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Diet-Induced Obesity Alters Dendritic Cell Function in the Presence and Absence of Tumor Growth

Britnie R. James,* Ann Tomanek-Chalkley,† Eric J. Askeland,‡ Tamara Kucaba,§ Thomas S. Griffith,*‡§ and Lyse A. Norian‡

Obesity is a mounting health concern in the United States and is associated with an increased risk for developing several cancers, including renal cell carcinoma (RCC). Despite this, little is known regarding the impact of obesity on antitumor immunity. Because dendritic cells (DC) are critical regulators of antitumor immunity, we examined the combined effects of obesity and tumor outgrowth on DC function. Using a diet-induced obesity (DIO) model, DC function was evaluated in mice bearing orthotopic RCC and in tumor-free controls. Tumor-free DIO mice had profoundly altered serum cytokine and chemokine profiles, with upregulation of 15 proteins, including IL-1α, IL-17, and LIF. Tumor-free DIO mice had elevated percentages of conventional splenic DC that were impaired in their ability to stimulate naive T cell expansion, although they were phenotypically similar to normal weight (NW) controls. In DIO mice, intrarenal RCC tumor challenge in the absence of therapy led to increased local infiltration by T cell-suppressive DC and accelerated early tumor outgrowth. Following administration of a DC-dependent immunotherapy, established RCC tumors regressed in normal weight mice. The same immunotherapy was ineffective in DIO mice and was characterized by an accumulation of regulatory DC in tumor-bearing kidneys, decreased local infiltration by IFN-γ-producing CD8 T cells, and progressive tumor outgrowth. Our results suggest that the presence of obesity as a comorbidity can impair the efficacy of DC-dependent antitumor immunotherapies. The Journal of Immunology, 2012, 189: 1311–1321.
Materials and Methods

Animals and diets

Female BALB/c mice were purchased (Harlan Laboratories) at 7–8 wk of age, maintained on standard chow for 1 wk after receipt, and then randomly assigned to either standard chow or HFF (Research Diets number 12492, 60% kcal from fat) for 20 wk. Mice were housed five to a cage under pathogen-free conditions at the University of Iowa Animal Care Facility, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. All animal procedures were approved by the University of Iowa Institutional Animal Care and Use Committee. After 20 wk on feed, all mice were weighed, and the mean and SD of the NW (standard chow) group were calculated. Mice in the HFF group were defined as being DIO if their body weight at this time point was greater than 3 SD above the mean of the NW group.

Tumor cell line and tumor challenge

The murine renal adenocarcinoma cell line Renca was obtained from Dr. R. Wiltrout (National Cancer Institute, Frederick, MD) and was authenticated in 2010 by microsatellite marker analysis (Research Animal Diagnostic Laboratory, Columbia, MO). Renca cells were maintained in complete RPMI 1640 medium as described previously (18). Renca-Luc is a variant that stably expresses firefly luciferase; it was generated via retroviral transduction as described previously (19, 20). Renca-Luc cells were maintained in complete RPMI 1640 medium supplemented with 0.05 μg/ml puromycin.

For intrarenal (IR) tumor challenge, a skin incision was made on the left flank, and 2 × 10^5 Renca-Luc cells were injected through the intact peritoneum into the left kidney. On day 7 following tumor challenge, mice were reinfected with the same kidney with either sterile PBS or 10^7 PFU adenovirus5-encoded murine TRAIL (18) plus 100 μg CpG1826 (Coley Pharmaceuticals, Wellesley, MA) in a 100-μl volume. Renal tumor growth in vivo was measured via BLI (University of Iowa Central Microscopy Core Facility). For experiments on DC function in tumor-bearing mice, renal tumor growth was validated by BLI prior to sacrifice, typically between days 21 and 28. For s.c. tumor growth, 2 × 10^5 parental Renca tumor cells were injected on day 0, followed by sterile PBS or adenovirus5-encoded murine TRAIL + CpG1826, as above, given peritumorally on day 7.

BLI of tumor growth

BLI was done using an IVIS 200 (Caliper Life Sciences, Hopkinton, MA) as described previously (19, 20). Briefly, 10 min prior to imaging, mice were injected i.p. with 100 μl of a 15 mg/ml solution of D-Luciferin (GoldBio, com, St. Louis, MO) and then anesthetized via inhalation of oxygenated isoflurane. Live mice were imaged for 1 min each for dorsal and ventral aspects. Renca-Luc generated photon flux (photons per second) was calculated within a defined region of interest using Living Image software (version 2.5, Caliper Life Sciences). The mean total flux per mouse (averaged of dorsal and ventral images) was determined at the indicated time points and used to calculate an overall mean for each treatment group.

DC and T cell enrichment and isolation

DC were harvested from spleens or tumor-bearing kidneys, as indicated. Organ samples were removed, manually disrupted, and then digested for 15–30 min in HBSS containing 0.56 Wuensch U/ml Liberase Blendzyme 3 (Roche, Branford, CT) and 0.15 mg/ml DNAse I (Sigma-Aldrich, St. Louis, MO). Organs were removed, manually disrupted, and then digested for 15–30 min (average of dorsal and ventral images) was determined at the indicated time points and used to calculate an overall mean for each treatment group.

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T cell proliferation and inhibition assays

T cells were prepared from DUC18 TCR transgenic mice (22, 23), incubated with CD80 microbeads (Miltenyi Biotec), and purified over two sequential MACS columns. The percentage of DUC18 T cells was determined via flow cytometry, based on surface coexpression of CD80 and Vβ8.3. T cells were used in proliferation assays with sort-purified splenic DC (spDC) from either NW or DIO tumor-free mice, which were set up as described previously (21). DC inhibition of T cell proliferation was assessed by culturing naive DUC18 T cells (5 × 10^5 cells/well) with tERK peptide-pulsed spDC (21–23) from control tumor-free BALB/c mice (5 × 10^5 cells/well) and sort-purified renal DC from tumor-bearing mice (5 × 10^5 cells/well). [3H]Thymidine was added on day 3 for the final 18 h of culture. T cell inhibition was calculated as the percent decrease in T cell proliferation with renal DC present relative to that seen for DUC18 T cells cultured with control, peptide-pulsed spDC alone (21).

Surface staining for flow cytometry

Spleens and tumor-free or tumor-bearing kidneys were harvested, manually disrupted, and then digested as above. Cells were stained with combinations of the following mAbs, and results were acquired using multiparameter flow cytometry on a BD LSRII (BD Biosciences, San Diego, CA) and then analyzed with FlowJo software (Tree Star, Ashland, OR). For DC: CD1c, biotin, streptavidin-APC/Cy7, CD11b-PE/Cy5, Gr1-APC, I-A^b-PE, CD4-PE, CD8-PerCP-Cy5.5, CD40-APC, CD86-APC, CD80-FITC, CD44-PE, and Hoechst; for T cells: CD3-PE, CD8-APC, CD4-PE/Cy7, CD11a-FITC, IFNγ-PerCP-Cy5.5, and Hoechst; and for Renca tumor cells: Rael1-A647 and purified anti-coxsackie adenosirus receptor (CAR) plus goat anti-mouse A488. mAbs were from eBioscience (San Diego, CA), BioLegend (San Diego, CA), or Millipore (Billerica, MA).

Cytokine and chemokine evaluation by ELISA and BioPlex

Serum samples were obtained from NW or DIO mice and frozen at −80°C until use. DC were purified from spleens or tumor-bearing kidneys and cultured on 5 × 10^5 cells/ml in complete medium in the presence or absence of 1 μg/ml LPS (Sigma-Aldrich). Supernatants were harvested and frozen at −80°C until use. Leptin serum concentrations were determined by ELISA (ChrysalChem) according to the manufacturer’s protocol. Serum and supernatant concentrations of the following cytokines and chemokines were determined via Multiplex analysis (Milliplex MAP kits; Millipore) on a Bio-Rad BioPlex: etoxacin, G-CSF, IFNγ, IP-10, KC, LIF, LIX, MIP-2, TNFα, VEGF, macrophage-derived chemokine, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-23, and IL-27.

Statistical analyses

Statistical significance between experimental groups was determined by using paired or unpaired Student t tests, with Welch’s corrections for unequal variances, as appropriate (Prism; GraphPad Software, La Jolla, CA). Throughout the paper, significance at p < 0.05 is indicated by one asterisk; p < 0.01 is indicated by two asterisks.

Results

Profoundly altered serum cytokine profile in female BALB/c DIO mice

The majority of prior studies on murine DIO used the C57BL/6 strain, because these mice rapidly become obese after being placed on HFF (9–11, 13, 17, 24). Because our goal was to determine the combined effects of renal tumor outgrowth and DIO on DC function, our experimental model necessitated using the BALB/c strain for Renca RCC tumor challenge. We found that BALB/c mice are more resistant to DIO than are C57BL/6 mice, and BALB/c mice placed on HFF for 10 wk did not develop the systemic inflammation that normally accompanies DIO (data not shown). Consequently, we modified our protocol so that BALB/c mice were fed HFF for 20 wk and performed a thorough characterization of the resulting DIO mice.

We observed that 45–55% of BALB/c mice on HFF showed increased weight gain relative to age-matched mice fed standard chow over the same period of time (Fig. 1A). Therefore, we defined DIO mice as those having a final weight of greater than the mean weight + 3 SD of age-matched NW mice that had been fed standard chow for 20 wk. The mean body weights of one cohort of 13 NW and 13 DIO mice are shown in Fig. 1B. Compared with NW mice, DIO mice had increased percentages of visceral body fat and increased concentrations of serum leptin (Fig. 1C, 1D), both of which are hallmarks of obesity.

We next tested serum from NW and DIO mice for concentrations of 35 individual cytokines and chemokines via multiplex array. Of these, only IL-5 and VEGF were elevated in NW versus DIO serum (Table I). In contrast, a large number of analytes showed statistically significant increases in DIO versus NW serum, including IL-1α, IL-7, IL-15, IL-17, IFNγ, IP-10, LIF, LIX, and TNFα.
CD11c expression, which differentiates them from other cell populations that can express intermediate to low levels of this integrin (25, 26). Fig. 2A shows the gating strategy used to identify spDC. We observed increased percentages of CD11c\textsuperscript{high} spDC in DIO mice (Fig. 2B). An analysis of CD11c\textsuperscript{high} DC subsets revealed equivalent percentages of CD8\textsuperscript{+} DC, CD4\textsuperscript{+} DC, CD11b\textsuperscript{+} DC, and CD8\textsuperscript{−}/CD4\textsuperscript{−} double-negative DC in the spleens of NW and DIO mice (Supplemental Fig. 1A). In addition, we also detected higher percentages of CD11c\textsuperscript{low}/MHC II\textsuperscript{−}/B220\textsuperscript{+} plasmacytoid DC in the spleens of DIO mice (Supplemental Fig. 1B). Of note, total live splenocyte counts were nearly identical in NW and DIO mice (Fig. 2C), indicating that the obesity-associated inflammation had not resulted in overall increases in splenic cell populations that can express intermediate to low levels of this integrin (25, 26).

Table I. Serum cytokine and chemokine profiles for NW versus DIO mice

<table>
<thead>
<tr>
<th>Serum Analyte (pg/ml)</th>
<th>NW Mean ± SEM</th>
<th>DIO Mean ± SEM</th>
<th>p</th>
<th>p &lt; 0.05</th>
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<tbody>
<tr>
<td>IL-1α</td>
<td>51.0 ± 15.6</td>
<td>328 ± 241.8</td>
<td>*</td>
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</tr>
<tr>
<td>IL-1β</td>
<td>1.0 ± 0.71</td>
<td>3.4 ± 1.5</td>
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<tr>
<td>IL-2</td>
<td>1.5 ± 0.71</td>
<td>14.3 ± 5.6</td>
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</tr>
<tr>
<td>IL-3</td>
<td>6.8 ± 1.1</td>
<td>19.3 ± 13.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>6.5 ± 5.0</td>
<td>10.8 ± 5.7</td>
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<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>41.5 ± 2.8</td>
<td>23.7 ± 6.2</td>
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</tr>
<tr>
<td>IL-6</td>
<td>35.0 ± 7.1</td>
<td>26.0 ± 7.3</td>
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</tr>
<tr>
<td>IL-7</td>
<td>7.5 ± 2.1</td>
<td>55.8 ± 11.9</td>
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<tr>
<td>IL-10</td>
<td>4.0 ± 0.71</td>
<td>15.3 ± 9.3</td>
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<tr>
<td>IL-12p40</td>
<td>12.3 ± 0.35</td>
<td>27.6 ± 11.1</td>
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<tr>
<td>IL-12p70</td>
<td>4.3 ± 0.71</td>
<td>8.6 ± 6.8</td>
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<tr>
<td>IL-13</td>
<td>56.0 ± 11.0</td>
<td>105 ± 23.7</td>
<td>*</td>
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<tr>
<td>IL-15</td>
<td>28.8 ± 1.4</td>
<td>98.5 ± 26.9</td>
<td></td>
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</tr>
<tr>
<td>IL-17</td>
<td>3.0 ± 1.4</td>
<td>37.1 ± 15.8</td>
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<tr>
<td>Eotaxin</td>
<td>13,391.8 ± 612.7</td>
<td>18,897 ± 875.4</td>
<td>*</td>
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<tr>
<td>G-CSF</td>
<td>538.0 ± 21.2</td>
<td>552 ± 54.0</td>
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<tr>
<td>IFN-γ</td>
<td>6.0 ± 4.6</td>
<td>31.5 ± 7.1</td>
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<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>711.3 ± 1.06</td>
<td>1,726 ± 510</td>
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<tr>
<td>KC</td>
<td>1,208 ± 205.1</td>
<td>1,156 ± 246.5</td>
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<tr>
<td>LIF</td>
<td>10.0 ± 2.1</td>
<td>52.9 ± 13.0</td>
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<tr>
<td>LIX</td>
<td>5,731 ± 1,289.1</td>
<td>13,461 ± 2,038</td>
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<tr>
<td>TNF-α</td>
<td>2.5 ± 0.71</td>
<td>13.5 ± 9.16</td>
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</tr>
<tr>
<td>VEGF</td>
<td>3,011 ± 4,141</td>
<td>184.9 ± 291</td>
<td>*</td>
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</table>

Serum was harvested from NW or DIO mice (n = 6 NW; n = 7 DIO) after 20 wk on feed, frozen, and analyzed via MultiPlex array for the above analytes.
lularity. Thus, our findings in BALB/c DIO mice support those reported earlier by Macia et al. (12) during their study of leptin-deficient obese mice.

LN DC subset composition has not been thoroughly examined in DIO mice. We isolated brachial, axillary, and inguinal LNs from DIO and NW mice and determined the frequencies of total DC in pooled LNs, as well as percentages of the three main DC sub-populations present, based on expression of CD11c and MHC class II (MHC II) (I-A\textsuperscript{d}). Tissue-derived migratory DC are defined as CD11c\textsuperscript{+}/MHC II\textsuperscript{high} (subset i), inflammatory DC as CD11c\textsuperscript{+}/MHC II\textsuperscript{+} (subset ii), and LN-resident DC as CD11c\textsuperscript{high}/MHC II\textsuperscript{+} (subset iii) (Supplemental Fig. 1C) (27). DIO mice had increased percentages of total LN DC as compared with NW mice (Supplemental Fig. 1C). No statistically significant alterations were observed in the percentages of any of the LN subsets in DIO versus NW mice, although there was a trend in each case toward increased percentages with obesity (Supplemental Fig. 1D). Taken together, these results show that DC subset composition is similar between NW and DIO mice but that greater percentages of conventional spDC, plasmacytoid spDC, and LN DC are present in DIO mice.

**Decreased stimulatory capacity but increased CXCL10 production in DIO spDC**

Previous investigations into the effects of obesity on DC stimulatory capacity produced conflicting results. Studies on naive animals had found that bulk splenic APCs from DIO mice and bone marrow-derived DC from leptin-deficient ob/ob mice were less able to stimulate naïve T cell proliferation than were cellular counterparts from NW mice (12, 14). In contrast, another report found that during influenza infection, lung DC from DIO mice retained the ability to induce IFN-\gamma production in T cells (17). Therefore, our next set of experiments addressed whether DIO impacted the steady-state stimulatory capacity of highly purified spDC.

For these experiments, spDC were sort purified from NW or DIO mice by gating on CD45\textsuperscript{+}/Gr-1\textsuperscript{-}/CD11c\textsuperscript{high}/CD11b\textsuperscript{+} cells. We focused on CD11b\textsuperscript{+} DC, because we had determined previously that this subpopulation accumulated within murine fibrosarcomas and mammary carcinomas (21); we therefore wanted to determine whether baseline differences were present in the stimulatory capacity of this DC subset in tumor-free DIO versus NW mice. Following purification, spDC were pulsed with tERK peptide and used to stimulate CD8\textsuperscript{+} TCR-transgenic DUC18 T cells in vitro (21–23, 28). The results of four independent experiments illustrate that spDC from DIO mice induced less T cell proliferation than their NW counterparts (Fig. 3A, 3B). Overall, mean T cell proliferation in the presence of DIO spDC was 58% of that seen with NW spDC.

To determine whether obvious phenotypic differences might account for the decreased stimulatory capacity observed in DIO spDC, bulk splenocytes from NW and DIO mice were stained as indicated (Fig. 3C). Gating on the CD11c\textsuperscript{high}/Cd11b\textsuperscript{+}/I-Ad\textsuperscript{+} spDC, the mean fluorescent intensity (MFI) of the indicated surface proteins on gated live CD11c\textsuperscript{high}/I-Ad spDC are shown. Bars indicate mean ± SEM. (D) Cytokine and chemokine concentrations are shown for six NW and six DIO spDC samples, obtained from individual mice. Analyte concentrations were determined via Multiplex array. **p ≤ 0.01.
population revealed no significant alterations in surface expression of MHC I, MHC II, CD86, or CD40 between NW and DIO mice.

We then asked whether DIO spDC possessed an altered cytokine/chemokine profile as compared with NW spDC, because DC-derived cytokines can profoundly alter T cell differentiation outcomes (29). Following an overnight DC culture with LPS, supernatants were harvested from either DIO or NW spDC cultures and analyzed via multiplex array for a panel of 24 cytokines and chemokines. The only significant difference found was in the production of the chemokine IP-10, which was higher in DIO spDC than NW spDC (Fig. 3D, Supplemental Table I). For many analytes, such as IL-6, the expression patterns were nearly identical between the two groups, whereas for others, such as MIP-2, trends were visible but did not reach statistical significance. Overall, the phenotypes and chemokine/ cytokine profiles in DIO and NW spDC were largely comparable; thus, the major difference we detected was a defect in DIO spDC stimulatory capacity.

Increased numbers of inhibitory DC infiltrate renal tumors from DIO mice

Once a baseline examination of steady-state spDC function had been performed in tumor-free DIO versus NW mice, we examined the combined effects of tumor outgrowth and obesity on DC function. We had found previously that tumor-infiltrating CD11c⁺/CD11b⁻/Gr-1⁻ DC (TIDC) from NW mice acted as regulatory cells that suppressed T cell proliferation ex vivo and impeded antitumor immunity in vivo, leading to enhanced tumor outgrowth (21). Currently, the impact of obesity on antitumor immunity is unclear, and it is not known how or whether obesity and tumor growth interact to further alter DC function.

Our experiments made use of an orthotopic RCC model, in which luciferase-expressing Renca tumor cells were injected directly into the left kidney of DIO or NW mice after 20 wk of feeding either HFF (DIO) or standard chow (NW). This system allowed us to measure renal tumor burdens over time in live mice via BLI. BLI revealed that early tumor growth through day 7 was more rapid in DIO mice as compared with NW counterparts (Fig. 4A, Supplemental Fig. 2A). Despite this fact, kidney masses remained comparable at day 7 (Fig. 4B), because renal tumors are not yet macroscopically visible at this time point in our model system (30). Therefore, to validate our BLI observation at day 7, we performed a complementary analysis by performing flow cytometry on dissociated kidneys from NW and DIO mice. Mice were challenged IR with parental Renca, and then, both tumor-bearing
and contralateral kidneys were isolated from each animal and stained for Rae1γ, an NKG2D ligand known to be expressed on Renca cells (31). Although Rae1γ is expressed on cell types other than Renca tumors, the low baseline staining in tumor-free contralateral kidneys (30) from both NW and DIO mice shows that Rae1γ can be used to assess Renca tumor burdens in murine kidneys (Fig. 4C). This staining approach validated our BLI results by showing increased tumor burdens in DIO kidneys at day 7 relative to NW kidneys and also illustrated that early increases in DIO tumor burdens are not sustained over time.

To determine whether obesity fundamentally altered the DC response to progressing solid tumor development, we examined DC infiltration into tumor-bearing kidneys over time in NW and DIO mice. First, however, we examined the percentages of CD11c<sup>hi</sup>/MHC II<sup>+</sup> renal DC (Supplemental Fig. 2B) and the total numbers of live cells per kidney (data not shown) in tumor-free NW and DIO mice and found them to be equivalent. An examination of Renca tumor-bearing kidneys illustrated that DIO and NW mice had comparable percentages of DC at both days 7 and 13 after tumor challenge (Fig. 5A). The percentages of DC in Renca-positive kidneys were increased at day 7, relative to tumor-free contralateral kidneys, in both DIO and NW mice. As an additional control, we injected some mice IR with sterile PBS to evaluate whether local inflammation caused by the IR injection itself led to local DC infiltration or whether the responses observed were specific to tumor growth. The percentages of DC in PBS-injected kidneys were comparable to those seen in tumor-free contralateral kidneys, indicating that heightened DC infiltration in tumor-bearing kidneys was a response to local tumor growth. Phenotypic analysis of renal DC at day 7 illustrated that DIO mice had fewer CD83<sup>+</sup> DC, suggesting that DC were less mature (32). Expression of CD86 was similar on NW and DIO renal DC at both days 7 and 13 and was elevated on DC from tumor-bearing kidneys relative to contralateral or PBS-injected kidneys (Supplemental Fig. 2C).

We then evaluated DC infiltration after renal tumor growth had continued unchecked for more than 3 wk. Advanced tumor outgrowth was accompanied by increased percentages of DC in the tumor-bearing kidneys of DIO mice (Fig. 5B). Again, DC infiltration of contralateral kidneys and spleens remained at low levels in both NW and DIO mice (data not shown), indicating that the response was tumor specific. Therefore, obesity led to increased local DC infiltration in response to advanced renal tumor growth, but did not differentially impact systemic DC that were distal to the tumor site.

On the basis of our prior work showing that TIDC from multiple tumor types functioned as suppressor cells (21), we evaluated the inhibitory capacity of renal TIDC from DIO and NW mice. To accomplish this, CD11c<sup>+</sup>/CD11b<sup>-</sup>/Gr-1<sup>-</sup> DC were sort purified from the kidneys of mice with advanced renal tumor growth, as evidenced by BLI. Performing DC isolations in this manner allowed us to control for variations in tumor outgrowth rates and to isolate DC when tumor burdens were similar. Following sort purification, renal DC from tumor-bearing kidneys were put into culture with naive DUC18 T cells (21) and stimulatory tERK peptide-pulsed spDC from young, NW tumor-free mice. We then determined DC-suppressive capacity by measuring decreases in T cell proliferation, relative to control wells that contained only T cells and stimulatory spDC. Mean results from three independent experiments showed a significantly stronger inhibitory capacity in DC from tumor-bearing kidneys of DIO mice, as compared with counterparts from NW mice (Fig. 5C). The latter showed no ability to suppress naive T cell proliferation. Although the suppressive capacity of DIO TIDC was moderate on a per cell basis, increased frequencies of these cells were present in the tumor-bearing kidneys of DIO mice. This raised the possibility that the net effect might be a noticeable inhibition of tumor clearance in vivo.

**Diminished responsiveness to immunotherapy in DIO mice is accompanied by local increases in suppressive DC and decreased T cell infiltration**

The endogenous immune response to Renca tumors is not protective, but we had determined previously that administration of immunotherapy could induce DC- and T cell-dependent Renca eradication in NW mice (18, 30). The therapy we used consisted of adenovirus-encoded TRAIL (AdTRAIL) (33) plus Cpg 1826. TRAIL has been explored as an anticancer agent because of its ability to preferentially induce tumor cell death, whereas Cpg oligodeoxynucleotides promote DC maturation and enhanced Ag presentation (34–37). Successful AdTRAIL + Cpg therapy relies upon DC presentation of Ag derived from apoptotic tumor cells to CD8 T cells, which then become the main effectors of tumor clearance (18, 30). Given the elevated percentages of suppressive DC in tumor-bearing kidneys from DIO mice (Fig. 4), we predicted that DIO mice would have a reduced ability to clear Renca luciferase tumors following administration of AdTRAIL + Cpg immunotherapy.
DIO and NW mice were challenged IR with Renca-luciferase on day 0, followed by either AdTRAIL + CpG therapy or PBS on day 7, at a time when tumor growth was already evident (Fig. 4, Supplemental Fig. 2A). By day 28, both DIO and NW PBS-treated mice had equivalently large renal tumor burdens (Fig. 6A). NW mice that had received AdTRAIL + CpG on day 7 showed a substantial reduction in tumor growth as compared with PBS-treated NW mice (Fig. 6A, □). In fact, the total light flux in treated NW mice approximated the background flux seen in tumor-free controls \((p > 0.05)\), indicating that few tumor cells remained in these kidneys. In contrast, DIO mice that received AdTRAIL + CpG on day 7 showed no decrease in tumor burden relative to PBS-treated DIO mice (Fig. 6A, ■). To determine whether AdTRAIL + CpG therapy would show greater efficacy in DIO mice if a different tumor challenge model were used, we injected NW and DIO mice with parental Renca s.c. on the hind flank. Again, mice were given AdTRAIL + CpG or sterile PBS peritumorally on day 7. In this model, AdTRAIL + CpG was highly effective at reversing tumor growth in NW mice but produced little benefit in DIO mice (Fig. 6B). An examination of survival in s.c.-challenged DIO versus NW mice revealed that whereas 80% of NW mice survived long-term after AdTRAIL + CpG therapy, all treated DIO mice succumbed to their tumors (Supplemental Fig. 3A). Thus, an immunotherapy that was able to reverse uncontrolled tumor outgrowth in NW mice was ineffective in DIO mice.

We wanted to explore possible mechanisms that might account for this therapeutic failure, and began by assessing expression of the CAR in the kidneys of DIO and NW mice at day 7 after IR tumor challenge. Decreased CAR expression in DIO mice could explain the therapeutic failure we observed, as this would diminish local adenoviral entry into cells and preclude expression of TRAIL. However, DIO mice had increased CAR expression at day 7 in tumor-bearing kidneys, negating this possibility (Supplemental Fig. 3B). We then examined the degree of DC infiltration into tumor-bearing kidneys following therapy. We found that tumor-bearing kidneys from DIO mice treated with AdTRAIL + CpG had increased percentages of DC relative to PBS-treated DIO mice (Fig. 6C). Of note, the percentages of DC present in tumor-bearing kidneys from NW AdTRAIL + CpG-treated mice were greatly reduced compared with what we had seen in untreated tumor-bearing NW mice (Fig.

**FIGURE 6.** Immunotherapeutic failure in DIO mice is associated with increased percentages of suppressive DC in tumor-bearing kidneys. (A) IR tumor growth at day 28 postchallenge, as measured by BLI in live mice that received either PBS or AdTRAIL + CpG on d7. Bars indicate the mean ± SEM of \(n = 5–8\) tumor-bearing NW or DIO mice in each treatment group, combined from three independent experiments. For tumor-free controls, \(n = 3\) mice. \(*p < 0.05.\) (B) SC Renca tumor growth in NW and DIO mice treated with either AdTRAIL + CpG or PBS on day 7. (C) The mean percentages (±SEM) of CD11c\(^{high}\)/CD11b\(^{+}\) myeloid DC are shown for NW or DIO AdTRAIL + CpG-treated mice, grouped by organ. Data are cumulative from two independent experiments, where \(n = 2\) for TR + CpG NW c-Kid and spleen, and \(n = 4–8\) individual mice for all other groups. The right panels illustrate naive DUC18 T cell proliferation in the presence of control spDC, with or without sort-purified renal DC (upper panel) or spDC (lower panel) from tumor-bearing DIO mice that were treated with AdTRAIL + CpG on day 7. x-axis numbers indicate ratios of the indicated cell populations present in culture. (D) Phenotype and cytokine production in renal DC from day 21 tumor-bearing kidneys of mice treated with AdTRAIL + CpG on day 7. Combined results from three experiments are shown. c-Kid, Tumor-free contralateral kidney; spln, spleen; tumor, tumor-bearing kidney. \(*p < 0.05,\) \(**p < 0.001.\)
5B). In treated DIO mice, the increased percentage of renal DC was again specific to the tumor site, because increased DC percentages were not evident in non–tumor-bearing contralateral kidneys. The spleens of AdTRAIL + CpG-treated DIO mice did trend toward having an increased percentage of DC relative to similarly treated NW spleens, but this difference was not significant (Fig. 6C).

We next assessed DC function from DIO tumor-bearing kidneys to determine whether administration of immunotherapy had diminished the suppressive capacity of these cells. Experiments similar to those described in Fig. 5C were performed, this time using sort-purified renal DC from DIO AdTRAIL +CpG-treated mice. Even in the presence of immunotherapy, renal DC from tumor-bearing DIO mice inhibited T cell proliferation (Fig. 6C, upper right panel). Suppressive function was specific to DC from tumor-bearing kidneys, because DC isolated from the spleens of AdTRAIL + CpG-treated tumor-bearing DIO mice showed no inhibition of T cell proliferation (Fig. 6C, lower right panel). Our attempts to evaluate the suppressive function of DC from NW AdTRAIL + CpG-treated kidneys were unsuccessful, because we were unable to obtain sufficient numbers of these DC for functional evaluation (data not shown). We then stained renal DC from the tumor-bearing kidneys of AdTRAIL + CpG-treated DIO and NW mice at day 21 and again found decreased percentages of CD8<sup>+</sup> DC (Fig. 6D). To further investigate the function of DC from tumor-bearing kidneys, we sort purified DC from NW and DIO mice that had received AdTRAIL + CpG therapy and evaluated their cytokine production. Renal DC from DIO mice produced less IL-12p70 and TNF-α than did their NW counterparts, although their production of IL-1β was equivalent. Both IL-12p70 and TNF-α are known to promote protective immunity (29, 38), so a loss in their expression would be predicted to diminish local T cell responses.

To determine whether a decreased local T cell response in DIO mice contributed to immunotherapeutic failure, we evaluated the percentages of CD8<sup>+</sup> effector T cells present in the kidneys at day 21 after AdTRAIL + CpG treatment of DIO and NW mice. Tumor-bearing kidneys of treated NW mice showed a significant increase in the percentage of CD8<sup>+</sup> T cells, relative to tumor-free contralateral kidneys of the same mice (Fig. 7A). In contrast, the T cell response was modest in tumor-bearing kidneys from DIO mice and was not significantly elevated with respect to contralateral kidneys. Interestingly, the impaired T cell influx observed in DIO mice at day 21 was not present at earlier time points; at both days 7 and 13 after IR tumor challenge, the percentages of Ag-experienced CD11a<sup>high</sup>CD8<sup>+</sup> T cells (39) in tumor-bearing kidneys from DIO and NW mice were equivalent (Supplemental Fig. 3C). We then examined effector function in CD8<sup>+</sup> T cells from AdTRAIL + CpG-treated NW and DIO mice by staining for intracellular IFN-γ. Doing so again revealed a strong T cell

![Figure 7](http://www.jimmunol.org/)
response in tumor-bearing kidneys from NW mice (Fig. 7B). Although DIO mice had nearly comparable percentages of CD8 T cells that produced IFN-γ (Fig. 7B), the low percentages of T cells present meant that the overall frequency of effector CD8+ T cells present within tumor-bearing kidneys was no greater than that seen in tumor-free contralateral kidneys (Fig. 7C). Finally, to determine whether the cytolytic activity of DIO effector CD8+ T cells were also diminished, we performed an in vivo cytolytic assay using NW and DIO influenza-infected mice, because this model system allowed us to readily evaluate site-specific cytolytic activity in vivo. Decreased cytolytic activity was observed in DIO mice (Supplemental Fig. 3D), complementing the results we obtained in our IR Renca tumor model. Thus, a robust, local effector T cell response was lacking in DIO mice.

Collectively, these results show that DIO renders a normally efficacious immunotherapy largely ineffective, leading to uncontrolled tumor outgrowth. The loss of responsiveness to immunotherapy in DIO mice was accompanied by increased percentages of suppressive DC in tumor-bearing kidneys and diminished effector T cell responses at the tumor site.

Discussion

Understanding the influence of obesity on immune function, tumor outgrowth, and the efficacy of administered antitumor immunotherapies is of critical importance, given the widespread prevalence of obesity in the United States today. In the current study, we used a mouse model of DIO to identify DC functional alterations, both in the absence and presence of orthotopic RCC, a cancer for which obesity is a major risk factor (3). We found that a DC-based immunotherapy was largely ineffective in DIO mice with RCC, although the same therapy was able to control RCC outgrowth in NW mice. This loss of efficacy was accompanied by increased percentages of suppressive DC in tumor-bearing kidneys of DIO mice and a decreased influx of CD8 T cells into the tumor site.

We identified several obesity-related alterations in DC function in DIO mice. During steady-state conditions, we found that DIO spDC had elevated production of IP-10 (CXCL10), a chemokine that normally regulates local recruitment of macrophages, T cells, NK cells, and DC. Because concentrations of this chemokine were also elevated in the serum of DIO mice, this raised the possibility that site-specific T cell trafficking might be impaired in DIO mice. For example, we found decreased CD8+ T cell infiltration of tumor-bearing kidneys after administration of immunotherapy. If elevated systemic IP-10 were causing dysregulated T cell trafficking, we would have expected to see increased percentages of CD8+ T cells in the tumor-free contralateral kidneys of DIO mice, which we did not. Recently, Krinninger et al. (40) reported that adipocytes from mice and humans produce IP-10, which may explain the accumulation of CD8+ T cells seen in obese adipose tissue in other models. In addition, increased systemic leptin concentrations have been associated with increased numbers, percentages, and suppressive activities of CD4+ regulatory T cells during obesity (41). Therefore, it is possible that decreased T cell infiltration into the tumor site was due to a combination of regulatory T cell-mediated inhibition of effector T cell expansion and IP-10–mediated abnormalities in T cell trafficking. We did not examine adipose tissue in our DIO mice to determine whether CD8+ T cells were also accumulating there during tumor outgrowth, rather than trafficking to the tumor site.

We also found that spDC from DIO mice had a reduced ability to stimulate naive T cell proliferation, a fact that also could have contributed to the decreased T cell response we observed in tumor-bearing DIO mice receiving immunotherapy. One explanation for the observed changes in DIO spDC function is elevated systemic lipids, because intracellular lipid accumulation has recently been shown to impair DC function in mice with tumors; in that study, normalization of DC lipid content via pharmacological inhibition improved DC function (42). Another possibility is that DC function is impaired because of the unique cytokine milieu present systemically in DIO mice. Several of the factors that were elevated in the serum of DIO mice are known to impact the function of DC and other leukocytes. For example, leptin is a protein that functions both as a hormone and a cytokine and is increased at elevated levels by visceral adipocytes in the obese. Leptin is structurally similar to IL-6 and has a wide range of effects on the immune system: it enhances phagocytosis in macrophages, it supports the differentiation and cytolytic activity of NK cells, it promotes Th differentiation toward a Th1 state, and it has been shown to enhance the maturation of bone marrow–derived DC in culture (43, 44). From these findings, one might predict that DIO mice would have DC with enhanced stimulatory capacities. However, leptin is just one cytokine that was elevated in DIO mice; other factors acting singly or in combination may counteract its effects. One example is eotaxin (CCL11), which was increased in the serum of DIO mice and has been shown to impede DC differentiation through activation of the suppressor of cytokine signaling (45).

Our study illustrated that the combination of obesity and tumor outgrowth leads to a markedly impaired DC response to RCC in this orthotopic murine model. We observed increased infiltration of DC into tumor-bearing kidneys of DIO mice in the presence and absence of immunotherapy. Rather than inducing T cell proliferation, the DC that infiltrated tumor-bearing kidneys in DIO mice inhibited T cell expansion (Figs. 5C, 6C). This was in contrast to the DC from tumor-bearing kidneys in NW mice, which did not show any ability to suppress T cell proliferation. In DC mice, suppression of T cell expansion occurred even after administration of a combinatorial therapy that contained CpG1826. CpG1826 is an unmethylated oligonucleotide known to induce DC maturation and Ag presentation in NW mice, and it is being investigated as an anticancer therapeutic for several tumor types (34). Despite this, treated DIO mice showed decreased percentages of CD83+ mature DC in tumor-bearing kidneys (Fig. 6D) and also produced fewer of the immunoprotective cytokines IL-12 and TNF-α (Fig. 6D). These results highlight the necessity of examining immunotherapeutic efficacy in both NW and obese mice, because the altered physiology of the latter may negatively impact results.

Using two murine breast cancer models and a fibrosarcoma model, we previously found that TIDC from young NW mice were able to inhibit T cell expansion ex vivo (21). Although the degree of inhibition varied according to the tumor model being examined, all TIDC showed some ability to suppress T cell expansion. Because of this, we were initially surprised to find that DC from RCC-bearing kidneys in NW mice did not inhibit T cell proliferation. Several factors may account for this finding. First, there were low percentages of DC present in tumor-bearing kidneys from NW mice. Second, it was impossible for us to determine whether those low numbers of renal DC were actually infiltrating tumor masses within kidneys or were located in normal tissue adjacent to malignant areas. The use of BLI allowed us to monitor tumor growth in live mice, so we were able to objectively determine that all mice used for experiments had progressively growing tumors by day 7. However, even after several weeks of tumor growth, when most DC experiments were performed, it was frequently impossible to identify distinct tumor masses upon gross examination of excised kidneys. To validate our BLI results, several tumor-bearing kidneys were sectioned and stained for histological evaluation, and all were found to contain renal tumors.
(data not shown). Consequently, we could not determine the relative ratios of true “TIDC” versus the broader population of renal DC. It is possible that many of the renal DC from tumor-bearing NW mice had resided in healthy kidney tissue, rather than in the developing tumor mass.

In our study, BLI and flow cytometric analyses revealed that early RCC outgrowth was more rapid in DIO mice than in NW counterparts (Fig. 4A, 4C), but these differences were not sustained (Fig. 5A). Although accelerated tumor outgrowth has been reported in other models of murine obesity, this trend is not universally observed (46–49). The variable responses of tumor growth to ongoing obesity likely stem from the unique responses of each tumor cell type to the cytokine/chemokine environment present in the host. Leptin is known to promote angiogenesis through increased expression of VEGF, to promote tumor cell proliferation in vitro, and to increase tumor cell resistance to apoptosis (50). These actions could have promoted the increased early tumor growth we observed and could also have contributed to the decreased efficacy of AdTRAIL + CpG therapy in DIO mice. It is also likely that elevated concentrations of inflammatory mediators influenced the rapid, early tumor outgrowth in DIO mice. Previous studies in a mouse that lacks white adipose tissue, and therefore lacks leptin, but still develops insulin resistance and chronic inflammation, implicated inflammation as a key factor in accelerated tumor outgrowth (51). In agreement with this idea, numerous studies in NW mice have demonstrated that chronic inflammation supports tumor outgrowth and suppresses antitumor immunity (52–56). Because PGE2 is a critical mediator of inflammation and is elevated during obesity in response to high levels of cytokines such as IL-1β and IL-18 (57), it is possible that blocking this pathway might have benefits in terms of reducing tumor burdens and/or normalizing antitumor immunity in obese individuals, an idea that could be addressed in future experiments.

We show in this study that tumor-free DIO BALB/c mice have increased serum concentrations of multiple proinflammatory cytokines (Table I). Several of these have been previously shown to augment tumor outgrowth. For example, IL-13 and IFN-γ were shown previously to support breast cancer growth in mice (58). IL-10 has been linked to breast cancer metastasis (59). LIF has been shown to promote accelerated growth of kidney, breast, and prostate cancer cells (60–62). The net effect of these and other factors on tumor growth is not known. Interestingly, the pattern of cytokine/chemokine alterations we observed in BALB/c DIO versus NW mice were distinct from those previously reported for DIO C57BL/6 mice (11). For example, we found higher serum concentrations of IL-1α, IL-2, IL-12p40, IL-13, IL-17, and eotaxin in DIO mice, whereas these same proteins were expressed at equal or lower levels in the serum of DIO C57BL/6 mice, relative to NW C57BL/6 mice (11). Therefore, underlying strain differences may affect not only serum cytokine/chemokine profiles but might also influence tumor outgrowth rates.

DC responses to tumor outgrowth are critical factors that impact the success or failure of many immunotherapeutic strategies being used or developed today. New treatment options are illustrating the potential for DC-based immunotherapies to prolong the lives of cancer patients. One such example is Provenge, a biologic therapy the success or failure of many immunotherapeutic strategies being carried out in preclinical studies in mice and clinical trials in cancer patients may benefit from examining the efficacy of DC-based immunotherapies in the presence of obesity and its associated inflammation.

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References


