A fundamental bimodal role for neuropeptide Y1 receptor in the immune system

Julie Wheway,[1] Charles R. Mackay,[1,3] Rebecca A. Newton,[1] Amanda Sainsbury,[2,3] Dana Boey,[2] Herbert Herzog,[2,3] and Fabienne Mackay[1,3]

[1]The Arthritis and Inflammation Research Program and [2]The Neurobiology Research Program, The Garvan Institute of Medical Research, Darlinghurst NSW 2010, Australia
[3]The University of New South Wales, Sydney NSW 2052, Australia

Psychological conditions, including stress, compromise immune defenses. Although this concept is not novel, the molecular mechanism behind it remains unclear. Neuropeptide Y (NPY) in the central nervous system is a major regulator of numerous physiological functions, including stress. Postganglionic sympathetic nerves innervating lymphoid organs release NPY, which together with other peptides activate five Y receptors (Y1, Y2, Y4, Y5, and y_6). Using Y1-deficient ($Y1^{-/-}$) mice, we showed that $Y1^{-/-}$ T cells are hyperresponsive to activation and trigger severe colitis after transfer into lymphopenic mice. Thus, signaling through Y1 receptor on T cells inhibits T cell activation and controls the magnitude of T cell responses. Paradoxically, $Y1^{-/-}$ mice were resistant to T helper type 1 (Th1) cell–mediated inflammatory responses and showed reduced levels of the Th1 cell–promoting cytokine interleukin 12 and reduced interferon γ production. This defect was due to functionally impaired antigen-presenting cells (APCs), and consequently, $Y1^{-/-}$ mice had reduced numbers of effector T cells. These results demonstrate a fundamental bimodal role for the Y1 receptor in the immune system, serving as a strong negative regulator on T cells as well as a key activator of APC function. Our findings uncover a sophisticated molecular mechanism regulating immune cell functions that can lead to stress–induced immunosuppression.

CORRESPONDENCE
Fabienne Mackay:
f.mackay@garvan.org.au
OR
Herbert Herzog:
h.herzog@garvan.org.au

Abbreviations used: CFSE, carboxyfluorescein diacetate succinimidyl ester; DSS, dextran-sulfate sodium; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; mBSA, methylated BSA; NP, nitrophe-nyl; NPY, neuropeptide Y; PGN, peptidoglycan; TLR, Toll-like receptor.

Psychological conditions, such as stress or de-pression, are known to compromise immune defenses and increase the likelihood of infec-tions, autoimmunity, or cancer (1). Although this concept has been well accepted for decades, the molecular mechanisms controlling this inter-play remain unclear. Neuropeptides, neurotrans-mitters, hormones, and cytokines are thought to participate in the modulation of immune func-tions by the central or peripheral nervous system (1). Studies looking at the effect of a defective sympathetic nervous system on immune func-tions and autoimmunity have focused on cate-cholamines as they are viewed as the major sympathetic transmitters (2). Although cate-cholamines have some effects on immune func-tions and can modulate clinical manifestations in some animal models of autoimmune diseases (3), their function does not explain other major im-mune effects associated with altered sympathetic nervous system functions.

Postganglionic sympathetic nerves that inner-vate primary and secondary lymphoid organs also release neuropeptide Y (NPY; reference 4). Five Y receptors (Y1, Y2, Y4, Y5, and y_6) are known to mediate the actions of NPY together with two other family members, peptide YY and pancreatic polypeptide (5). NPY is widely expressed in the central and peripheral nervous system and is a major regulator for many im-portant physiological functions, including the regulation of food consumption and energy homeostasis (6). In addition, NPY controls many behavioral aspects, such as anxiety and other depression-related disorders (7), and recent findings point to a role for this neuropeptide in immune functions (3, 8). NPY appears to be particularly important in modulating cytokine release from leukocytes and affecting Th1 T cell function (8).

The polarization of T cell responses to Th1 or Th2 cells is central for T cell effector responses and also has relevance for the development of autoimmune and allergic diseases (9). Th1 T cells secrete IFN-γ, whereas Th2 T cells pro-

H. Herzog and F. Mackay contributed equally to this work.
The online version of this article contains supplemental material.

duce predominantly IL-4 and IL-5 (9). Th1 T cells specifically contribute to the progression of several inflammatory and autoimmune disorders, such as delayed-type hypersensitivity (DTH), colitis, rheumatoid arthritis, and multiple sclerosis (10). In contrast, Th2 T cells participate in allergic reactions such as asthma (11). Activation of APCs is a critical event for determining the polarization of T cell responses, and production of IL-12 by DCs, for instance, is required for Th1 T cell differentiation (12). A recent study showed that treatment of mice with NPY suppressed experimental autoimmune encephalomyelitis (EAE), a model of Th1 cell–driven autoimmune disease in mice (13). Using agonist and antagonist reagents, this study showed that this effect was triggered through the Y1 receptor expressed on T cells, suggesting that the NPY–Y1 axis represses Th1 T cell effector functions (13). Yet, opposite results were obtained in a Th1 cell–mediated experimental model of colitis in mice (14). In this model, $Y1^{-/-}$ mice, or normal mice treated with a Y1 receptor antagonist, were protected against colitis compared with control animals, suggesting that signaling through the Y1 receptor contributed to disease progression (14). Therefore, the major challenge in this system is to explain how signals through Y1 receptors on T cells can inhibit their activation and protect mice in a Th1 cell–mediated EAE model while lack or inhibition of Y1 signaling protects mice against Th1 cell–mediated colitis.

In recent years, various Y receptor–deficient mice have been generated, and these have proved invaluable for dissecting the physiological roles of this complicated receptor–ligand system (6). Here, using $Y1^{-/-}$ mice, we show that

$Y1^{-/-}$ T cells were hyperresponsive to activation and trigger severe colitis after transfer into lymphopenic mice. Paradoxically, $Y1^{-/-}$ mice were resistant to Th1 cell–mediated inflammatory responses, which was due to a functional defect in $Y1^{-/-}$ APCs that masked the hyperreactivity of $Y1^{-/-}$ T cells. Thus, Y1 signaling serves a bimodal role in the immune system as an activator of APC function and a repressor of T cell activation.

RESULTS
Altered lymphocyte numbers in the spleen and LNs of $Y1^{-/-}$ mice

To ascertain the precise functional role and mechanisms for NPY in the immune system, we assessed mice deficient in various NPY receptors and noted significantly reduced spleen sizes in $Y1^{-/-}$ mice (Fig. 1 A), but not in $Y2^{-/-}$ and $Y4^{-/-}$ mice (not depicted). Smaller $Y1^{-/-}$ spleen sizes correlated with reduced B cell numbers as assessed by FACS analysis of anti-B220–stained splenocytes (Fig. 1 B). Splenocytes were also stained with antibodies to IgM, CD23, CD21, and CD24, and B cell subsets were gated by FACS as described previously (15). Absolute numbers of cells in all B cell subsets were reduced, including immature transitional type 1 and 2 B cells and marginal zone B cells, but particularly mature B cells (Fig. 1 C). B cell numbers were also reduced in LNs (not depicted). Numbers of $CD8^+$ T cells were also slightly reduced in the spleen (Fig. 1 D), whereas numbers of splenic $CD4^+$ T cells were normal (not depicted). Interestingly, a profound alteration of the T cell compartment was seen in peripheral LNs with significantly
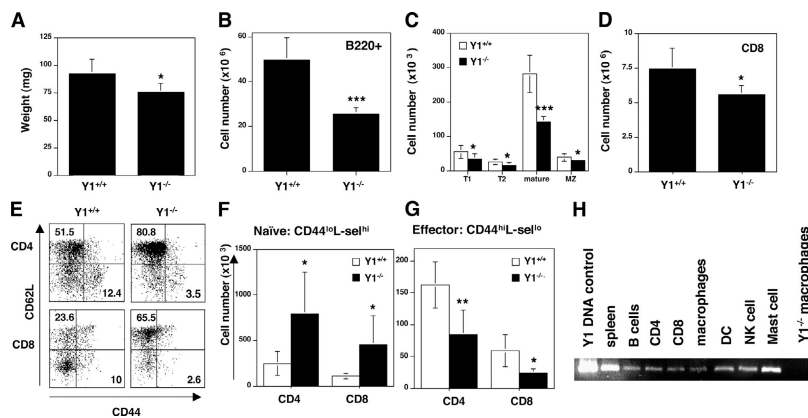
Figure 1. Altered T and B cell numbers in Y1$^{-/-}$ mice and Y1 expression in immune cells. (A) Spleen weight of Y1$^{+/+}$ and Y1$^{-/-}$ mice. (B) Absolute numbers of B220$^+$ B cells in the spleen of Y1$^{+/+}$ and Y1$^{-/-}$ mice. (C) Absolute numbers of splenic B cell subsets (immature transitional type 1 [T1], type 2 [T2] mature, and marginal zone [MZ] B cells) in Y1$^{+/+}$ (white bars) and Y1$^{-/-}$ mice (black bars) as indicated. (D) Absolute numbers of CD8$^+$ T cells in the spleen of Y1$^{+/+}$ and Y1$^{-/-}$ mice. (E) Representative FACS plots illustrate the increased proportions of naive T cells (top left quadrant) and decreased numbers of effector T cells (bottom right quadrant) in peripheral LNs of Y1$^{-/-}$ mice compared with Y1$^{+/+}$ mice (percent shown in each quadrant). Absolute numbers of naive T cells (F) and effector memory T cells (G) in the peripheral LNs of Y1$^{-/-}$ mice (black bars) and Y1$^{+/+}$ (white bars). Cells were stained and analyzed as described previously (reference 15). (E) RT-PCR for murine Y1 using cDNA from tissues, sorted immune cells, and control DNA as indicated. High expression in the spleen, mast cells, NK cells, and DCs, and B cells and reduced expression in CD4 and CD8 T cells as well as macrophages. A Y1 cDNA construct was used as a positive control and cDNA prepared from Y1$^{-/-}$ macrophages was used as a negative control. Mean ± SD of eight animals are shown in A–D, F, and G. ***, P < 0.001; **, P < 0.005; and *, P < 0.05 as determined by t test (ANOVA).

increased numbers of naive CD4$^+$ and CD8$^+$ T cells (Fig. 1, E and F) and decreased numbers of effector T cells (Fig. 1, E and G) when compared with Y1$^{+/+}$ mice. This phenomenon was clearly apparent from the greatly increased proportion of naive CD44^{lo} L-selectin^{hi} CD4$^+$ or CD8$^+$ T cells on representative dot plot histograms (Fig. 1 E, top left quadrant of dot plots). T cell development in the thymus was normal (not depicted).

We prepared RNA from normal splenocytes and cell-sorted immune cell subsets and assessed expression of the Y1 receptor in these samples using quantitative real-time PCR. We detected high levels of Y1 expression in the spleen, DCs, NK cells, and mast cells, and lower levels in T cells, B cells, and macrophages (Fig. 1 H). Expression of other Y receptors

was unaffected in the same cells/tissue isolated from Y1$^{-/-}$ mice (not depicted). In sum, Y1 deficiency leads to significant alterations of the lymphocyte compartments, and the relatively widespread expression of Y1 receptor in immune cells suggests an important role for Y1 in immune functions.
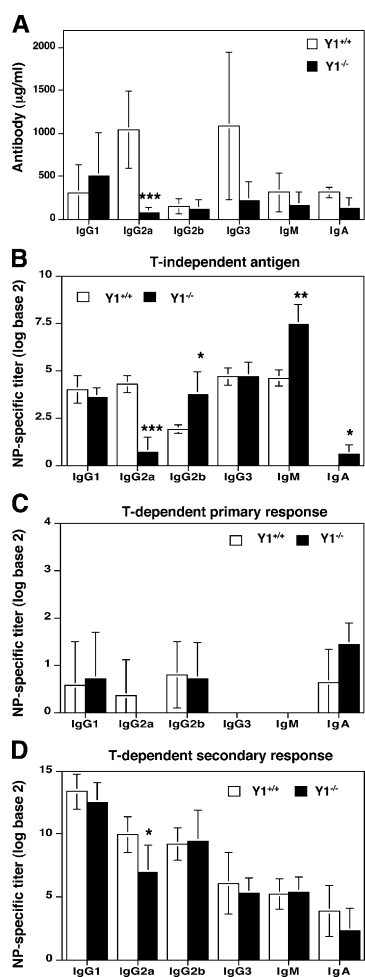
Impaired production of IgG2a in Y1$^{-/-}$ mice

Reduced B cell numbers in Y1$^{-/-}$ mice prompted us to measure by ELISA total serum Ig levels in Y1$^{-/-}$ mice. A specific and significant reduction of total IgG2a levels was observed in resting Y1$^{-/-}$ mice compared with Y1$^{+/+}$ mice (Fig. 2 A). Immunization of Y1$^{-/-}$ mice with the T-independent antigen nitrophenyl (NP)-Ficoll or the T-dependent antigen NP-OVA to elicit primary and secondary responses revealed defective antigen-specific IgG2a responses (Fig. 2, B–D). Although secondary responses to NP-OVA in Y1$^{-/-}$ mice led to a higher NP-specific IgG2a response compared with a primary response in these mice, the secondary NP-specific IgG2a response in Y1$^{-/-}$ mice still remained significantly inferior to that of Y1$^{+/+}$ mice (Fig. 2 D). In contrast to the IgG2a response, the IgG2b, IgM, and IgA response to NP-Ficoll was higher in Y1$^{-/-}$ mice compared with Y1$^{+/+}$ mice (Fig. 2 B). Interestingly, despite a 50% reduction in mature B cell numbers (Fig. 1 B), antigen-specific IgG1 secretion was not affected, suggesting possible compensatory mechanisms for some aspects of the antibody production (Fig. 2). Thus, Y1$^{-/-}$ mice display a general defect in IgG2a production, which was not corrected upon immunization.

Impaired Th1 cell–mediated DTH and dextran–sulfate sodium (DSS)–induced colitis in Y1$^{-/-}$ mice

Ig class switching to the IgG2a isotype requires Th1 cell cytokines, particularly IFN-γ, and is viewed as a hallmark of a Th1 cell response (16). The consistent, general, and selective reduction of IgG2a levels in Y1$^{-/-}$ mice led us to infer that Y1 expression might be important for Th1 cell responses. Therefore, we tested the response of Y1$^{-/-}$ mice to methylated BSA (mBSA), which is used to elicit a classic DTH response that is Th1 cell–mediated (17). Swelling in Y1$^{-/-}$ mice was significantly reduced to 55% of the average response seen in Y1$^{+/+}$ mice 24 h after challenge with mBSA (Fig. 3, A and B). Levels of mBSA-specific IgG2a were also reduced in Y1$^{-/-}$ mice (not depicted). When lymphocytes were prepared from the draining inguinal LNs of these mice and restimulated with mBSA in vitro, the proliferation of Y1$^{-/-}$ LN cells and the production of IFN-γ was reduced compared with that of control cells (Fig. 3, C and D, respectively). In contrast, IL-4 levels were unchanged (Fig. 3 D), suggesting that Y1 receptor signaling was essential for optimal Th1 T cell responses, but Y1 deficiency did not skew the T cell response toward a Th2 T cell response.

To verify whether this phenomenon could be reproduced in a mucosal Th1 cell disease model, we fed Y1$^{-/-}$ and Y1$^{+/+}$ mice with DSS, an irritant that induces colitis by destroying the intestinal lining (18). Under these condi-

Figure 2. Impaired IgG2a production in Y1$^{-/-}$ mice. (A) Total serum Ig levels in Y1$^{+/+}$ and Y1$^{-/-}$ mice. (B) NP-specific antibody titers 7 d after immunization with NP-Ficoll. (C) NP-specific antibody titers 7 d after NP-OVA immunization and (D) 14 d after a secondary immunization with NP-OVA. Mean and SD for six animals per group are shown in B–D, and eight animals are shown in A. IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA levels, total or antigen-specific, are shown in Y1$^{+/+}$ (white bars) and Y1$^{-/-}$ (black bars) mice. p-values as in Fig. 1.
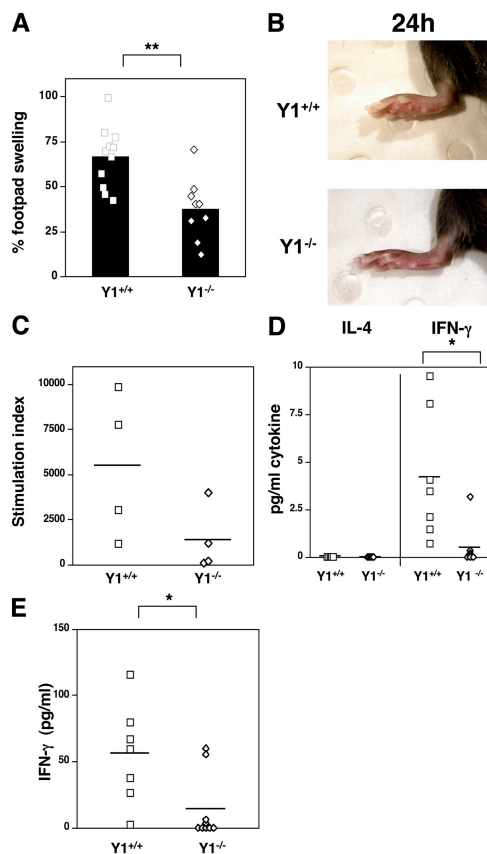
Figure 3. Impaired Th1 cell inflammatory responses in Y1$^{-/-}$ mice.
(A) Footpad swelling in Y1$^{+/+}$ and Y1$^{-/-}$ mice 24 h after secondary challenge with mBSA ($n = 10$ per group). Mean values are represented by black bars. (B) Pictures of an inflamed paw of Y1$^{+/+}$ (top) and a protected paw from a Y1$^{-/-}$ mouse representative of A. (C) Proliferation of Y1$^{-/-}$ LN cells after a 72-h mBSA restimulation ex vivo. Four mice were analyzed and data is representative of two independent experiments. (D) IFN-γ and IL-4 levels in the medium from cultures of ex vivo mBSA-restimulated LN cells (stimulated for 72 h). Each symbol represents an individual animal: squares, Y1$^{+/+}$; diamonds, Y1$^{-/-}$. Average of triplicates are shown in D for each animal ($n = 7$). (E) Serum levels of IFN-γ in Y1$^{-/-}$ mice 7 d after inducing DSS-triggered colitis (eight animals analyzed per group). p-values as in Fig. 1.

tions, our Y1$^{-/-}$ mice were protected against Th1 cell–mediated DSS-induced colitis (not depicted). The specific benefit from Y1 deficiency in DSS-mediated colitis was associated with strongly reduced IFN-γ serum levels (Fig. 3 E). These results demonstrate that Y1 deficiency protects against Th1 cell–mediated inflammatory disorders via suppression of T cell activation and reduction of IFN-γ production. Therefore, signaling through the Y1 receptor is essential for optimal Th1 cell immune responses.
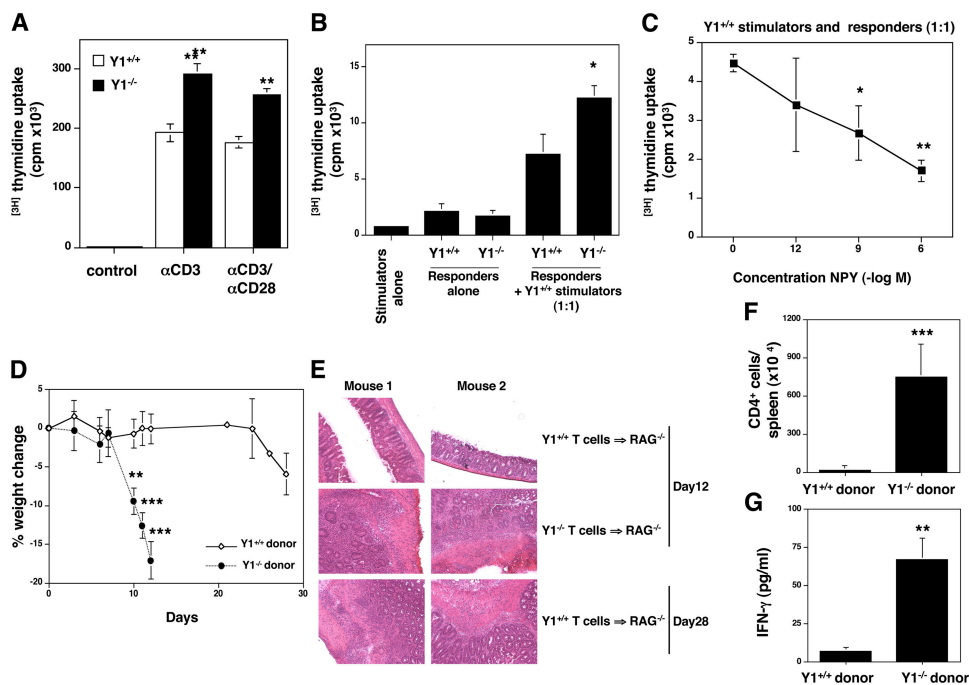
Y1$^{-/-}$ T cells are hyperresponsive to activation
Th1 cell responses are complex and involve several cell types as well as the production of various factors (10). APCs, such as DCs and macrophages, that produce IL-12 are the key el-

ements that drive the differentiation of activated T cells into Th1 effector T cells (12). Studies using a mouse Th1 cell–mediated model of EAE suggested that Y1 reduced EAE symptoms and triggered repressive signals in T cells that inhibited T cell activation (13). Similarly, when we treated normal mice with NPY this also prevented mBSA-mediated DTH responses (not depicted). To explain these seemingly conflicting results, we hypothesized that signaling through Y1 may indeed trigger inhibitory signals in T cells but may also be essential for the activation of APCs. Defective Y1$^{-/-}$ APC functions would prevent T cell activation and Th1 cell–mediated disease in Y1$^{-/-}$ mice, whereas the administration of NPY or Y1 agonists in a normal mouse would trigger inhibitory signals in T cells, suppressing their activation and polarization into Th1 T cells in vivo. To test this hypothesis, we analyzed the activation of Y1$^{-/-}$ T cells and Y1$^{-/-}$ APCs separately.

First, we checked the function of cell-sorted Y1$^{-/-}$ T cells in various in vitro and in vivo models for T cell activation. Interestingly, Y1$^{-/-}$ T cells were hyperresponsive to stimulation with anti-CD3 or anti-CD3$^+$ anti-CD28 compared with Y1$^{+/+}$ T cells (Fig. 4 A). A dose-response analysis of Y1$^{-/-}$ and Y1$^{+/+}$ T cell responses to anti-CD3 stimulation confirmed the increased sensitivity of Y1$^{-/-}$ T cells to low-dose anti-CD3 stimulation (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20051971/DC1), and this correlated with a faster rate of cell division as assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) staining (Fig. S1 B). Production of IL-2 and expression of the activation marker CD69 were normal in Y1$^{-/-}$ T cells (Fig. S1, C and D, respectively). Similarly, Y1$^{-/-}$ T cells were much better responders in MLR compared with Y1$^{+/+}$ T cells when inactivated Y1$^{+/+}$ splenocytes were used as stimulators (Fig. 4 B). Conversely, the addition of increasing amounts of NPY to an MLR using Y1$^{+/+}$ T cells as responders inhibited proliferation triggered by inactivated Y1$^{+/+}$ stimulators (Fig. 4 C). Thus, Y1 signaling negatively regulates T cell activation. Moreover, Y1$^{-/-}$ T cells are not intrinsically defective but are in fact hyperresponsive to activation in vitro.

To confirm this result in vivo, we used a colitis model in RAG1$^{-/-}$ mice induced by the transfer of cell-sorted naive CD45RB^{hi} CD4$^+$ T cells as described previously (19–21). This model provides for a host with Y1$^{+/+}$ APCs into which Y1$^{+/+}$ or Y1$^{-/-}$ CD4$^+$ CD45RB^{hi} CD4$^+$ naive T cells are transferred. If Y1$^{-/-}$ T cells are not defective, as our in vitro assessment would suggest (Fig. 4, A and B), there should be the usual development of colitis after the transfer of Y1$^{-/-}$ CD4$^+$ CD45RB^{hi} CD4$^+$ T cells into RAG1$^{-/-}$ mice. Indeed, such a transfer did induce colitis, but it was of a more rapid and severe type as soon as 10 d after transfer (Fig. 4 D). In fact, mice receiving Y1$^{-/-}$ T cells were killed around day 15 due to disease severity and excessive weight loss associated with this disease (Fig. 4 D), whereas RAG1$^{-/-}$ mice that received Y1$^{+/+}$ T cells retained normal weight at day 15 (Fig. 4

Figure 4. Y1–deficient T cells are hyperresponsive in vitro and in vivo. (A) Proliferation of Y1$^{+/+}$ and Y1$^{-/-}$ T cells 72 h after stimulation with anti-CD3 or anti-CD3 plus anti-CD28 as indicated. (B) T cell proliferation in an MLR with Y1$^{+/+}$ APCs as stimulators. (C) Dose-dependent inhibitory effect of NPY on an MLR with Y$^{+/+}$ responder and stimulator cells. Mean counts ± SE of triplicates are shown, representative of five experiments in A–C. (D) Naive Y1$^{-/-}$ T cell induction of early onset of weight loss and colitis after transfer to RAG1$^{-/-}$ mice. Y1$^{+/+}$ donor T cells, white diamonds;

Y1$^{-/-}$ donor T cells, black circles. (E) Histological analysis of hematoxylin and eosin–stained colon sections of RAG1$^{-/-}$ mice 12 (top) and 28 d (bottom) after the transfer of Y1$^{+/+}$ T cells and 12 d after the transfer of Y1$^{-/-}$ T cells (middle). Two representative mice shown out of eight mice analyzed. Magnification, ×100. (F) Absolute numbers of splenic Y1$^{+/+}$ and Y1$^{-/-}$ CD4$^+$ T cells 15 d after transfer to RAG1$^{-/-}$ mice. (G) Serum levels of IFN-γ in RAG$^{-/-}$ mice 15 d after receiving Y1$^{+/+}$ or Y1$^{-/-}$ naive T cells as indicated. $n = 5$ mice per group in D–F. p-values as in Fig. 1.

D) and were typically killed between day 21 and 28 (Fig. 4 D). Weight loss in these animals correlated with severe inflammation of the colon as shown by histopathologic analysis (Fig. 4 E). At day 12, the colon of RAG1$^{-/-}$ mice that received Y1$^{-/-}$ T cells showed a massive infiltration of leukocytes in the lamina propria and, occasionally, in the submucosa, prominent epithelial hyperplasia, and loss of mucin-containing vesicles in the goblet cells (Fig. 4 E). In contrast, RAG1$^{-/-}$ mice that received Y1$^{+/+}$ T cells and were killed on the same day (day 12) showed little if any inflammation (Fig. 4 E). Severe colonic inflammation in these mice was only evident when the animals were killed on day 28 (Fig. 4 E). The exacerbated colitis that occurred after the transfer of Y1$^{-/-}$ naive CD4$^+$ T cells into RAG1$^{-/-}$ mice was accompanied by a massive expansion of the splenic CD4$^+$ T cell population (Fig. 4 F) and very high serum levels of IFN-γ (Fig. 4 G). Thus, Y1$^{-/-}$ T cells are functional in vivo, and signaling through Y1 receptors in T cells is an essential regulatory feature that prevents T cell hyperactivity. Moreover, Y1$^{-/-}$ T cells were able to differentiate to Th1 effector T cells and produce IFN-γ in this model as well as after activation with IL-12 plus anti–IL-4 in vitro (not depicted). Therefore, reduced Th1 T cell responses, such as DTH (Fig. 3) or DSS-induced colitis observed in Y1$^{-/-}$ mice (Fig. 3; refer-

ence 14), might be attributable to a defect in APC function, but not to an intrinsic defect of Y1$^{-/-}$ T cells.

Impaired macrophage function in Y1$^{-/-}$ mice

To test for a possible defect in Y1$^{-/-}$ APC function, we first tested inactivated Y1$^{-/-}$ splenocytes as stimulators with Y1$^{+/+}$ T cells as responders in an MLR, and, as suspected, the response was significantly reduced, indicating that antigen presentation by Y1-deficient APCs is impaired (Fig. 5 A). To understand the nature of the defect in Y1$^{-/-}$ APCs, we first measured by ELISA serum levels of IL-12, a key cytokine driving the differentiation of activated CD4$^+$ T cells into Th1 effector cells. IL-12 is produced primarily by APCs, such as macrophages and DCs (12). IL-12 levels were significantly lower in the serum of Y1$^{-/-}$ compared with Y1$^{+/+}$ mice (Fig. 5 B). Mouse macrophages were isolated from peritoneal lavage and activated with LPS to trigger cytokine production, and the secretion of IL-12 and TNF into the culture medium was measured by ELISA. Peritoneal Y1$^{-/-}$ macrophages produced reduced amounts of IL-12 and TNF in response to LPS (Fig. 5, C and D, respectively). As LPS activates Toll-like receptors (TLRs), particularly TLR4, we checked whether activation through other TLRs was also affected in Y1$^{-/-}$ mice. Peritoneal macrophages
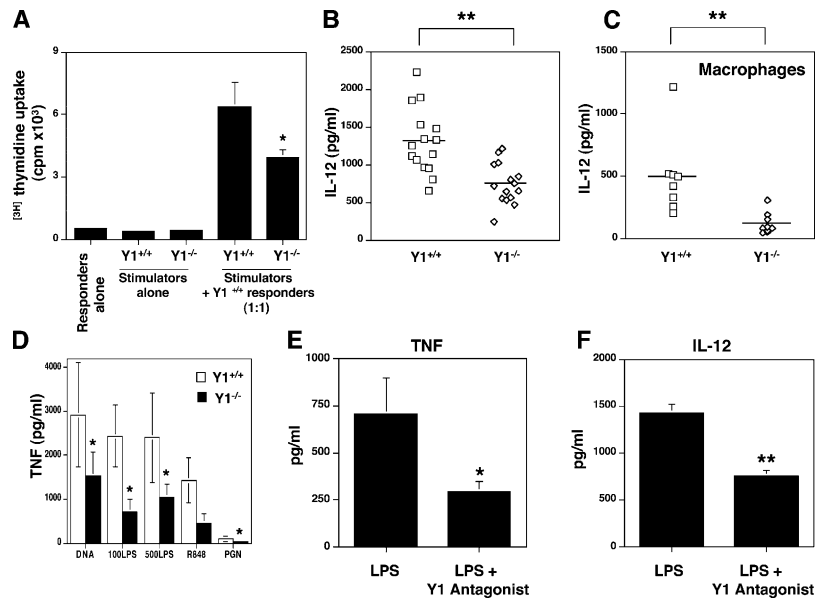
Figure 5. Impaired macrophage function in Y1$^{-/-}$ mice. (A) Y1$^{+/+}$ T cell proliferation in an MLR with Y1$^{-/-}$ stimulators. Mean counts $\pm$ SE of triplicates are shown, representative of three experiments. (B) Serum IL-12 levels in Y1$^{+/+}$ (squares) and Y1$^{-/-}$ (diamonds) mice ($n = 15$). (C) Production of IL-12 by Y1$^{+/+}$ (white squares) and Y1$^{-/-}$ (white diamonds) peritoneal macrophages after 4 h of stimulation with LPS. Seven Y1$^{+/+}$ and nine Y1$^{-/-}$ mice were analyzed and the black bars indicate the mean levels. (D) TNF production by Y1$^{+/+}$ (white bars) and Y1$^{-/-}$ (black bars) peritoneal macrophages after 4 h of LPS (100 and 500 µg/ml), R848, and PGN stimulation. Five mice per group were analyzed. Production of TNF (E; left) or IL-12 (F; right) by LPS-stimulated (for 4 h) Y1$^{+/+}$ peritoneal macrophages in the presence or absence of a Y1 peptide antagonist as indicated. Mean and SD of triplicates are shown. (D–F) are representative of three independent experiments. p-values as in Fig. 1.

were isolated and stimulated in culture with various TLR ligands, such as LPS, R848, and peptidoglycan (PGN) specific for TLR4, TLR7/8, and TLR2, respectively. Y1$^{-/-}$ macrophages produced significantly less TNF than Y1$^{+/+}$ macrophages in response to stimulation through all these different TLRs (Fig. 5 D).

Both APCs and lymphocytes produce NPY after activation (22). Therefore, because the response of Y1$^{-/-}$ macrophages was impaired ex vivo, NPY production by these cells after activation might provide an autocrine signal via the Y1 receptor necessary to tune APCs into effective activators of T cells. To test this hypothesis, we activated Y1$^{+/+}$ macrophages with LPS in the presence or absence of a Y1-specific antagonist peptide and measured the production of TNF and IL-12 in the supernatants of these cultures by ELISA. Inhibition of Y1 signaling after the addition of the Y1 receptor antagonist led to reduced production of TNF and IL-12 by normal LPS-activated peritoneal macrophages (Fig. 5, E and F, respectively). These results suggest that NPY produced by activated macrophages provides an autocrine signal through their Y1 receptor to promote an optimal response and demonstrates that signaling through Y1 is required for normal macrophage function.

Impaired DC function in Y1$^{-/-}$ mice

Mature DCs are very potent APCs driving T cell activation and differentiation. We prepared BM-derived mouse DCs in culture with GM-CSF and IL-4, and CD11c$^+$ cells were used in activation assays. In contrast to the activation of Y1$^{-/-}$ macrophages, the activation of BM-derived Y1$^{-/-}$ DCs with LPS led to normal expression of activation markers (not depicted). However, uptake of an antigen, such as OVA-FITC, by immature Y1$^{-/-}$ DCs was reduced compared with that of Y1$^{+/+}$ immature DCs as determined by FACS analysis (Fig. 6, A and B). The same result was obtained using FITC-dextran (not depicted). We prepared BMDCs from Y1$^{+/+}$ or Y1$^{-/-}$ mice and used these as immature cells, or cells activated with LPS or anti-CD40 mAb. After inactivation, the stimulatory activity of these purified DC preparations was tested in an MLR using Y1$^{+/+}$ T cells as responders. The results showed that activated Y1$^{-/-}$ DCs were poor stimulators compared with activated Y1$^{+/+}$ DCs (Fig. 6 C). In addition, IL-12 production by these DC preparations was also impaired (Fig. 6 D). Taken together, these results and those obtained with macrophage preparations (Fig. 5) showed a marked impairment of Y1$^{-/-}$ APC function, in particular IL-12 production, consistent with defective Th1 cell responses in Y1$^{-/-}$ mice.

Next, we checked whether Y1$^{-/-}$ APC function was affected in vivo. For this we used two strategies. First, we crossed Y1$^{-/-}$ mice onto RAG1$^{-/-}$, generating double KO mice. This model provides for a host with Y1$^{-/-}$ APCs in which Y1$^{+/+}$ or hyperresponsive Y1$^{-/-}$ CD45RB^{hi} CD4$^+$ T cells can be injected to induce colitis as is shown in Fig. 4. We injected Y1$^{-/-}$ T cells (shown to be very potent colitis inducers; Fig. 4) into RAG1$^{-/-}$ × Y1$^{-/-}$ mice and RAG1$^{-/-}$ control littermates. Fig. 6 E shows that injec-
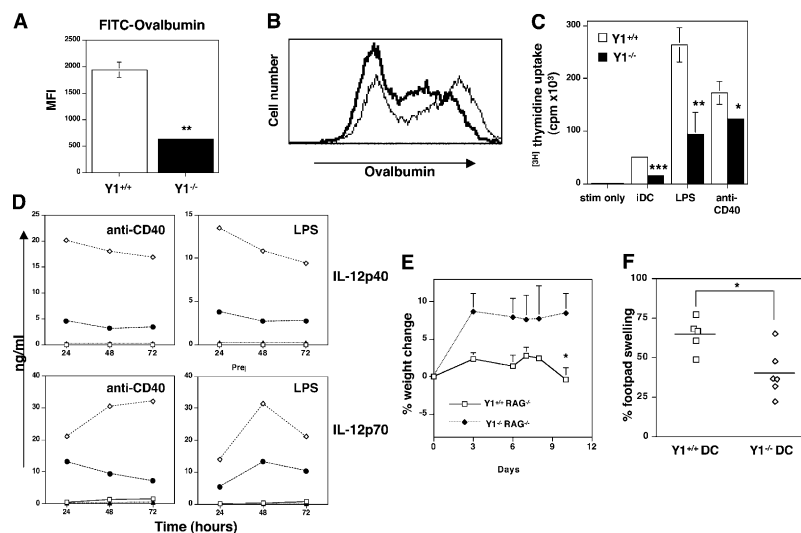
Figure 6. Impaired DC function in Y1^{−/−} mice. (A) OVA-FITC uptake by Y1^{+/+} (white bar) and Y1^{−/−} (black bar) immature DCs. Flow cytometry was used to quantitated FITC-OVA uptake by DC, and results were plotted as mean fluorescence intensity (MFI). Four animals were analyzed per group. (B) Representative FACS plot showing OVA-FITC uptake by Y1^{+/+} (thin line) and Y1^{−/−} (thick line) immature DCs. (C) Both Y1^{−/−} immature DCs (iDC) or DCs stimulated with LPS or anti-CD40 antibody as indicated (black bars) have impaired stimulatory functions in MLR compared with Y1^{+/+} cells (white bars). Y1^{+/+} T cells were used as responders. (D) IL-12p40

(top) and IL-12p70 (bottom) production by anti-CD40– (left) and LPS-stimulated (right) Y1^{+/+} (white diamonds) and Y1^{−/−} (black circles) DCs measured by ELISA. The results are representative of two separate experiments. White and black squares represent control unstimulated Y1^{+/+} and Y1^{−/−} cells, respectively. (E) Body weight of RAG1^{−/−} or RAG1^{−/−} × Y1^{−/−} mice days after transfer with Y1^{−/−} CD45RB^{hi} CD4⁺ T cells as indicated. Four animals per group. (F) DTH response induced after injection of Y1^{+/+} mice with mBSA-pulsed Y1^{+/+} (white squares) or Y1^{−/−} (white diamonds) DCs. p-values as in Fig. 1.

tion of Y1^{−/−} T cells into RAG1^{−/−} mice, but not into RAG1^{−/−} × Y1^{−/−} mice, induced colitis as soon as day 10 after T cell transfer, leading to weight loss of these animals compared with RAG1^{−/−} × Y1^{−/−} mice that in fact gained weight normally through the course of the experiment. RAG1^{−/−} mice, but not RAG1^{−/−} × Y1^{−/−} mice, injected with Y1^{−/−} T cells developed diarrhea by day 15 and had to be killed. This suggests that Y1^{−/−} T cells failed to be activated in RAG1^{−/−} × Y1^{−/−} mice due to a lack of Y1 expression on APCs and demonstrates that Y1 is critical for proper APC function in vivo.

To further confirm the defect of Y1^{−/−} APCs in vivo, we set up a DC transfer system adapted from studies described previously (23, 24). BM-derived Y1^{+/+} and Y1^{−/−} DCs were prepared and pulsed with mBSA. These cells were injected in two sets of Y1^{+/+} mice. 1 wk later, mBSA was injected in the footpad to trigger DTH. The results show that the injection of mBSA-pulsed Y1^{+/+} DCs was very efficient at priming the recipient's Y1^{+/+} T cells, which led to a robust DTH response (Fig. 6 F). This is in contrast to mBSA-pulsed Y1^{−/−} DCs, which showed impaired DTH responses in Y1^{+/+} mice (Fig. 6 F). Taken together, in vitro and in vivo results show that Y1^{−/−} APCs are defective and fail to provide optimal stimulation to T cells.

Immune defects in Y1^{−/−} mice are immune cell–specific

Because the Y1 receptor has a wide range of important physiological functions in addition to its role in the immune sys-

tem (6, 25), it was important to check whether impaired Th1 cell responses in Y1^{−/−} mice were due solely to a defect in APC function and not to secondary effects resulting from the lack of the Y1 receptor in the peripheral and central nervous systems. We used BM chimera animals and transferred Y1^{+/+} BM into lethally irradiated Y1^{−/−} mice and Y1^{−/−} BM into Y1^{+/+} mice, creating Y1^{−/−} mice with Y1^{+/+} immune cells and Y1^{+/+} mice with Y1^{−/−} immune cells, respectively. The absence of Y1 from immune cells was sufficient to impair DTH responses (Fig. 7 A), mBSA-specific IgG2a secretion (Fig. 7 B), and levels of total serum IgG2a (Fig. S1 E). Thus, the Y1 receptor has an important activating/regulatory role in the immune system, independent of its functions in other tissues, such as the central nervous system.

DISCUSSION

Neuroscience and immunology are two areas of research that historically have evolved separately despite decades of evidence for bidirectional cross talk. A number of studies have shown the effects of neurotransmitters and neuropeptides on immune cell function in vitro and in vivo (4, 26). However, very few studies have attempted to pinpoint the physiological significance of these effects and/or the molecular basis behind them. Recent studies have established that a number of factors and receptors, originally described in the nervous system, are also expressed by immune cells. Moreover, work on the immune system of mice deficient for certain neuronal molecules has uncovered unexpected roles for
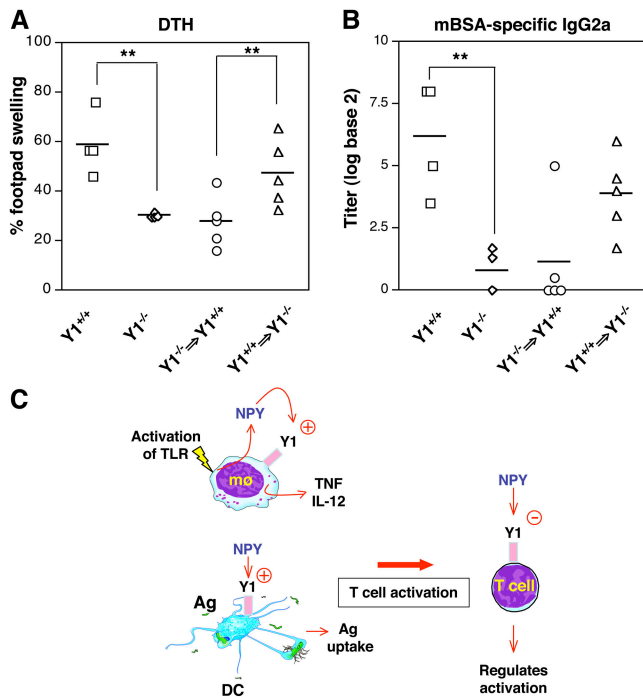
Figure 7. BM chimeras revealed an immune–specific role for Y1: a proposed NPY/Y1 immune model. (A and B) $Y1^{+/+}$ and $Y1^{-/-}$ mice were lethally irradiated and reconstituted with $Y1^{-/-}$ (white circles) and $Y1^{+/+}$ (white triangles) BM cells, respectively. $Y1^{+/+}$ (white squares) and $Y1^{-/-}$ (white diamonds) mice are shown as reference. (A) The DTH response to mBSA and (B) the mBSA-specific IgG2a response are shown. Six animals per group. (C) A proposed model: Upon activation, macrophages secrete NPY (reference 13), which through Y1 receptor promotes the secretion of TNF and IL-12 important for T cell differentiation into Th1 effector cells. Y1 expression on DCs is important for optimal antigen uptake and presentation to T cells. In contrast, signaling through Y1 receptor on T cells inhibits T cell activation. p-values as in Fig. 1.

immune function. For instance, semaphorins, originally described as guidance molecules for developing neuronal axons, are now emerging as molecules with powerful effects on T, B and DC activation and appear necessary for optimal T cell–APC interactions (27). Interestingly, similar to the Y1 receptor in this study, the expression of the semaphorin Sema4A on DCs has also been shown to be critical for T cell priming (24). From this recent work emerged an interesting parallel between the immunological and neurological synapses and a new basis for immune regulation at the interface of the nervous and immune systems (27).

Our study highlights the important role of the NPY/Y receptor system for immune cell function. NPY is produced by the central and peripheral nervous system as well as by immune cells in response to activation (8, 22). NPY has a number of pleiotropic effects in the immune system, ranging from the modulation of cytokine release to cell migration and innate immune cell activity (8). Recently, treatment with NPY has been shown to protect mice against disease in models of EAE (13) and DTH (unpublished data), suggesting

important functions for NPY receptors as regulators of immune responses in inflammation and autoimmunity. The use of a Y1 agonist and antagonist suggested that NPY–mediated protection in EAE was triggered via repressive signals through the Y1 receptor on T cells (13). Although these results suggest an important role for the NPY–Y1 axis in the immune system, the true significance of this function emerged from our study of $Y1^{-/-}$ mice. Indeed, initial observations were confusing as $Y1^{-/-}$ mice were protected from colitis and DTH, which appeared to contradict the notion of a protective role for Y1 receptor signaling in inflammation (14). Our study of $Y1^{-/-}$ T cells and $Y1^{-/-}$ APCs put these observations into a proper perspective and uncovered a sophisticated bimodal role for Y1 in the immune system. Isolated $Y1^{-/-}$ T cells are clearly hyperreactive, responding better in alloreactions and inducing severe colitis upon transfer into lymphopenic mice. Conversely, the addition of NPY in a T cell assay suppresses T cell proliferation in an MLR. We showed that the addition of NPY at the onset of T cell activation, but not 24 h after activation, in vitro was able to trigger this inhibitory effect, suggesting that signaling through Y1 on T cells inhibited T cell activation/effector differentiation but was not able to shut off the function of preexisting proliferating effector T cells. In addition, these results confirmed that $Y1^{-/-}$ T cells are not only capable of differentiating into active IFN-γ–producing Th1 T cells, but are also intrinsically hyperreactive. The results also confirmed that the Y1 receptor is a key negative regulator on T cells, controlling the magnitude of T cell responses. The realization that a problem with $Y1^{-/-}$ T cell function could not account for the protection seen in $Y1^{-/-}$ mice in models of DTH and DSS-induced colitis suggested that a defect in APCs in these mice might explain this protected phenotype. Indeed, the activation and production of cytokines critical for Th1 cell responses were clearly impaired in $Y1^{-/-}$ macrophages and in $Y1^{-/-}$ mice, as well as antigen uptake by $Y1^{-/-}$ DCs, which was markedly reduced and suggested reduced antigen-presenting capacity in $Y1^{-/-}$ mice. $Y1^{-/-}$ APCs provided reduced proliferative stimuli to $Y1^{+/+}$ T cells in the MLR, which was consistent with the reduced number of effector T cells detected in $Y1^{-/-}$ mice. In addition, our two in vivo systems providing $Y1^{-/-}$ APCs in the presence of $Y1^{+/+}$ or hyperreactive $Y1^{-/-}$ T cells confirmed that expression of Y1 on APCs is critical for optimal T cell priming. Therefore, the key immunological protection in $Y1^{-/-}$ mice derives from a defect in APC function, which masked the hyperactive nature of the $Y1^{-/-}$ T cell compartment. Thus, the Y1 receptor fulfils two important roles in the immune system: one as an activator of APC function, and the other as a regulator of T cell responses (see model in Fig. 7 C).

The powerful effects of Y1 deficiency on both APC and T cell functions suggest that signaling through Y1 on APCs and T cells is normally finely balanced to ensure proper immune responses. Our reciprocal BM transfer experiment

clearly showed that when endogenous expression of NPY is physiological, the function of Y1 in the immune system is independent of its function in the central and peripheral nervous systems. However, excessive and/or chronic systemic release of NPY as a result, for example, of psychological stress or depression (28) would strongly inhibit T cell activation and predictably increase the vulnerability of the stressed individual to infections and cancer (1) or other forms of immune dysfunction. Interestingly, a recent report showed that Y1 receptor expression is reduced in T cells from schizophrenic patients (29). Immune defects and in particular defects in Th1 T cell responses are well documented in schizophrenic patients (30). Schizophrenic patients share a few common features with $Y1^{-/-}$ mice, such as a greater proportion of circulating naive T cells (31), increased in vitro T cell responses, but reduced T cell activation to tuberculin in vivo (32). It will therefore be interesting to examine the expression of Y1 on APCs from schizophrenic patients.

Currently, the natural advantages of the bimodal mechanism of immune cell regulation through Y1 are not entirely clear, and it will be important to integrate this effect within a general schema of immune regulation by neurotransmitters (1). Most likely, the inhibition of T cell activation by excess NPY is a physiological counterbalance that prevents adverse concurrent effects from APCs overstimulated by NPY, which might otherwise corrupt T cell tolerance (33). Indeed, the treatment of macrophages with NPY elevates cytokine production and inflammatory responses in vitro (34); therefore, elevated serum NPY levels in response to psychological stress (in addition to NPY produced locally by immune cells) would likely trigger inappropriate APC activation and inflammation if a concomitant antagonistic activity of NPY in T cells was not in place. However, as NPY can trigger proinflammatory effects in macrophages, we cannot exclude lymphocyte-independent inflammatory disorders triggered by excess production of NPY. This may explain why Y1 antagonists provide an effective protection against DSS-induced colitis. This model of colitis is highly dependent on the activation of macrophages (18) and can be triggered in lymphopenic mice (35). Therefore, blocking Y1 on macrophages was a key event in protecting mice against DSS-induced colitis. This is in contrast to DTH, a model highly dependent on T cell activation (36), and therefore, protection in this model relied strongly on agonist signals through Y1 on T cells. A number of inflammatory conditions, such as asthma, correlate with elevated serum levels of NPY, and production was not explained by the increased formation of NPY-producing nerve terminations in inflamed tissues (25). This suggests that immune cells rather than nerves might be the source of additional NPY (22, 34). It is possible that the immune system adopted the Y1/NPY receptor–ligand system for immune cell regulation and that central nervous system–derived NPY has inadvertent effects on immune function. Another possibility is that central nervous system peptides, produced as a result of stress, etc., play

a meaningful role in modulating immune cell function for unknown reasons. Regardless, increased production of NPY in response to psychological stress does modulate immune responses (1), and it will be important to understand the circumstances of NPY production by both neurones and immune cells, and how increased NPY production by activated immune cells during inflammation may affect other NPY-mediated physiological and psychological functions.

In conclusion, this work uncovers a powerful new mechanism for Y1 neuropeptide receptor in regulating both APC and T cell functions. This bimodal role for Y1 receptor likely represents an important interface between the peripheral nervous system and the immune system. Understanding signaling events through the Y1 receptor on immune cells may disclose novel strategies for immunomodulation and improve our understanding of the effect of psychological stress on immune functions.

MATERIALS AND METHODS

Mice. Mice lacking the Y1 receptor ($Y1^{-/-}$) have been described (37). 8–12-wk-old male and female mice were used for the studies. 12–14-wk-old $RAG1^{-/-}$ and BALB/c mice were obtained from Animal Resources Center. Homozygote $Y1^{-/-}$ and $RAG^{-/-}$ mice were crossed, and seven pairs of these double heterozygote mice were used to generate double $Y1^{-/-} \times RAG^{-/-}$ KO and control mice. From the generated 110 offspring of these breeding pairs, four double KO mice were identified, slightly less than the expected seven according to Mendelian rules. Animals were housed under conventional barrier protection and handled in accordance with the Animal Experimental and Ethic Committee, which complies with the Australian code of practice for the care and use of animals for scientific purposes. The response of these mice to T-dependent and –independent antigens was conducted as described previously (38).

Flow cytometry. T and B lymphocyte subsets from the LNs, spleen, peritoneal lavage, BM, and thymus were isolated and analyzed by four-color flow cytometry as described previously (15, 39). Lymphocytes from the LNs, spleen, BM, and thymus were isolated by mechanical disruption of the organs followed by a 5-min incubation in RBC lysis buffer (0.156 M ammonium chloride, 0.01 M sodium bicarbonate, and 1 mM EDTA) on ice. For multicolor flow cytometric analysis, cells were incubated in the presence of fluorochrome- and biotin-conjugated mAbs against CD21/CD35 (7G6), CD23 (B3B4), B220 (RA3-6B2), CD43 (1B11), CD4 (L3T4), CD8 (53–6.7), CD44 (IM7), L-selectin (MEL-14), CD5 (Ly-1), IgD (11-26c.2a), IgM (R6-60.2), CD25 (PC61), CD69 (H1.2F3), CD45RB (16A), CD11b (M1/70), CD11c (HL3), MHC class II (I-A/I-E, 2G9), CD80 (16-10A1), CD86 (B7-2), and CD40 (3/23; BD Biosciences). When biotinylated antibodies were used, strepatvidin-PerCP (BD Biosciences) or streptavidin-Cy5 (Jackson ImmunoResearch Laboratories) allowed detection. For labeling with CFSE (Invitrogen), T cells at a density of 10^7 cells/ml were incubated for 10 min at room temperature in 5 μM CFSE in PBS containing 0.1% BSA. The labeling reaction was stopped by the addition of serum. Cells were analyzed by FACS analysis. Reduction in CFSE mean of fluorescence is indicative of cell division.

ELISA. For detection of serum Ig, plates were coated with 2 μg/ml goat anti–mouse Ig (H+L; Southern Biotechnology Associates, Inc.) at 4°C overnight. Plates were then blocked with 3% skim milk powder for 1 h at 37°C. Serum serial dilutions were incubated for 1 h at 37°C with serial dilutions of serum. Alkaline phosphatase–labeled goat anti–mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM (Southern Biotechnology Associates, Inc.) were used as secondary antibodies, diluted at 1:2,000, and incubated at 37°C for

1 h. The reaction was developed using *p*-NP phosphate in 10% diethanolamine (Sigma-Aldrich) and stopped with 3 M NaOH. The optical density was measured at 405 nm. The detection of T-dependent and –independent NP-specific antibodies was performed as described previously (38). Anti-mBSA–specific antibodies were captured by coating the ELISA plate with 10 μg/ml mBSA (Sigma-Aldrich) and detected as described above. The titer (log base 2) for anti-NP antibodies was defined as the serum dilution giving an OD four times higher than that of the background (1 = 1/100 dilution). Cytokine ELISAs for TNF, IL-12p40, IL-12p70, IL-4, and IFN-γ were performed using kits purchased from BD Biosciences, and the IL-2 cytokine ELISA was performed using a kit supplied by PeproTech. All kits were used according to the manufacturers' specifications.

Preparation and stimulation of BM-derived DCs. BM was flushed with a syringe from the tibias and femurs of Y1$^{-/-}$ and Y1$^{+/+}$ mice, and red cells were lysed. The remaining cells were depleted of erythroid precursors and granulocytes as well as T, B, and NK cells using magnetic cell sorting according to the manufacturer's instructions (MACS; Miltenyi Biotec). After depletion, 2×10^5 cells were seeded in nontreated 10-cm dishes, cultured in RPMI plus 10% fetal bovine serum, 2% penicillin/streptomycin, and 1% of 200 mM glutamine containing 20 ng/ml GM-CSF and 20 ng/ml IL-4, and incubated at 37°C/5% CO_2. Medium was replenished on days 3, 5, and 7. At day 8, nonadherent cells were collected and adherent cells were discarded. These cells were immature DCs, and purity was confirmed by FACS analysis using anti-CD11c mAb (BD Biosciences). Mature DCs were obtained after stimulation with either 1 mg/ml LPS or 10 μg/ml anti-CD40 (BD Biosciences) for 24 h. The ability of immature DCs to take up fluorescently labeled protein was assessed using FACS analysis after the cells were incubated with 1 mg/ml FITC-OVA or FITC-dextran (Invitrogen) at 37°C for 30 min and washed three times with PBS. For MLR, immature and mature DCs were inactivated by γ irradiation, washed, and mixed with CD3$^+$ BALB/c-derived splenocytes. The optimal ratio of DCs to T cells was determined to be 1:10. Proliferation after 3 d was measured by pulsing cells with 1 μCi/well [^{3}H]thymidine for the final 18 h of stimulation.

Preparation and stimulation of thioglycollate–elicited macrophages. Y1$^{-/-}$ and Y1$^{+/+}$ mice were injected i.p. with 2 ml 3% thioglycollate (Sigma-Aldrich) 3 d before they were killed. Peritoneal macrophages were collected by peritoneal lavage with 5 ml of sterile RPMI 1640 medium. Macrophages were purified by adherence to tissue culture plates for 2 h. The macrophage population was ∼92–95% Mac-1$^+$ (CD11b antibody; BD Biosciences) by FACS analysis. Peritoneal macrophages plated at 2×10^6 cells/ml were treated with 100 ng/ml LPS (Sigma-Aldrich) or 1 μg/ml R848 (Invivogen) or 10 μg/ml PGN (Invivogen) for 4 h. The Y1-specific antagonist peptide GW1229 was designed as described previously (40) and custom made by AUSPEP. The antagonist Y1-specific peptide was used at 10^{-8} M in culture.

DTH model. mBSA-induced DTH was performed as described previously (41). In brief, mice were sensitized with 1.25 mg/ml mBSA (Sigma-Aldrich) in CFA, injected subcutaneously at the base of the tail. 7 d after the sensitization, mice were challenged with 200 μg/20 μl mBSA in the right footpad and 20 μl PBS was injected into the left footpad. Footpad swelling was measured using a calliper and calculated as follows: (footpad swelling [%]) = [(thickness of mBSA-injected footpad) [mm]) − (thickness of PBS-injected footpad [mm])] / (thickness of PBS-injected footpad [mm]) × 100. For the DC-induced DTH responses (adapted from references 23 and 24), day 8 immature BM-derived immature DCs (prepared as described above) were pulsed with 50 μg/ml mBSA plus 0.2 μg/ml anti-CD40 in X-Vivo 15 serum-free media (Cambrex) for 24 h. 10^6 pulsed DCs were injected subcutaneously at the base of the tail. 7 d after the sensitization, mice were challenged with 600 μg mBSA in 30 μl PBS in the right footpad. 30 μl PBS was injected into the left footpad for control. Footpad swelling was measured and calculated as described above.

T cell stimulation assays. For recall responses, lymphocytes were isolated from inguinal LNs from mice 14 d after the initial tail-base injection of mBSA. T cell numbers were normalized and cells were plated at 2×10^5 T cells/well. Cells were stimulated with 4 μg/ml mBSA for 72 h and supernatant was collected for ELISA. For measurement of cell proliferation, cells were pulsed with 1 μCi/well [^{3}H]thymidine for 18 h before harvest. Stimulation index equals the counts from activated cells/counts from nonstimulated cells. T cells were isolated from Y1$^{+/+}$ and Y1$^{-/-}$ mice using a MACS Pan T cell negative selection kit (Miltenyi Biotec) according to the manufacturer's specifications. 95% purity was confirmed by FACS analysis. In vitro T cell stimulations (with anti-CD3$^{+/-}$ anti-CD28 antibodies; BD Biosciences) were performed as described previously (42). For MLR, using BALB/c-derived splenocytes for MHC mismatch, stimulator cells were first inactivated for 20 min with 200 μg/ml mitomycin C (Sigma-Aldrich), washed, and mixed at a ratio of 1:1 with CD3$^+$ responder cells. Proliferation after 6 d was measured by pulsing cells with 1 μCi/well [^{3}H]thymidine for the final 18 h of stimulation.

Models of colitis. Acute colitis was induced by administering 3.5% (wt/vol) DSS (mol wt: 36,000–50,000; ICN Biochemicals Inc.) for 7 d in males and 8 d in females ad libitum. Mice were weighed every day and percent weight change for each mouse was calculated as follows: % weight change = (weight at specific day − weight on day 0) / weight on day 0) × 100. The CD4$^+$ CD45RB^{hi} transfer model of colitis was induced in Y1$^{+/+}$ RAG1$^{-/-}$ and Y1$^{-/-}$ RAG1$^{-/-}$ mice by injecting i.v. 2×10^5 sorted CD4$^+$ CD45RB^{hi} cells purified as described previously (21) from either Y1$^{+/+}$ or Y1$^{-/-}$ mice. Animal body weight was monitored weekly for 2 wk and then daily. Upon loss of 20% original body weight, mice were culled for organ collection. Weight loss appeared simultaneously with diarrhea. % weight change = (weight at specific day − weight on day 0) / weight on day 0) × 100.

Immunohistochemistry. Colons were collected from mice undergoing colitis models and frozen in OCT compound (Tissue-Tek; Sakura). 7-μm-thick tissue sections were stained with hematoxylin and eosin for histologic examination as described previously (43).

Quantitative PCR. Total RNA was isolated from cells or tissue using TRIzol reagent (Invitrogen). After cDNA synthesis using the Reverse-IT RTase Blend Kit (ABgene), real-time PCR was conducted using the Light Cycler–FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals) according to the manufacturer's specifications. The following primers were used: mY1 forward: 5′-CAAATTCTGACCGACGAGCC-3′, reverse: 5′-TAAAGCAGAGTGGGCCGAAA-3′; and mGAPDH forward: 5′-CTCATGACCACAGTCCATGC-3′, reverse: 5′-CACATTG-GGGGTAGGAACAC-3′.

Whole spleen and thymus homogenized for total RNA preparation. B220$^+$/CD3$^-$ (B cells), CD4$^+$ and CD8$^+$ T cells, NK1.1$^+$ CD3$^-$ NK cells, and CD11c$^+$ DCs were sorted using a FACSVantage SE DiVa (Becton Dickinson). All antibodies were purchased from BD Biosciences. Macrophages were thioglycollate-elicited and lymphocyte-depleted as described above. Sorted cell preparations were always >95% purity. Mouse mast cells (prepared as described elsewhere; reference 44) and mouse DCs (see protocol above) were BM cell–derived and had a purity of between 90 and 95%.

BM chimeras. 8–10-wk-old recipient mice were lethally irradiated (950 rad) and rescued 6–8 h later with an i.v. injection of 2×10^7 BM cells. Chimeras were analyzed 6–8 wk after reconstitution.

Online supplemental material. Fig. S1 shows that T cells from Y1$^{-/-}$ mice responded with much higher proliferation to suboptimal doses of anti-CD3 than did WT T cells (Fig. S1, A). This was also confirmed by CFSE staining in Y1$^{+/+}$ and Y1$^{-/-}$ T cells after activation with anti-CD3 (Fig. S1

B). However, this increased capacity for proliferation did not extend to IL-2 production as T cells from Y1$^{+/+}$ and Y1$^{-/-}$ mice showed equivalent IL-2 production after stimulation (Fig. S1 C). In addition, expression of the activation marker CD69 72 h after activation with anti-CD3 or anti-CD3 plus anti-CD28 was equivalent in Y1$^{+/+}$ and Y1$^{-/-}$ T cells (Fig. S1 D). Lastly, total IgG2a was measured in Y1$^{+/+}$ and Y1$^{-/-}$ mice lethally irradiated and reconstituted with Y1$^{-/-}$ or Y1$^{+/+}$ BM cells. This established that effects of Y1 on IgG2a production could be attributed to transferred BM-derived cells.

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