by APCs to $CD4^+$ T cells in draining lymph nodes, which will become effector cells. These $CD4^+$ effector cells are recruited at the site of inflammation and secrete Th1 cytokines such as IFNγ, which play a role in activating and attracting other inflammatory cells such as macrophages at this site [4]. The magnitude of the DTH response is measured as footpad swelling.

Both male and female C57/B6 WT mice injected daily with 50 μg/kg body weight NPY were protected against DTH compared to the PBS-treated control mice as shown by 68% reduction in footpad swelling (Fig. 1). This result suggested that NPY signaling on T cells inhibits the Th1 T cell response that drives DTH. Surprisingly, $Y_1^{-/-}$ mice subjected to this DTH response were protected against footpad swelling. Furthermore, when $Y_1^{-/-}$ LN cells were restimulated *ex vivo*, a reduction in proliferation and IFNγ production was observed [10]. In this case, the data suggests that Y_1 is an important inflammatory mediator in DTH.

In addition, our $Y_1^{-/-}$ mice were subjected to the DSS-induced colitis model, and were also protected against weight loss and disease activity (Fig. 2). The protection against colitis seen in $Y_1^{-/-}$ mice correlated with reduced levels of serum IFNγ [10] and a reduction in cell infiltrates and inflammation in the colon. Thus, in two independent models of Th1-mediated inflammation, a loss of signaling through the Y_1 receptor on immune cells results in protection against disease, hence contradicting the above findings suggesting that signaling through Y_1 has an protective effect when triggered using agonist reagents.

As no possible flaws could account for the disparities of the findings, we decided to dissect the immune response and control all events step by step to clearly address the exact role of Y_1 in the immune system.

The induction of Th1 responses is complex and involves multiple cell types. T cells require appropriate activation from APCs to become effector cells. To examine which defective immune cell type contributed to the reduction in Th1-mediated inflammation in $Y_1^{-/-}$ mice, it became critical to examine $Y_1^{-/-}$ T cells and APCs separately. This approach was critical and finally provided the answers to interrogations in this system.

2.1. $Y_1^{-/-}$ T cells are hyper-responsive to activation and normally differentiate into Th1 cells in vitro and in vivo

To examine T cell responses in the presence of excess NPY or the absence of Y_1, classical *in vitro* T cell activation assays were performed on T cells isolated from WT and $Y_1^{-/-}$.

Firstly, the ability of naïve $Y_1^{-/-}$ T cells to become Th1 cells was examined in an *in vitro* Th1 cell differentiation assay. In the presence of IL-12 (driving Th1 development) and anti-IL-4 antibody (preventing Th2 cell development), $Y_1^{-/-}$ cells were able to become IFNγ-producing Th1 T cells (Fig. 3). The percentage of IFNγ-producing $Y_1^{-/-}$ T cells (Fig. 3A) and the amount of IFNγ secreted was similar to that of WT Th1 T cells (Fig. 3B). Thus, $Y_1^{-/-}$ T cells are functional and their ability to be activated and differentiate into Th1 T cells is intact *in vitro*.

The *in vitro* effect of NPY on T cell activation was examined in two T cell activation assays. Stimulation of T cells with an agonistic anti-CD3 antibody in the presence of nanomolar concentrations of NPY inhibited T cell proliferation (Fig. 4A). Interestingly, NPY-induced inhibition of T cell proliferation occurs at the initiation stage of activation. When NPY was added to the reaction 24 h after stimulation, no reduction in proliferation is observed (Fig. 4A). Furthermore, in a mixed lymphocyte reaction (MLR) in which T cells proliferate in response to an allogeneic APC, NPY was able to inhibit T cell proliferation in a dose-dependent manner [10]. Thus NPY inhibits proliferation of T cells *in vitro*, presumably via signaling through the Y_1 receptor at an early stage of T cell priming.

WT and $Y_1^{-/-}$ T cells were isolated and subjected to activation *in vitro*. Intriguingly, Y_1-deficient T cells were

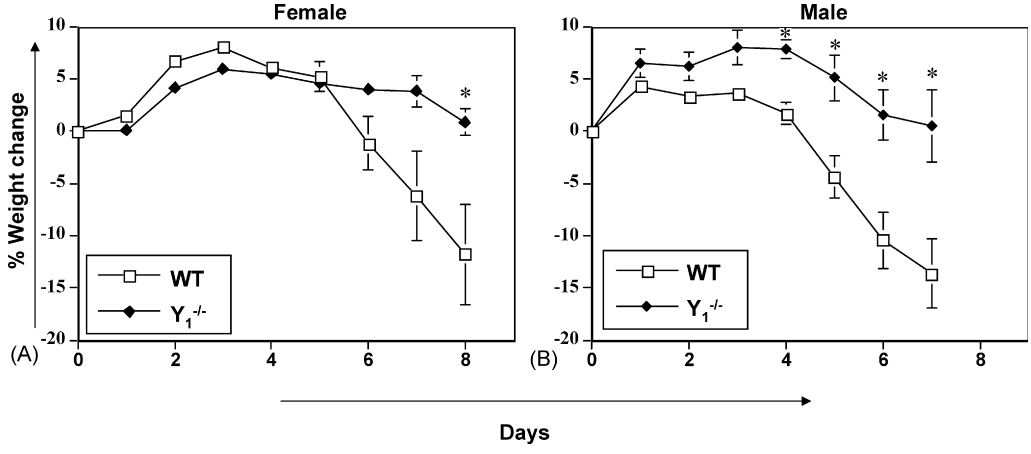

Fig. 2 – $Y_1^{-/-}$ mice are protected against weight loss as a consequence of DSS-induced colitis and have reduced inflammation of the colon. Percentage weight change in female (A) and male (B) mice, following induction of colitis using DSS. The change in weight over time is expressed as percentage of original body weight. Data are representative of two independent experiments (n = 5–8 mice per group per experiment). Mean ± S.E.M. is shown for WT (white squares) and $Y_1^{-/-}$ (black diamonds) mice. $^*P < 0.05$ as determined by t-test.

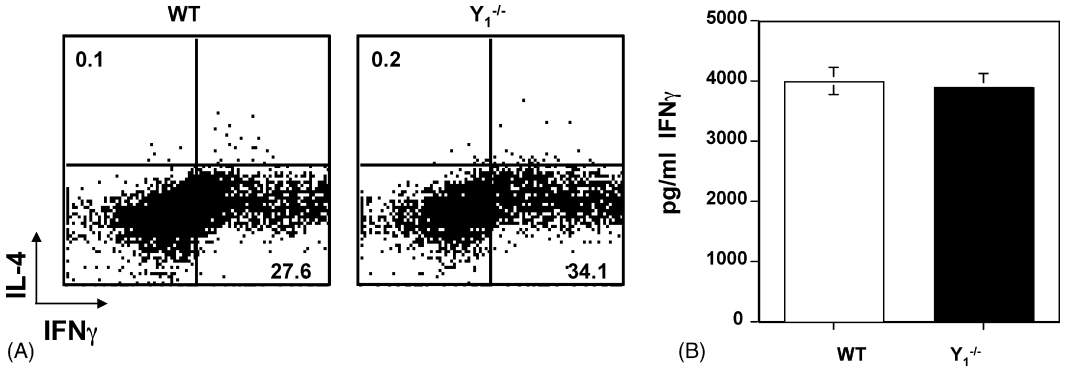

Fig. 3 – $Y_1^{-/-}$ T cells can become Th1 cells *in vitro*. (A) Percentage of IFNγ-producing cells generated from WT (left) and $Y_1^{-/-}$ (right) naïve T cells at day 5 in a Th1 differentiation assay. (B) Cytokine production of IFNγ from naïve T cells differentiated for 5 days under Th1 conditions from WT and $Y_1^{-/-}$ mice as determined by ELISA. Mean ± S.E.M. is shown for WT (white) and $Y_1^{-/-}$ (black) mice.

hyper-responsive to activation. Stimulation with anti-CD3 resulted in increased proliferation of Y_1-deficient T cells, and this corresponded with an increased recruitment into cell division of these T cells [10]. Similarly, in an MLR using allogeneic APCs as stimulators, $Y_1^{-/-}$ T cells responded better than WT T cells [10]. At this stage a new pattern is emerging with a role for Y_1 signaling as a negative regulator of T cell activation, hence the inhibitory role of NPY in T cell activation assays and, conversely, the hyper-reactivity of Y_1-deficient T cells in the same assays.

To determine whether this exciting observation is meaningful *in vivo*, we employed the $CD45RB^{hi}$ naïve $CD4^+$ T cell transfer model to drive autoimmune colitis in lymphopenic mice [6]. In the absence of regulatory cells, the $CD45RB^{hi}$ naïve $CD4^+$ T cells proliferate unchecked and populate the colon where they drive severe inflammation and tissue destruction. Three to 5weeks following adoptive transfer, mice succumb to severe colitis characterized by the onset of diarrhea and weight loss [6]. The colitis induced by the $CD45RB^{hi}$ population is due to a proinflammatory, Th1-type immune response [7].

Interestingly, the adoptive transfer of Y_1-deficient naïve T cells into $RAG1^{-/-}$ hosts resulted in an earlier onset of disease (days 10–12), compared with mice receiving WT naïve T cells (days 25–28) [10]. This early onset of disease corresponded with increased serum IFNγ and gut inflammation [10]. Thus, Y_1-deficient T cells are hyper-responsive to activation *in vitro* and *in vivo* and are able to differentiate into effector Th1 cells. This data in combination with the ability of NPY to inhibit T cell activation suggests that NPY via the Y_1 receptor is a negative regulator of T cell activation and presumably explains reduced T cell responses observed *in vivo* in the EAE model [1]. Moreover, the ability of Y_1-deficient T cells to respond to activation and normally differentiate into Th1 T cells suggests that the reduction in Th1 T cell responses observed in $Y_1^{-/-}$ mice in DTH- and DSS-induced colitis may be the result of functionally defective APCs.

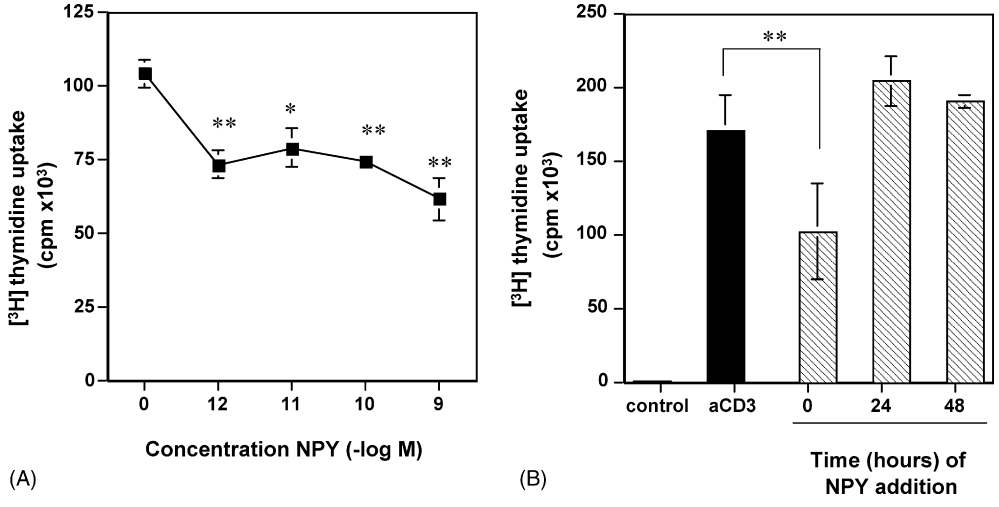

Fig. 4 – NPY inhibits T cell proliferation in response to anti-CD3. (A) Proliferation of WT $CD3^+$ T cells 72 h after stimulation with 1 μg/ml anti-CD3 in the presence of NPY. (B). Proliferation of $CD3^+$ T cells 72 h after stimulation with 1 μg/ml anti-CD3 (black bar) in the presence of 10^{-10} M NPY (hashed bar). Mean counts ± S.E.M. of triplicates is shown, representative of three experiments. $^{**}P < 0.005$, $^{*}P < 0.05$.

2.2. $Y_1^{-/-}$ APCs are functionally impaired

To test the function of $Y_1^{-/-}$ APCs, peritoneal macrophages were isolated and bone marrow-derived DCs were generated from WT and $Y_1^{-/-}$ mice. Similar numbers of macrophages and DCs were isolated, and the phenotype of these cells was similar. However, both Y_1-deficient macrophages and DCs produced fewer of the Th1 cytokines IL-12 and TNF following activation using a number of different Toll-like receptor (TLR) stimuli [10]. The control of cytokine production by Y_1 ligands is an autocrine mechanism, as the addition of a Y_1 receptor antagonist to cultured activated macrophages resulted in the reduction of cytokine production [10]. This data suggests that Y_1 ligand(s), produced by activated APCs, are required for optimal cytokine production by these cells.

$Y_1^{-/-}$ splenocytes (containing B cells and macrophages) and prepared $Y_1^{-/-}$ DCs used as stimulators in an allogeneic MLR were less effective stimulators when compared to WT cells [10]. Also, the ability of $Y_1^{-/-}$ DCs to take up fluorescently labeled antigen as measured by flow cytometry was significantly reduced compared to that of WT DCs [10].

Another key issue was to demonstrate *in vivo* the defect of $Y_1^{-/-}$ APCs. To address this point two key experiments were performed. In the first, antigen-pulsed DCs isolated from WT and $Y_1^{-/-}$ mice were transferred into WT mice to induce a DTH response *in vivo*. Mice receiving antigen-pulsed $Y_1^{-/-}$ DC developed significantly less footpad swelling than mice receiving antigen-pulsed WT DC [10], confirming *in vivo* the functional defect of $Y_1^{-/-}$ DCs. A second approach to confirm this point was performed using the $CD4^+CD45RB^{hi}$ transfer model to trigger autoimmune colitis. We propose that the early onset of colitis seen in mice receiving $Y_1^{-/-}$ T cells is due to the presence of functional WT APCs activating hyper-responsive $Y_1^{-/-}$ T cells. However, when we transferred hyper-reactive $Y_1^{-/-}$ T cells into $Y_1^{-/-}$ $RAG1^{-/-}$ mice in which all APC are $Y_1^{-/-}$ the early onset of disease did not occur [10], confirming that $Y_1^{-/-}$ APCs are unable to initiate optimal T cell responses.

Thus $Y_1^{-/-}$ APCs are functionally impaired in their ability to produce Th1-promoting cytokines and present antigens to T cells. This observation was important as it explains the protection of $Y_1^{-/-}$ mice in Th1-mediated inflammatory models not a defect in T cells but a defect in APC preventing T cell activation.

2.3. A bimodal role for the Y_1 receptor in the immune system

Detailed analysis of all immune players in $Y_1^{-/-}$ mice allowed us to first reconcile seemingly conflicting original data and uncover a sophisticated bimodal role for the Y_1 receptor in the regulation of immune responses. $Y_1^{-/-}$ T cells, either isolated in culture or in the presence of WT APCs, are unable to respond to suppressive signals provided by NPY and as such are hyper-responsive to activation. Conversely, $Y_1^{-/-}$ APCs are functionally impaired in their ability to take up antigen and produce Th1 cytokines. This can be viewed as a step 1 (APC activation) and step 2 (T cell activation by APC) model (Fig. 5). Activation of Y_1 inhibits step 2 and protects mice against Th1-mediated inflammation, whereas absence of Y_1 prevents step 1, without

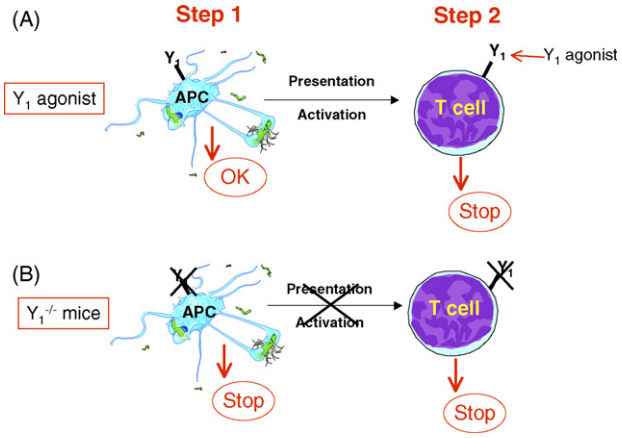

Fig. 5 – Two step model of Y_1 regulation in immune responses. (A) A Y_1 receptor agonist suppresses T cell activation, resulting in impaired T cell responses observed in EAE model. (B) In $Y_1^{-/-}$ mice, a deficiency of Y_1 on APC results in a reduced ability of these cells to prime T cells, resulting in reduced Th1 responses *in vivo*.

which step 2 cannot occur and this also protects mice against inflammation (Fig. 5). Our results, which helped us draw this model, very nicely reconcile previous seemingly conflicting observations. However, a number of key issues remain to be addressed. First of all, as Y_1 has many other functions outside the immune system, one can easily argue that the effect seen in $Y_1^{-/-}$ may be the indirect result of Y_1 deletion in tissues other than immune cells. To address this point we did reciprocal bone marrow transfers into irradiated WT and $Y_1^{-/-}$ hosts for the purpose of reconstituting a WT immune system in $Y_1^{-/-}$ mice and a $Y_1^{-/-}$ immune system in the WT mice.

Specifically, $Y_1^{-/-}$ mice sublethally irradiated and reconstituted with WT bone marrow respond normally in a DTH model and produce normal amounts of IgG2a antibodies. Conversely, WT mice reconstituted with $Y_1^{-/-}$ bone marrow have reduced DTH responses and isotype switching to IgG2a antibodies [10]. This result indicates that the immune defect observed in $Y_1^{-/-}$ mice is immune cell-specific and is not the result of Y_1 deficiency in non-immune tissues.

3. Conclusions

We have shown a novel bimodal role for NPY and its Y_1 receptor in the regulation of immune responses. NPY via Y_1 is a negative regulator on T cells, whereby Y_1 signaling acts as a key activator of APC function. These results have changed the way we look at the immune system. Historically, research has focused on the role of molecules specifically expressed in the immune system to explain immune functions. These recent findings tell us that mechanisms regulating the immune system are far from fully understood. As NPY and PYY show very similar pharmacological profiles an extension of these studies incorporating mice deficient in both PYY and NPY ($NPY^{-/-}/PYY^{-/-}$), will provide further insight into the role this complicated system plays in regulating immune responses. The finding that the NPY system may play a central role in

immuno-regulation may lead to novel strategies for therapeutic intervention in several autoimmune and inflammatory conditions.

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