Microenvironment and Immunology

Adiponectin Receptor Signaling on Dendritic Cells Blunts Antitumor Immunity

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Abstract

Immune escape is a fundamental trait of cancer. Dendritic cells (DC) that interact with T cells represent a crucial site for the development of tolerance to tumor antigens, but there remains incomplete knowledge about how DC-tolerizing signals evolve during tumorigenesis. In this study, we show that DCs isolated from patients with metastatic or locally advanced breast cancer express high levels of the adiponectin receptors AdipoR1 and AdipoR2, which are sufficient to blunt antitumor immunity. Mechanistic investigations of ligand–receptor interactions on DCs revealed novel signaling pathways for each receptor. AdipoR1 stimulated IL10 production by activating the AMPK and MAPKp38 pathways, whereas AdipoR2 modified inflammatory processes by activating the COX-2 and PPARγ pathways. Stimulation of these pathways was sufficient to block activation of NF-kB in DC, thereby attenuating their ability to stimulate antigen-specific T-cell responses. Together, our findings reveal novel insights into how DC-tolerizing signals evolve in cancer to promote immune escape. Furthermore, by defining a critical role for adiponectin signaling in this process, our work suggests new and broadly applicable strategies for immunometabolic therapy in patients with cancer. Cancer Res; 74(20); 1–12. ©2014 AACR.

Introduction

It is increasingly recognized that adipocytes secrete molecules, termed as adipokines with wide-ranging metabolic effects (1, 2). Leptin has been shown to promote T-cell immunity (3) and inhibit regulatory T-cell proliferation (4). Contrarily, adiponectin (APN) has been shown to inhibit macrophage function (5, 6), but its far-reaching effects on the adaptive immune system, in particular in the context of cancer immunology are yet to be elucidated.

APN can mediate antidiabetic (7, 8), anti-inflammatory (6), and antiatherosclerotic (9, 10) processes. In our study, dendritic cells (DC) isolated from patients with breast cancer with metastatic or locally advanced disease express higher level of APN receptors. We have therefore sought to explain how APN regulates the DC function, hence modulating T-cell immunity against cancer. We show here that AdipoR1 signaling in DCs mediates T-cell anergy via IL10-dependent mechanism through activation of the AMPK and MAPKp38 pathways. IL10 promotes further anti-inflammatory (6), and antiatherosclerotic (9, 10) processes. In our study, dendritic cells (DC) isolated from patients with breast cancer with metastatic or locally advanced disease express higher level of APN receptors. We have therefore sought to explain how APN regulates the DC function, hence modulating T-cell immunity against cancer. We show here that AdipoR1 signaling in DCs mediates T-cell anergy via IL10-dependent mechanism through activation of the AMPK and MAPKp38 pathways. IL10 promotes further anti-inflammatory action via the IL10 receptor in a process that is dependent on the STAT3 and SOCS3 pathways. On the other hand, AdipoR2 activation induces T-cell anergy through a distinct mechanism that utilizes PPARγ. The anti-inflammatory actions of PPARγ are mediated through inhibition of intracellular free radicals in the DC and promotion of Th2-skewed cytokine production by the subsequent T-cell responses. All these mechanisms center on regulating the downstream NF-kB pathway, which in turn arrests DC maturation processes.

To test our hypothesis that APN receptor signaling on DCs can inhibit antitumor immunity in vivo, we went to overexpress either AdipoR1 or AdipoR2 on murine DCs and carried out vaccination study. We show here that AdipoR1/R2 signaling in DCs can interfere with the ability of T-cell clearance of cancer cells in a T-cell–mediated tumor protection model.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell line authorization statement and cell culture
All the cell lines used in this study were from Dr. SK Xue’s laboratory and have been tested, authenticated, and previously used in the peer-reviewed articles from the laboratory (11–14). Human and murine DCs were generated as previously described (15–17). In some cases, DCs were transduced with various vectors or siRNAs specific for AdipoR1/R2 (Santa Cruz Biotechnology) as indicated and previously described (13, 18). In APN-conditioning process, various concentrations of APN (as indicated in Figures) were added into DC culture on day 2 of its differentiation process unless indicated otherwise.

Flow cytometry
Flow-cytometric analysis was performed as previously described (15, 19). Murine intracellular cytokines were determined using an intracellular detection kit (Caltag).

Western blotting
Total proteins were isolated from various cell lysates and Western blotted as previously described (20, 21). The blots were probed with monoclonal antibodies as indicated in Figures and Supplementary Information. Cell-cycle analysis was determined as previously described (15).

Intracellular radical oxygen species and NF-κB activity
Measurement of free radical scavenging capacity and NF-κB activity was determined as previously described (20).

Mixed lymphocyte reactions and anergy assays
Purified allogeneic T cells (10^5/well) were cultured in the presence of 30-Gy irradiated DCs (10^5/well) in a mixed lymphocyte reaction (MLR) as described (20). When appropriate, 10 μg/mL anti-IL10R or neutralizing anti-IL10 was added into the cultures. For investigating anergy induction by APN-conditioned DCs or AdipoR1/R2-transduced DCs, T cells were recovered from the primary MLR and rested for 5 days. These resting T cells were then restimulated with allogeneic DCs from either the same donor as in the primary MLR or allogeneic third party DCs (as a control). The cultures were pulsed with 3H-thymidine after days 3, 5, and 7 (unless indicated otherwise), and T-cell proliferation was measured.

ELISA
To determine cytokine production and serum APN, ELISAs were carried out as described (15, 20, 22).

Cloning of human and murine AdipoR1/R2
Cloning strategies are described in Supplementary Information.

Cytotoxic T-cell killing assay
CTL killing assays were conducted as described (23) with the effector to target ratios indicated.

In vivo tumor protection model
C57BL/6 (Congenic Thy1.2) bone marrow–derived DCs were transduced with lentiviral vectors encoding for either murine AdipoR1/R2 or empty vector (mock) on day 2. The differentiated DCs were then activated by incubation with proinflammatory cytokines as described (20) on day 5. Congenic Thy1.2 C57BL/6 recipient mice were conditioned by 5-Gy irradiation. On following day, vaccination was carried out by coinjection of 10^6 flu peptide NP-specific T cells isolated from F5-TCR transgenic (Thy1.1) mice together with activated DCs (2 × 10^5/mouse) that had been transduced with either mAdipoR1/R2 or empty vector, respectively. In all cases, all activated DCs were loaded with 10 μmol/L NP peptide for 3 hours. Two days later, the vaccinated mice were inoculated with 2 × 10^6 EL4-NP lymphoma cells. Tumor growth was monitored by measuring the volume following the inoculation. At the end of the experiment, the adoptively transferred T cells were recovered from spleens and then used either in a CTL killing assay, intracellular cytokine staining assay, proliferation assay, or intra-cellular molecular analysis. Some of the tumors were subjected to frozen section analysis as described (24).

Patient selection
Patients were divided into early breast cancer group (stage I and II disease) and metastatic cohort (including locally advanced cases). As a control, healthy subjects were recruited. The Oxford Ethic Committee A has approved the clinical aspect of this project (REC ref: 07/H0604/114).

Isolation of DCs from patients with cancer and real-time PCR
DCs were isolated using BD IMag DC enrichment kit (BD Biosciences), and total RNAs were extracted using RNeasy Kit (Qiagen). The total RNAs were converted into cDNAs using random primers as described (12). Copies of AdipoR1/R2 were determined as described (25). The primers used for quantification of AdipoR1/R2 were described in Supplementary Table S1.

Analysis of cancer patients’ T-cell function following challenge with their autologous DCs
Peripheral bloods from HLA-A2–positive breast cancer patients were used in these experiments. DCs (5 × 10^5) and T cells (10^6) were isolated using BDIMagTM DC enrichment kit and anti-CD3 magnetic-beads (Miltenyl Biotech), respectively. The isolated T cells were transduced with an EBV LMP2-TCR and expanded by peptide stimulation (26). After coculturing with their activated autologous DCs (10:1 ratio) in the presence of LMP2 peptide, the LMP2-specific T cells were isolated and used either in CTL killing assays (23) against 10 μmol/L LMP2 or control peptide loaded P3HR1-A2 tumor cells or in proliferation assays (20). In some cases, the DCs were transected with siRNAs specific for AdipoR1/R2 or their control siRNAs. Alternatively, blocking antibodies for 10 μg/mL AdipoR1/R2 or their isotype control Ig were added into the coculture.

Statistical analysis
SPSS 21 was applied to do the statistical analysis. Data were tested for normal distribution (using Skewness–Kurtosis test)
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and homogeneity of variance (homogeneity of variance test) to evaluate whether they were parametric. If the data were parametric, the Student t test was used to determine the statistical significance. When the data were nonparametric, the Mann–Whitney U test was applied. When comparing the dataset for more than two groups, the one-way ANOVA test (for parametric data) or Kruskal–Wallis test (for nonparametric data) was used. When the overall P values were statistically significant, post hoc pairwise comparisons with the Tukey Honest Significant Difference (HSD) method were performed. P < 0.05 was considered to be statistical significant.

Results

DCs isolated from patients with cancer overexpress APN receptors and inhibit antitumor immunity

To elucidate the mechanisms by which cancer cells evade immune surveillance, we started from analyzing DCs derived from cohorts of patients with cancer and compared with healthy donor controls. We first searched for specific receptors on DCs that may be responsible for the tumor immune evasion. To our surprise, DCs isolated from the peripheral blood of patients with breast cancer with metastatic and locally advanced disease showed significantly higher levels of AdipoR1 and AdipoR2 expression in comparison with healthy controls (Fig. 1A).

To ask the question how overexpression of APN receptor on DCs can affect patients’ antitumor immunity, we isolated T cells from HLA-A2–positive patients and transduced them with a TCR specific for the Epstein–Barr virus (EBV) latent membrane protein-2 (LMP2-TCR; ref. 26). By stimulating these LMP2-TCR engineered T cells with activated patients’ autologous DCs loaded with LMP2 peptide, we found that in comparison with healthy controls, DCs isolated from patients with cancer with metastasis were unable to support the proliferation of the LMP2-specific T cells (Supplementary Fig. S1A). To ascertain the diminished T-cell proliferation was
due to overexpression of the APN receptor on DCs, we cotransfected the DCs with siRNAs specific for AdipoR1 and AdipoR2, and found that their ability to support T-cell proliferation was increased to levels comparable with the healthy controls (Supplementary Fig. S1A). When we isolated T cells from the primary cocultures after exposure to activated DCs from patients with metastasis, these T cells showed an impaired ability to kill tumor cells that were rescued by cotransfection of APN receptor-specific siRNAs into patients’ DCs before the primary coculture (Fig. 1B). The same effects were also achieved by blocking the interaction between APN and its receptors using antibodies against AdipoR1 and AdipoR2 (Supplementary Fig. S1B).

When the T cells isolated after exposure to patients’ DCs were restimulated with HLA-A2–matched, activated DCs from healthy donors in the presence of LMP2 peptide, they failed to respond at all concentrations of the peptide (Fig. 1C). However, this anergy was reversed by siRNA knockdown of AdipoR1 and AdipoR2 expression in the DCs isolated from patients with metastasis. The same effect was observed with antibody blockade experiments (Supplementary Fig. S1C).

**APN induces T-cell tolerance through NF-κB–dependent inhibition**

To define how APN receptors could regulate DC function, we first demonstrated that the APN receptors are also expressed on monocytic-derived DCs (Supplementary Fig. S2A). By exposing monocytic progenitors to high levels of APN, we found that APN did not affect their ability to differentiate into DCs (data not shown), but did retard the maturation of DCs in response to inflammatory stimuli, arresting DCs in immature states lacking in expression of costimulatory molecules (Supplementary Fig. S2C). Consistent with the role of NF-κB in regulating DC maturation, we found that APN inhibited NF-κB activation in a dose-dependent manner (Fig. 2A) when determined by assays using either levels of phosphorylated IkB or a NF-κB promoter-inducible reporter system.

When activated with inflammatory stimuli, APN-conditioned DCs showed reduced ability to stimulate allogeneic T-cell proliferation (Supplementary Fig. S2D). When we isolated the T cells from these primary cocultures, and restimulated them with fresh, unconditioned but activated DCs, we found that the T cells remained anergic with respect to the alloantigens to which they had originally been exposed (Fig. 2B). This hyporesponsiveness was antigen specific because the T cells responded normally to third party alloantigens, and was not due to apoptosis because it could be reversed by treatment with IL2 (Fig. 2B). The effect of APN on immune system is summarized in Fig. 2C.

**Interaction of AdipoR1 with APN mediates T-cell anergy mainly via an IL10-dependent mechanism**

To dissect the molecular mechanism by which APN receptor signaling mediates T-cell tolerance, we examined the consequences of siRNA-mediated knockdown of AdipoR1 and AdipoR2 (Supplementary Fig. S2B). Double depletion of both receptors restored NF-κB activation (Fig. 3A) and DC maturation (Supplementary Fig. S3A), and abrogated APN-induced T-cell anergy (Fig. 3B). APN treatment induced expression of anti-inflammatory factors, TRAF-1, Be3, and A20 (Fig. 3C), which are known to attenuate NF-κB signaling (27). Depletion of either receptor alone only achieved partial rescues of these phenotypes (Supplementary Fig. S3A) but could not reverse their tolerogenic abilities (Supplementary Fig. S3B); AdipoR2 depletion resulted in a greater restoration of oxygen-free radical species and TNF-α than AdipoR1 depletion (Fig. 3D), suggesting differences between the signaling activities of the two receptors.

We also noted that APN-conditioned DCs can induce IL10 production (Supplementary Fig. S4A). This effect was largely abrogated by AdipoR1 depletion (Fig. 4A), suggesting that AdipoR1 was primarily responsible. At the same time, we found that AdipoR1 depletion had a greater effect on levels of phosphorylated AMPK and MAPKp38 than AdipoR2 depletion (Fig. 4B), while treatment with either dominant negative AMPK or MAPKp38 inhibitor abolished APN-dependent IL10 induction (Supplementary Fig. S4B) in the DCs that were transfected with AdipoR2 siRNA. This suggested that IL10 induction was stimulated by AdipoR1 via the AMPK and MAPKp38 pathways.

When we treated DCs with neutralizing antibodies against IL10, we found that IL10 blockade could rescue APN-induced T-cell hypoproliferation (Fig. 4C) and anergy (Supplementary Fig. S4C) in conjunction with AdipoR2-depletion, but not with AdipoR1 depletion (data not shown). This indicated that IL10 could functionally replace AdipoR1, but not AdipoR2, and that AdipoR1 acted primarily through AMPK- and MAPKp38-dependent induction of IL10. To demonstrate that the IL10 effect is dependent on IL10 receptor signaling, we went to block IL10 receptor and similar results were obtained (Supplementary Fig. S4D). STAT3 and SOCS3 are known to be activated by IL10 (28), and we found that activation of both was reduced following AdipoR1 depletion (Fig. 4D), and that inhibiting either was sufficient in rescuing APN-induced anergy (Supplementary Fig. S4E) in AdipoR2-depleted DCs.

To demonstrate the direct downstream interaction of STAT3/SOCS3 with NF-κB pathways, we set up experiment using NF-κB-luciferase reporter system. As indicated, in the presence of the STAT3 blockade (Supplementary Fig. S5A) or in the knockdown by SOCS3 siRNA (Supplementary Fig. S5B), AdipoR2 siRNA-transfected DCs showed no inhibition of NF-κB activities when conditioned by APN, suggesting that STAT3 and SOCS3 play an integral part in AdipoR1/IL10-mediated suppression mechanism.

**AdipoR2 signals through PPARγ pathway and is dependent on COX-2 activation**

To address AdipoR2 downstream signaling, we used siRNA approach targeting AdipoR2 and investigated expression of various downstream signaling molecules by Western blotting. We found that APN can induce expression of COX-2 and PPARγ, which was abrogated by depletion of AdipoR2 (Fig. 5A), PPARγ expression was dependent on COX-2 activity (Fig. 5A), indicating that AdipoR2 activates the PPARγ pathway via COX-2. Indeed, siRNA-mediated knockdown of PPARγ was able to replace AdipoR2 depletion in rescuing APN-induced
are shown as the mean (count per minute) of AdipoR2 signaling.

We then generated AdipoR1/R2-overexpressing human DCs using cells derived from healthy donors. These cells were resistant to maturation (Supplementary Fig. S7A), less able to stimulate allogeneic T-cell proliferation (Supplementary Fig. S7B), and rendered T-cells anergic (Fig. 6A). Both receptor overexpression in the presence of physiologic level of APN and exposure of donor-derived DCs to high level of APN in the

We went on to demonstrate the direct relationship between PPARγ expression and NF-κB activities in the AdipoR2 signaling. In APN-conditioned DCs that were doubly transfected with AdipoR1 and PPARγ siRNA, NF-κB activities remained uninhibited (Supplementary Fig. S6C). This indicates that PPARγ expression is integral to the ability of AdipoR2 signaling to inhibit NF-κB activities.

Overexpression of AdipoR1/R2 in human DCs generates tolerogenic phenotypes

We then generated AdipoR1/R2-overexpressing human DCs using cells derived from healthy donors. These cells were resistant to maturation (Supplementary Fig. S7A), less able to stimulate allogeneic T-cell proliferation (Supplementary Fig. S7B), and rendered T-cells anergic (Fig. 6A). Both receptor overexpression in the presence of physiologic level of APN and exposure of donor-derived DCs to high level of APN in the

T-cell anergy (Supplementary Fig. S6A). Taken together, our results suggest that AdipoR1 and AdipoR2 modulate DC functions and mediate T-cell tolerance through distinct and separable mechanisms (Fig. 5B).

Interestingly, when we stimulated T cells with APN-conditioned but AdipoR1-depleted DCs, we observed a polarized response toward a Th2 phenotype, with higher IL-4, IL-5, and IL-13, but lower IL-12 and IFN-γ production (Fig. 5C), highlighting the differences between AdipoR1- and AdipoR2-dependent signaling. To explore that Th-2 cytokine expression is related to PPARγ, we knocked down PPARγ expression and observed a diminished production of IL-4, IL-5, and IL-13 (Supplementary Fig. S6B). A comparable reduction of Th2 cytokines was noted with DCs singly transfected with PPARγ siRNA or doubly transfected with PPARγ and AdipoR2 siRNAs; implying that PPARγ is a downstream effector of AdipoR2 signaling.
presence of physiologic level of receptors resulted in induction of A20, Bcl3, and TRAF-1 and inhibition of the NF-κB pathway (Fig. 6B), and AdipoR1 overexpression gave greater activation of the AMPK and MAPKp38 pathways, whereas AdipoR2 overexpression resulted in greater expression of PPARγ (Fig. 6C).

**AdipoR1/R2-overexpressing DCs modulate tumor clearance and promote antigen-specific T-cell tolerance in vivo**

To address the significance of APN receptor signaling in an *in vivo* model, we generated murine DCs overexpressing murine AdipoR1/R2 by lentiviral gene transfer (18). Consistent with our human data, these AdipoR1/R2-overexpressing murine DCs remained resistant to activation *in vitro* (Supplementary Fig. S8A). When we loaded these DCs with an immunogenic NP peptide (derived from influenza virus), we found that they were unable to stimulate T cells expressing the corresponding F5 TCR (Supplementary Fig. S8B). After coculture with AdipoR1/R2-modified DCs, the isolated T cells became anergic (Supplementary Fig. S8C), and were incapable of lysing lymphoma cells either loaded with, or endogenously expressing, the NP peptide (Supplementary Fig. S8D).

We then tested these receptor overexpressing DCs and their interaction with T cells in an *in vivo* tumor protection model.
After vaccination in the forms of coinjection of AdipoR1/R2-engineered mDCs that had been loaded with NP peptide and Thy1.1 F5 TCR-transgenic T cells, the Thy1.2 congenic hosts were inoculated with EL4-NP tumors. These mice showed greatly impaired capacity for achieving tumor clearance (Fig. 4A). The cytokine IL10 (ng/mL) was then used to calculate P values in the figure for different groups comparing with the control siRNA group. B, AMPK and MAPKp38 phosphorylation in APN and siRNA-treated (as indicated) mDCs were assayed by Western blotting. C, IL10 blockade restores the ability of AdipoR2-depleted mDCs to support allogeneic T-cell proliferation. APN and siRNA-treated mDCs were cocultured with allogeneic T cells in the presence of anti-IL10 antibody (10 μg/mL; right) or its isotype control Ig (left), then proliferation of T cells was determined. D, AdipoR1 signaling activates STAT3, SOCS3, and ETV3. Total proteins isolated from human mDCs that were untreated or treated with siRNAs and/or APN (as indicated) were analyzed for downstream signaling proteins or their phosphorylated forms, STAT3, SOCS3, and ETV3 (as indicated) by Western blotting.

Discussion

Here, we have investigated DCs from patients with breast cancer with advanced diseases and uncovered a novel mechanism by which cancer cells were evading immune surveillance. We found the upregulation of AdipoR1 and AdipoR2 on DCs may be responsible for the poor antitumor immune response. To test this hypothesis, we analyzed the expression and function of these receptors in cohorts of patients with breast cancer. Very high levels of expression of both receptors were noted in patients with advanced diseases. Challenging of patients’ T cells that have been engineered to express a LMP2-TCR, with autologous DCs loaded with LMP2 peptide indicated that there was a specific tolerance induction. However, these observations were only seen in patients with stage III and IV diseases (not observed in early breast cancer cases; Supplementary Fig. S10A).

To define the immunologic mechanisms, we demonstrated that interaction of APN with its receptors can arrest DCs in an immature state to suppress antigen-specific T-cell responses. Previous observational studies of epidemiologic cohorts indicate that serum APN is inversely correlated to the development of various solid cancers (29–31) and leukemia (32). Although serum APN levels were low in advanced cancer cases (Supplementary Fig. S10B), the expression of APN receptors on their peripheral DCs was greatly elevated and directly correlated with tumor progression. It seems plausible that this cohort of
patients may try to compensate for the reduced serum APN through elevated expression of APN receptors. Unfortunately, this metabolic compensation has a direct immunologic consequence that it is detrimental to antitumor immune response. Therefore, we here, provide a good example; correcting the sequence that it is detrimental to antitumor immune response.

Previous work has shown that APN may mediate anti-inflammatory effects via the NF-κB pathway (33), we confirmed that NF-κB inhibition is indeed responsible for immature DC phenotypes following the interaction of APN with its receptors; explaining the fact that APN-conditioned DCs are less able to stimulate T-cell responses. For the first time, we report that both AdipoR1 and AdipoR2 were able to modulate the NF-κB activation via completely different upstream signaling pathways.

AdipoR1 signals via its conventional pathways, using AMPK and MAPKp38 to upregulate IL10, which in turn acts in an autocrine manner to promote its downstream signaling pathways. Activation of the IL10 receptor induces the STAT3 and SOCS3 pathways, which is then rendered the DC to become anergic, leading to induction of T-cell anergy or immune tolerance. C, siRNAs leads to induction of T cells (1:10 ratio). After coculture for 24 hours, supernatants were collected. The levels of IL4, IL5, IL13, IL10, IL12, IFNγ, and IL8 were determined by ELISA. The results are expressed as the mean ± SD of triplicate wells. One-way ANOVA was applied to check the differentiation between groups (overall P values in all panels are less than 0.01). Tukey HSD test was then used to calculate P values in the figure for different groups comparing with the control siRNA group (P is not shown when >0.05).
It is, therefore, plausible that AdipoR1 signaling via MAPKp38 may have an additive role to promote SOCS3-mediated tolerance caused by IL10. Therefore, our work on AdipoR1 signaling suggests that AdipoR1-mediated tolerance acts primarily through AMPK and MAPKp38-dependent induction of IL10, which may explain why APN levels do not affect the development and progression of colitis in an IL10 knockout model (35). One study suggests that APN-mediated anti-inflammatory effect is independent of IL10 (27); but its effect may be dependent on upregulation of anti-NF-κB effectors, A20, Bcl3, and TRAF1. To this end, we also identified that A20, Bcl3, and TRAF-1, as the potential modulators of NF-κB signaling, can be induced by DC AdipoR1/R2 signaling. However, their depletions were not sufficient to abrogate APN-induced anergy (data not shown). On the other hand, many studies have supported the notion that APN-induced IL10 production is indeed crucial in mediating its anti-inflammatory effects (36–38).

Recent work on conditioning STAT3-knockout DC showed that STAT3 is crucial in mediating IL10-dependent tolerance (39). Indeed, our work demonstrates that the AdipoR1 signaling in DCs mediates IL10-dependent T-cell tolerance.

Figure 6. Overexpression of AdipoR1/R2 promotes T-cell anergy. A, overexpression of APN receptors induces T-cell anergy. mDCs that had been transduced with either AdipoR1 or AdipoR2 or mock-construct (indicated) were exposed to allogeneic T cells, and the T-cell anergy assay was performed (20). The results are expressed as the mean ± SD of triplicate wells. In experimental group indicated in panel i, Mann–Whitney test was used. **, P < 0.01 when comparing mock transduced with either AdipoR1 or AdipoR2 transduced groups. In control experiments (panels ii, iii, and iv), no statistical significance was detected. B and C, comparison of signaling molecule activation. Using the same healthy subject, DCs were either unconditioned or conditioned with APN (as indicated on top of each panel). Some unconditioned DCs were transduced with either AdipoR1 or AdipoR2 or mock construct (as indicated). After activation with inflammatory stimuli, total proteins were extracted. The activity of various signaling molecules was determined by Western blotting.
Interestingly, it is well recognized that STAT3 is the dominant mediator for IL10 functions, but STAT3 can also mediate anti-inflammatory effect in IL10-independent manner (40); however, in our model, we identified that IL10-mediated NF-kB inhibition is dependent on STAT3 activation (Supplementary Fig. S5). In the context of IL10-independent mechanism, STAT3 activation can lead to activation of downstream molecules such as ETV3 (Fig. 4D) and SBNO2 (data not shown) that can significantly repress NF-kB activation (41).

It is important to note that APN used in suboptimal level (at 10 μg/mL of human physiologic level) can induce the degradation of IkB and nuclear translocation of NF-kB that were capable of generating Th1 and Th17 responses (42). Contrastingly, others have reported that APN can induce the expansion of regulatory T cells via its interaction with DCs (43). Our work indicates that at least 40 μg/mL APN is required to induce IL10 production (Supplementary Fig. S4A).

AdipoR2, however, exerts its effects by a different mechanism as compared with AdipoR1. As tampering with AdipoR2 interaction has less effect on IL10 production (Supplementary Fig. S4A). AdipoR2 signaling using the AdipoR1-depleted DCs indicates that COX-2 pathway may be responsible for upregulation of PPARγ, which then acts to promote anergic phenotypes on the engaging T cells, as knockout of PPARγ abolishes its tolerogenic phenotypes. Others (44) have also reported that stimulation of PPARγ by its agonist may be responsible for DC tolerogenic behavior. In the DC-T-cell interaction model, we show that the dominant AdipoR2 signaling in DCs via PPARγ pathways predominantly drives Th2-cytokine production following its encounter with allogeneic T cells; this is consistent with the stimulation experiments, in which DCs were directly treated with PPARγ agonist (44). Significantly more Th2-skewing cytokine production is noted when the DC possesses a dominant AdipoR2 signaling; a small degree of Th2-skewing cytokine production is induced by AdipoR1 as this observation has been reported previously (45).

To elicit the importance of interactions between APN and its receptors in tumor immune regulation, we tested whether overexpression of APN receptors (as noted in our clinical observations) can render murine T-cell tolerogenic in vitro and in vivo. In an immunogenic lymphoma model, the ability of tumor-specific T cells to confer protection was totally abolished when mice were vaccinated with AdipoR1 or AdipoR2 genetically engineered DCs. These findings demonstrate the in vivo relevance of our ex vivo human experiments, lending support to our hypothesis that overexpression of APN receptors on DCs may represent one of key immune modulators on how cancer influences the antitumor immunity. We accept that the animal model utilized may be too simplistic in addressing the complexity of tumor immune surveillance. Nevertheless, it is a
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