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14. ABSTRACT Immune-based therapy for solid tumors is a promising area of research, providing the potential for cell-mediated immunotherapies to provide long-lived protection against various stage cancer. Unfortunately, even the most successful clinical trials using T cells or dendritic cells (DC) only show objective response rates in <50% of patients. This is due, in part, to a variety of tumor-derived immunosuppressive mechanisms that arise in cancer patients, rendering antitumor immune responses ineffective. In addition, epidemiological studies have demonstrated that obese individuals face an increased risk of developing cancers, including breast cancer. The reasons for this are likely complex and multi-factorial, but a state of generalized immune suppression may contribute to these findings. Regardless of the body-mass index of the patient, successful long-term treatment of breast cancer must not only reduce the localized tumor burden, but must also target undetected or known metastases that may exist at the time the primary tumor is identified and treated.					
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INTRODUCTION

Breast cancer affects more women than any other single type of cancer. New treatment options for women with metastatic breast cancer are needed, and several types of immunotherapy are currently being investigated as treatment options for advanced or metastatic breast cancer. Unfortunately, even the most successful immunotherapy-based clinical trials only show objective response rates in <50% of patients, which is partially due to a variety of tumor-derived immunosuppressive mechanisms that arise in cancer patients, rendering antitumor immune responses ineffective. In addition, epidemiological studies have demonstrated that obese individuals face an increased risk of developing cancers, including breast cancer. The studies outlined in the funded DOD grant application were designed to test the hypothesis that when compounded by obesity and its associated chronic inflammation, solid tumor outgrowth will lead to the formation of regulatory DC at tumor-distal sites, such as the spleen, which will profoundly affect the generation of TRAIL-induced antitumor immunity. Our interest in the present application to investigate the impact of obesity and chronic inflammation on the therapeutic efficacy of Ad5-TRAIL is quite clinically relevant, and the utilization of a preclinical model that addresses advanced breast cancer (with metastases) targets a potentially increasing number of women in the U.S. that could be facing the issues of obesity-induced co-morbidities associated with breast cancer.

BODY

Statement of Work (as listed in the original proposal)

The *objective of this application* is to understand how obesity and chronic inflammation affect the therapeutic potential of a recombinant adenovirus encoding the cDNA sequence for murine TNF-related apoptosis-inducing ligand (TRAIL; Ad5-TRAIL) in the treatment of metastatic breast cancer.

The majority of the work is taking place at the University of Minnesota, under the direction of Dr. Griffith. Some of the proposed work in Aim 2 (as indicated) is being conducted by Dr. Lyse Norian at the University of Iowa through subcontract. This division of labor was agreed upon by Drs. Griffith and Norian prior to Dr. Griffith's relocation to the University of Minnesota.

Task 1. Determine the extent to which obesity and systemic inflammation affect TRAIL-based immunotherapy in breast tumor-bearing mice

Evaluate the impact of obesity and chronic inflammation on Ad5-TRAIL-based immunotherapy.

1. Analysis of tumor outgrowth after immunotherapy – months 1-12
2. Assess cytokine, chemokine, and adipokine expression as an indication of obesity and chronic inflammation – months 1-12 (serum samples will be taken from the mice used above)

Outcomes and deliverables from this phase of the project: Determine the efficacy of Ad5-TRAIL/CpG therapy in mice with advanced breast cancer (primary tumor and metastases). Determine the extent to which obesity and chronic inflammation alter the therapeutic potential of Ad5-TRAIL/CpG therapy in mice with advanced breast cancer.

Task 2. Identify molecular and functional alterations in DC that arise during tumor outgrowth in obese mice, with particular focus on those properties that would promote tumor outgrowth and metastasis

- A. Assess DC subset phenotype and percentages via multiparameter flow cytometry – months 10-13 (Dr. Norian)
- B. Evaluate shifts in DC stimulatory vs. regulatory function – months 12-16 (Dr. Norian)
- C. Evaluate pro-tumorigenic DC cytokine production via Multiplex – months 16-20
- D. Determine the effects of adipocyte-derived cytokines on DC phenotype and function – months 16-20 (Dr. Norian)

Outcomes and deliverables from this phase of the project: The above tasks test the hypothesis that in obese mice with metastatic breast cancer, both T1DC and systemic DC will undergo changes in stimulatory capacities and cytokine production, resulting in their function as regulatory cells that simultaneously inhibit T cell function and promote tumor metastasis.

Results pertaining to Task 1.

Altered serum cytokine profile in female BALB/c diet-induced obese (DIO) mice

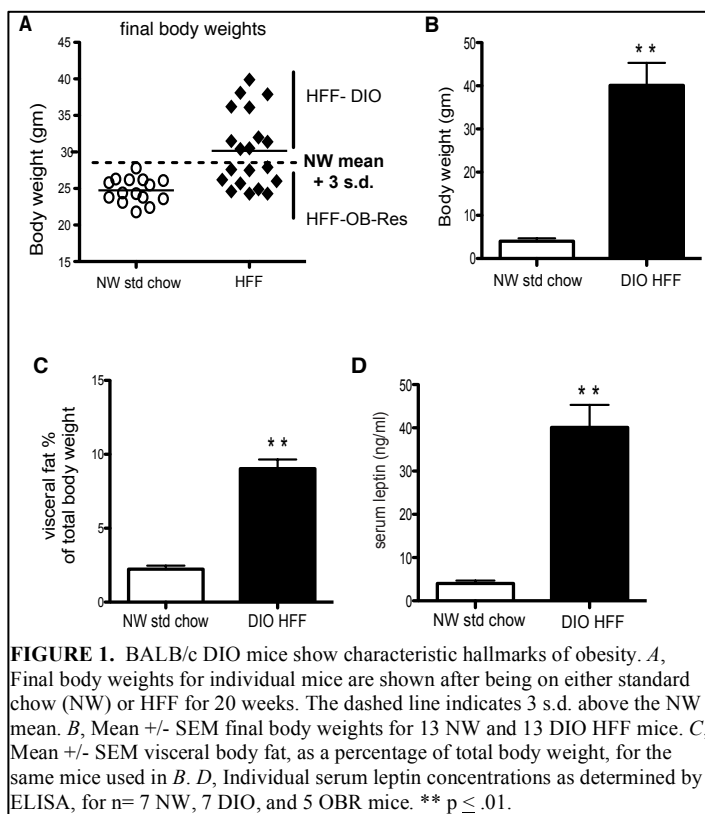
The majority of prior studies on murine DIO used the C57Bl/6 strain, as these mice rapidly become obese after being placed on HFF¹⁻⁶. As our goal was to determine the combined effects of breast tumor outgrowth and DIO on DC function, our experimental model necessitated using the BALB/c strain for challenge with the 4T1 murine breast tumor cell line. We found that BALB/c mice are more resistant to DIO than C57Bl/6 mice, and BALB/c mice placed on HFF for 10 weeks did not develop the systemic inflammation that normally accompanies DIO. Consequently, we modified our protocol so that BALB/c mice were fed HFF for 20 weeks, 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 (“NW” mice) over the same period of time (Fig. 1A). Therefore, we defined DIO mice as those having a final weight greater than the mean weight plus 3 s.d. of age-matched NW mice that had been fed standard chow for 20 weeks. The mean body weights of one cohort of 13 NW and 13 DIO mice is shown in Fig. 1B. Compared to NW mice, DIO mice had increased percentages of visceral body fat and increased concentrations of serum leptin (Fig. 1C and D), both of which are hallmarks of obesity.

We next measured the amount of 35 individual cytokines and chemokines in the serum of NW and DIO mice via multiplex array. Of these, only IL-5 and VEGF were elevated in NW vs DIO serum (Table 1).

Table I. Serum cytokine and chemokine profiles for NW versus DIO mice. Serum was harvested from NW or DIO mice (n= 6 NW, n= 7 DIO) after 20 wks on feed, frozen, and analyzed via MultiPlex array for the above analytes.

Serum Analyte (pg/ml)	NW mean +/- SEM	DIO mean +/- SEM	p < .05
IL-1 α	51.0 +/- 15.6	328 +/- 241.8	*
IL-1 β	1.0 +/- .71	3.4 +/- 1.5	
IL-2	1.5 +/- .71	14.3 +/- 5.6	*

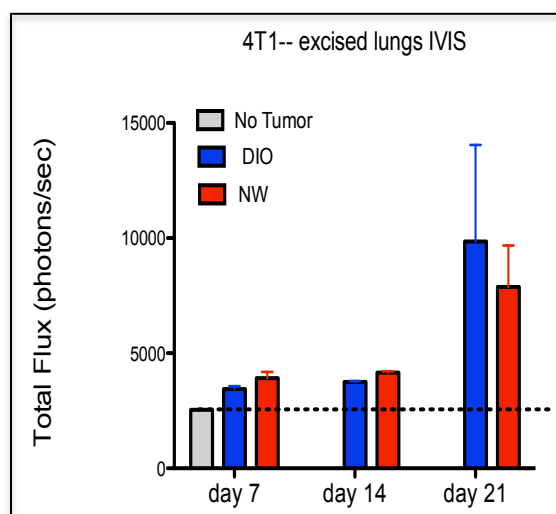
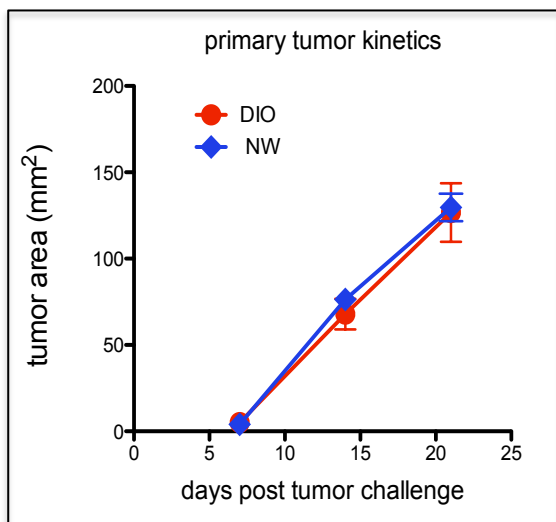


IL-3	6.8 +/- 1.1	19.3 +/- 13.5	
IL-4	6.5 +/- 5.0	10.8 +/- 5.7	
IL-5	41.5 +/- 2.8	23.7 +/- 6.2	*
IL-6	35.0 +/- 7.1	26.0 +/- 7.3	
IL-7	7.5 +/- 2.1	55.8 +/- 11.9	*
IL-10	4.0 +/- .71	15.3 +/- 9.3	
IL-12p40	12.3 +/- .35	27.6 +/- 11.1	*
IL-12p70	4.3 +/- .71	8.6 +/- 6.8	
IL-13	56.0 +/- 11.0	105 +/- 23.7	*
IL-15	28.8 +/- 1.4	98.5 +/- 26.9	*
IL-17	3.0 +/- 1.4	37.1 +/- 15.8	*
eotaxin	13391.8 +/- 612.7	18897 +/- 875.4	*
G-CSF	538.0 +/- 21.2	552 +/- 54.0	
IFN γ	6.0 +/- 4.6	31.5 +/- 7.1	*
IP-10	711.3 +/- 1.06	1726 +/- 510	*
KC	1208 +/- 205.1	1156 +/- 246.5	
LIF	10.0 +/- 2.1	52.9 +/- 13.0	*
LIX	5731 +/- 1289.1	13461 +/- 2038	*
TNF α	2.5 +/- .71	13.5 +/- 9.16	*
VEGF	3011 +/- 4141	184.9 +/- 291	*

In contrast, a large number of analytes showed statistically significant increases in DIO vs NW serum, including IL-1 α , IL-7, IL-15, IL-17, IFN γ , IP-10, LIF, LIX, and TNF α . Together, these data illustrate that placing BALB/c mice on HFF for 20 weeks generates a robust obesity characterized by systemic inflammation.

Efficacy of Ad5-TRAIL/CpG ODN therapy against 4T1 tumors

We first examine the growth of 4T1 tumors in lean and FIO mice to determine if there was any difference in tumor growth in the absence of therapy. In the figures below, obese mice have no changes in primary tumor outgrowth or metastatic lung tumor burdens as measured by calipers (primary tumors) or IVIS Bioluminescent imaging of excised lungs (lung metastases).



Ad5-TRAIL/CpG therapy

eradicates primary and metastatic breast tumors in lean mice. Lean mice were challenged with 4T1.2 cells in the mammary fat pad, followed by injection of PBS or Ad5-TRAIL/CpG (Rx) into the primary tumor site on d 7. When evaluated on d 28, Ad5-TRAIL/CpG therapy significantly reduced primary tumor mass and the number of lung metastases (using 6-thioguanine selection) vs. PBS-treated mice (Fig 2).

Depletion of CD4⁺ or CD8⁺ cells or use of CD8 α DC deficient *Batf3*^{-/-} mice significantly increased primary tumor mass and number of lung metastases, suggesting Ad5-TRAIL/ CpG therapy induces a systemic anti-tumor T cell response.

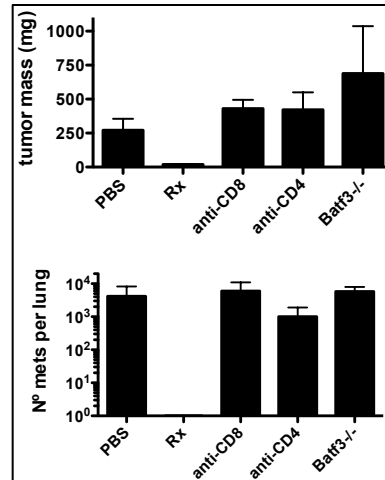


Fig. 2. Eradication of primary and metastatic 4T1.2 tumors after Ad5-TRAIL/CpG therapy. 4T1.2 cells were injected into the mammary fat pad, followed by PBS or Ad5-TR/CpG (Rx) on d 7. Some groups of Rx mice were depleted of CD4, CD8, pDC, or CD8 α DC (*Batf3*^{-/-}). Primary tumor mass and number of lung metastases were determined on d 28 (5 mice/group).

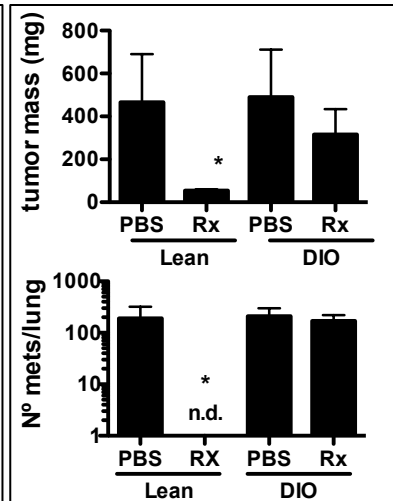


Fig. 3. Ad5-TRAIL/CpG therapy fails to control 4T1 tumor growth in DIO mice. 4T1.2 cells were injected into the mammary fat pad of lean or DIO mice, followed by PBS or Ad5-TRAIL/CpG (Rx) on d 7. Primary tumor mass and number of lung metastases were determined on d 28 for 5-8 mice/group. * $p < 0.05$; n.d. – none detected

Decreased efficacy of Ad5-TRAIL/CpG therapy in DIO mice. Genetically obese and DIO mice have impaired DC function. Given that DC are required for Ad5-TRAIL/CpG efficacy, we posited Ad5-TRAIL/CpG-treated DIO mice would have a reduced ability to clear 4T1 tumors. By d 28, both PBS-treated lean and DIO mice had similarly large tumor burdens, and Ad5-TRAIL/ CpG-treated lean mice had significantly reduced tumor burden (Fig 3). However, there was no decrease in tumor burden in Ad5-TRAIL/CpG-treated DIO mice relative to PBS-treated DIO mice.

Expanded populations of MDSC can be detected in the blood, liver, spleen, and tumor in mice. While MDSC in the spleen and blood of tumor-bearing mice are frequently studied, recent data suggest MDSC acquire their

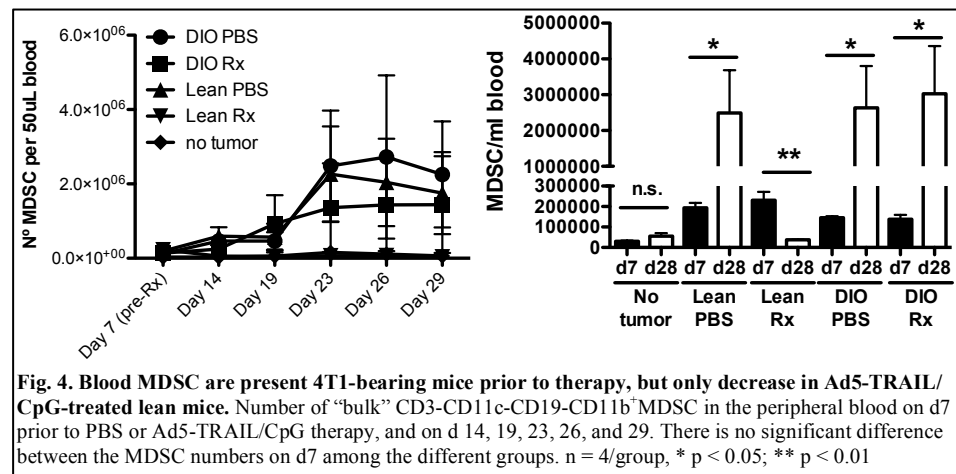


Fig. 4. Blood MDSC are present 4T1-bearing mice prior to therapy, but only decrease in Ad5-TRAIL/CpG-treated lean mice. Number of “bulk” CD3⁺CD11c⁻CD19⁻CD11b⁺MDSC in the peripheral blood on d7 prior to PBS or Ad5-TRAIL/CpG therapy, and on d 14, 19, 23, 26, and 29. There is no significant difference between the MDSC numbers on d7 among the different groups. n = 4/group, * $p < 0.05$; ** $p < 0.01$

suppressive function only after exposure to factors in the tumor microenvironment. One characteristic of the 4T1 model is the rapid and robust accumulation of MDSC that are readily detectable in peripheral lymphoid tissues. When we examined MDSC in our 4T1.2 model, we saw similar CD3⁺CD11c⁻CD19⁻CD11b⁺ MDSC numbers in the peripheral blood of the different groups of mice prior to PBS or Ad5-TRAIL/CpG therapy (d 7; Fig 4). MDSC numbers only decreased in the Ad5-TRAIL/CpG lean mice when measured on d 28.

Conclusions from Aim 1 experiments

1. 4T1 tumor growth is similar in lean and DIO mice, suggesting that the complications from obesity do not alter tumor growth (primary tumor and lung metastases).
2. Ad5-TRAIL/CpG therapy is effective in controlling 4T1 tumor outgrowth in lean mice, and this is dependent on several cells of the adaptive immune system – CD8 T cells, CD4 T cells, and CD8 α DC.
3. Tracking MDSC in the peripheral blood can be used as a surrogate marker of tumor burden.

Results pertaining to Task 2 – the following data was generated by Dr. Norian as part of the subcontract.

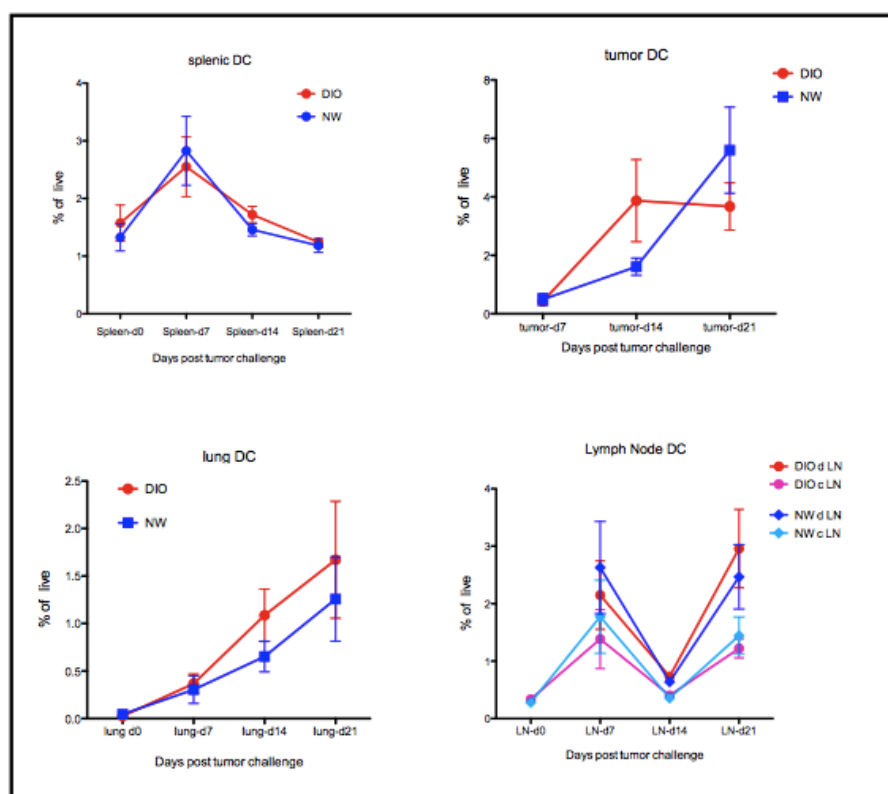
Aim 2. Identify molecular and functional alterations in DC that arise during tumor outgrowth in obese mice, with particular focus on those properties that would promote tumor outgrowth and metastasis. Original experimental rationale: DC maturation and function is strongly influenced by local cytokine and stromal cell composition. As visceral adipose tissue increases in obese mice and obesity triggers increased production of adipokines like IL-6 and leptin that can impact immune responses, it is likely that mammary DC function in DIO mice will be altered, even in the absence of tumor growth. In support of this idea, published studies in tumor-free mice have shown that obesity alters DC migration and stimulatory capacity^{5,7}.

Preliminary Studies: Evaluation of 4T1 tumor outgrowth kinetics and spontaneous lung metastases in obese versus lean mice.

A. Assess DC subset phenotype and percentages via multiparameter flow cytometry.

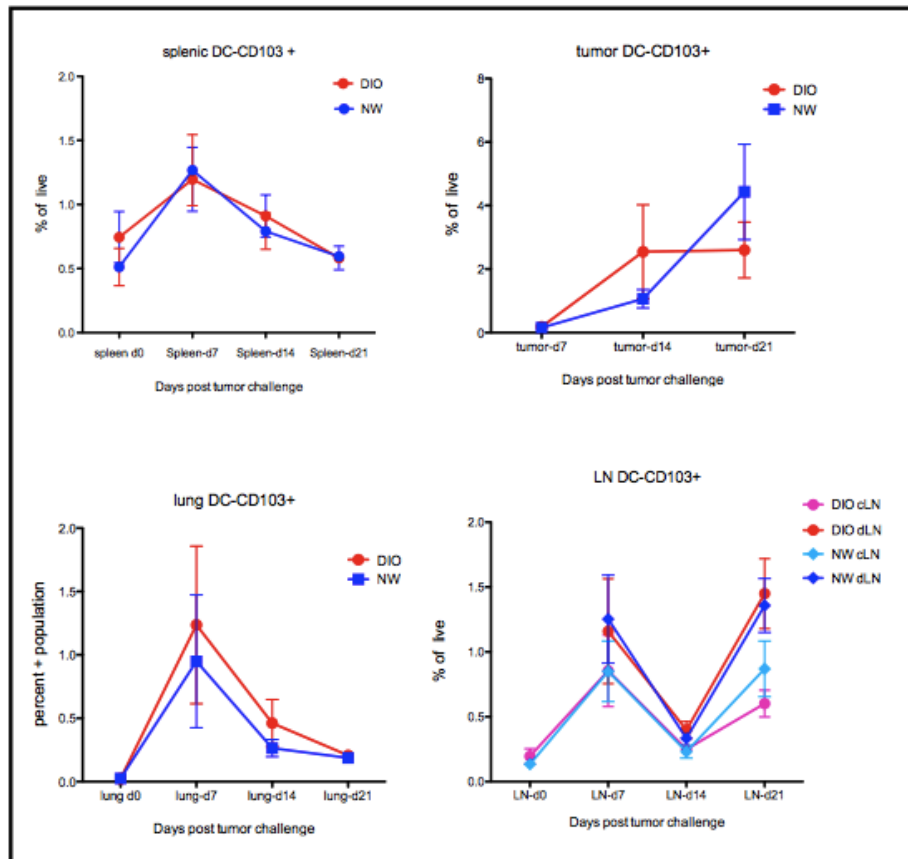
A series of experiments were performed to determine whether obesity was associated with changes in the percentages and/or phenotypes of DC in mice with 4T1 tumors in the absence of any therapy. Diet-induced obese mice were generated by feeding cohorts of animals high fat diet (RD #12492) for 20 weeks. Age-matched control mice were fed standard rodent chow for the same 20 weeks. When ready for use, mice were challenged with 4T1 tumors into mammary gland #9. Tumor-free mice were used as controls, and are indicated as “day 0” time points on the included graphs. Tumor-bearing mice were harvested on days 7, 14, 21 post-tumor challenge. DC subpopulations and percentages were analyzed in tumors, tumor-draining lymph nodes, contralateral lymph nodes, spleens, and lungs.

As shown below (pooled data from all experiments), obesity produced no changes in the percentages of DC present in the spleen, lung, tumor or LNs. DC percentages in the spleen peaked at day 7 post-tumor challenge and at day 21 in the lungs. In tumors from DIO mice, the

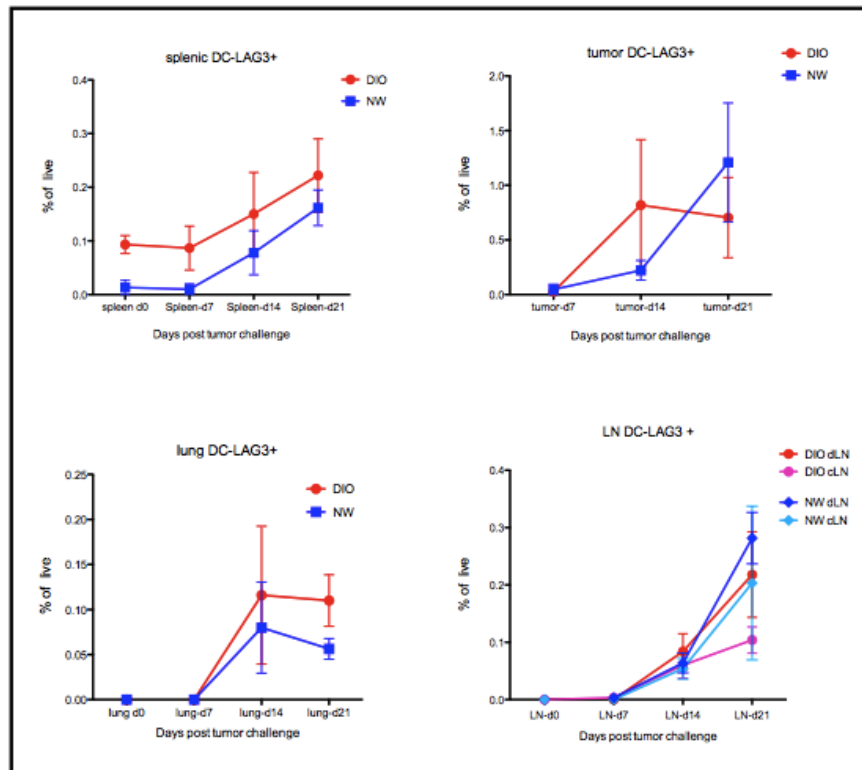


percentages of DCs present plateaued at day 14 post-tumor challenge, whereas in NW (normal weight) mice, the percentages of DC continued to increase through day 21. Given the large amount of variability in raw numbers between individual mice and individual experiments, there were no statistical differences in DC percentages in tumors from obese and lean mice. Lymph Node (LN) staining showed that DC frequency was bimodal, with equivalent peak values being present at days 7 and 21. In all cases, higher percentages of DC were found in tumor-draining LN (dLN) than in contralateral, non-draining LN (cLN).

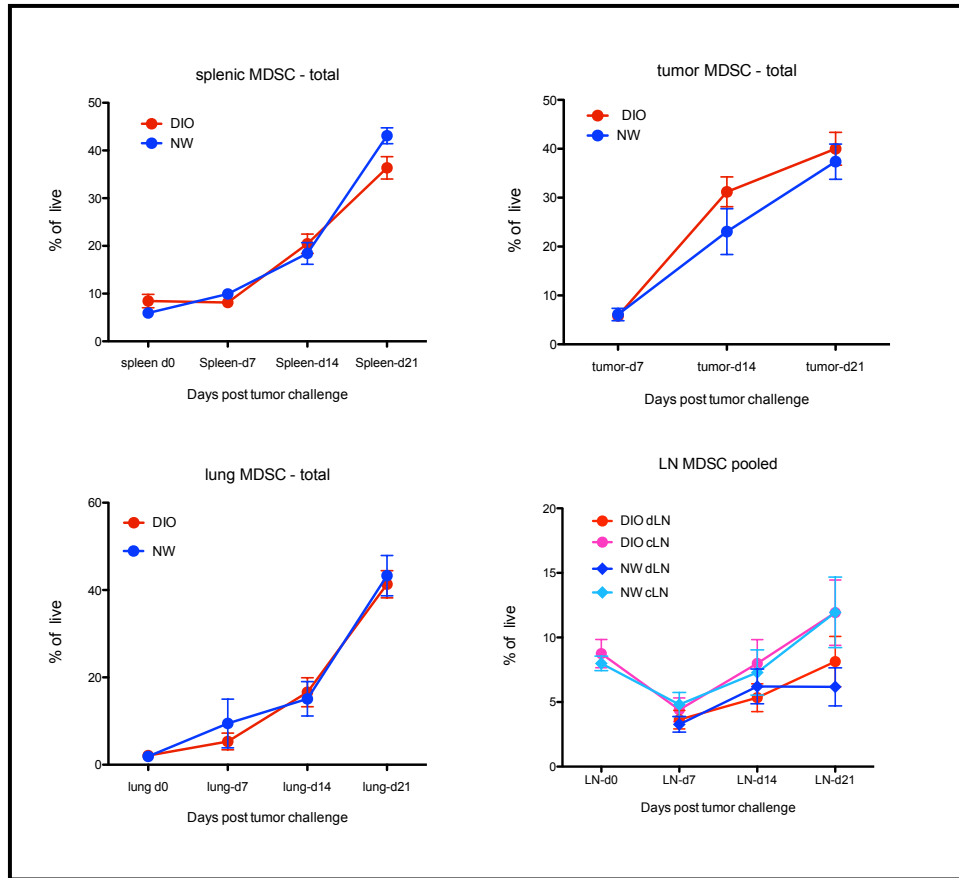
Our examination of DC phenotypes showed that obesity induced no changes in the relative frequencies of any marker examined (graph below). The percentages of CD11b+, CD103+, and PD1+ DC were unchanged in obese mice relative to lean mice.



In tumor-free mice, obese animals had lower percentages of LAG3+ DC than did lean mice, but the percentages of each were less than 0.1%, so this difference was negligible. With tumor growth, the percentages of LAG3+ DC became less pronounced (graph below).



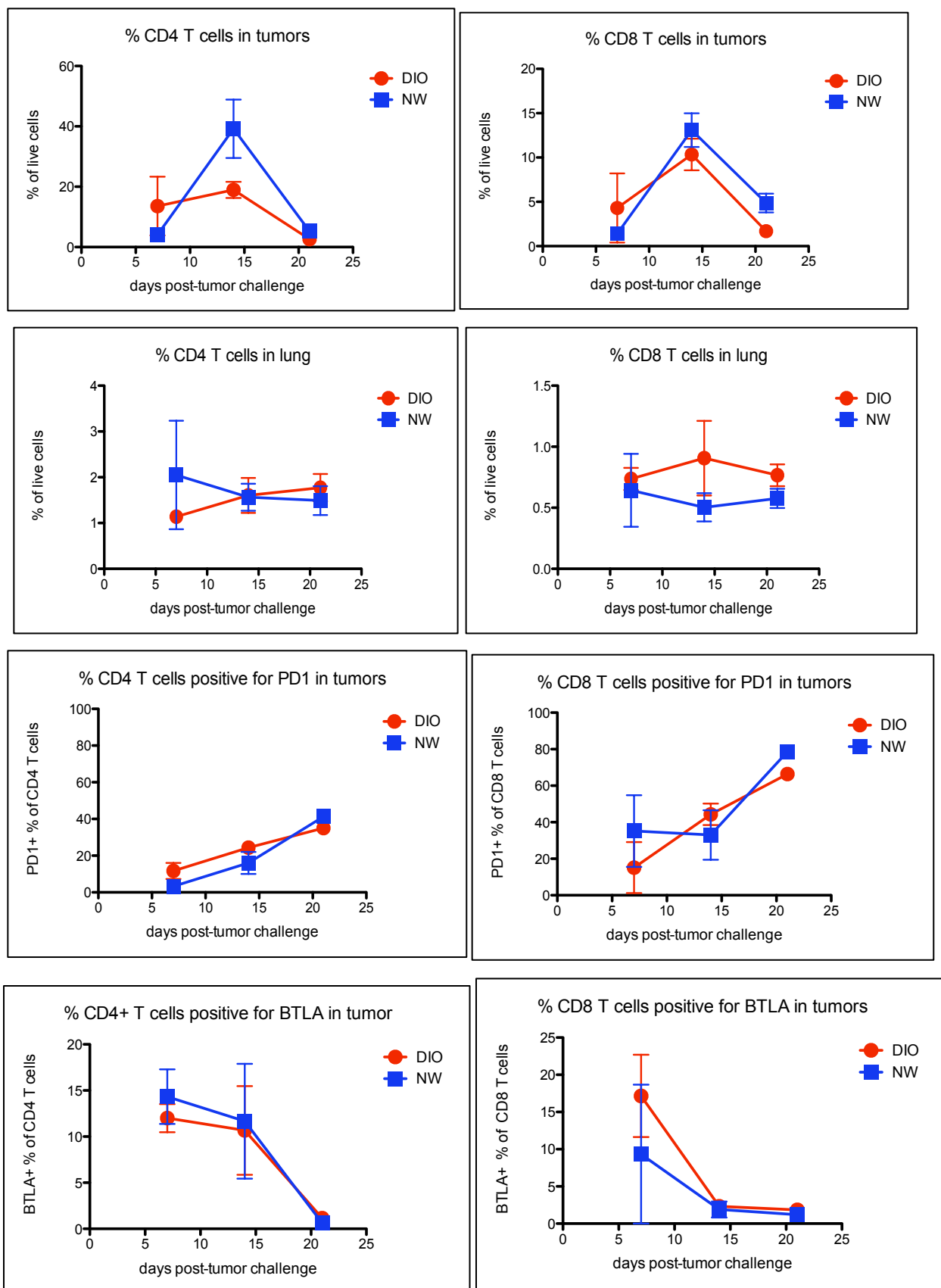
During our examination of DC percentages, we also examined the relative abundance of myeloid-derived suppressor cells (MDSC) in obese and lean mice, as this cell population is known to inhibit T cell-mediated tumor immunity. MDSC are comprised of two subpopulations, the LY6C⁺ monocytic MDSC, and the LY6G⁺ granulocytic MDSC. As shown below, we found no differences in the total percentages of MDSC in the spleen, tumor or lung (LNs-not shown) in obese versus lean mice. In the spleen, tumor, and lung MDSC percentages increased steadily from a low in tumor-free mice through day 21 post-tumor challenge. There were no differences observed in the relative abundance of LY6C⁺ and LY6G⁺ subtypes (not shown).

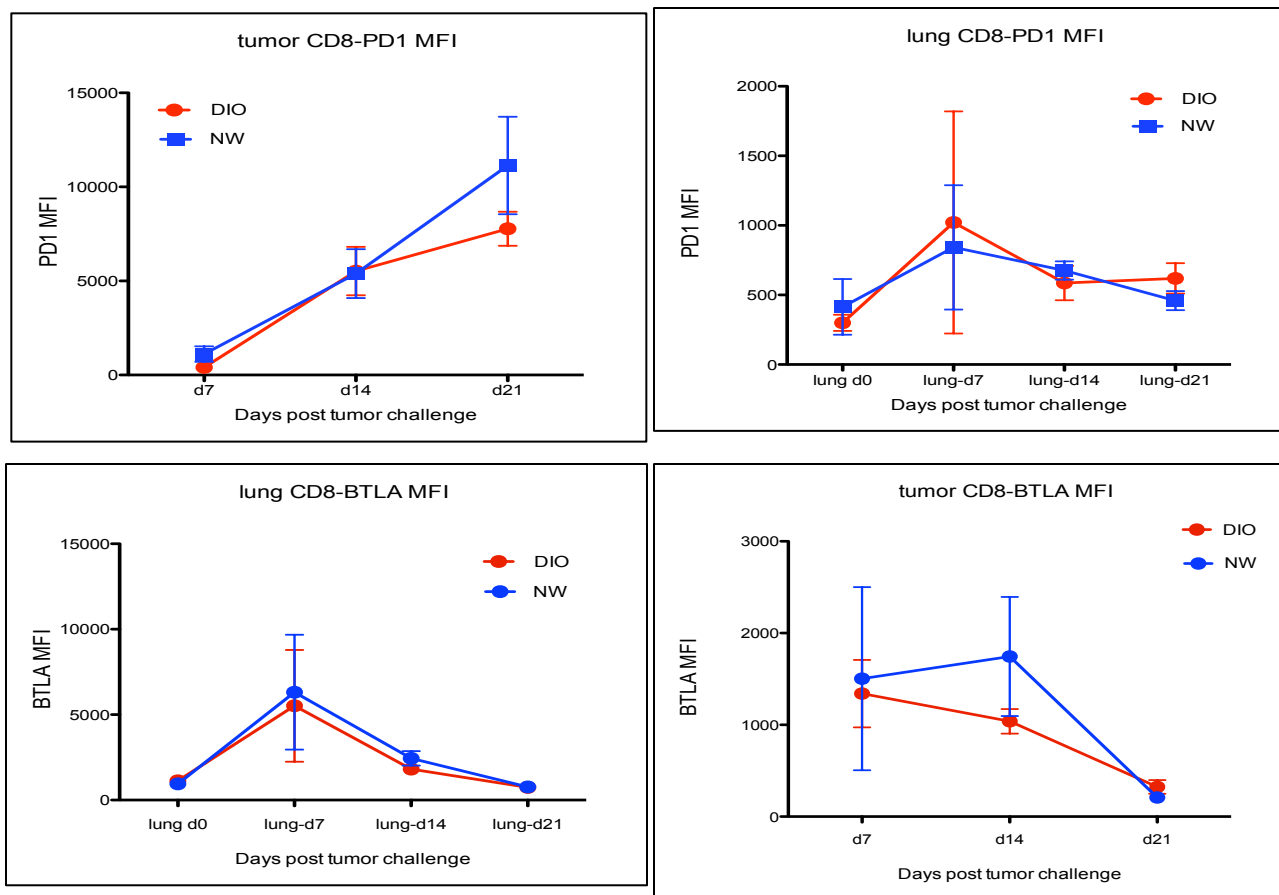


B. Evaluate shifts in DC stimulatory vs regulatory function.

DC are the primary stimulators of naive T cell activation *in vivo*. Therefore, if DC in obese mice with 4T1 tumors had a decreased stimulatory capacity or an increased suppressive function, this would have resulted in decreased percentages of CD4⁺ or CD8⁺ T cells in tumor-bearing mice.

However, we found no striking evidence that the percentages or phenotypes (using markers of activation or exhaustion) of T cells were different in DIO versus NW mice as 4T1 tumor outgrowth progressed. With the exception of decreased CD4⁺ T cell percentages in primary tumors at day 14 post-tumor challenge, there were no measurable differences in the T cell response, for either the CD4⁺ or CD8⁺ T cell subset. These data indicate that DC (and other antigen presenting cell) stimulatory capacity was unaltered by obesity in the presence of a 4T1 challenge.





C. Evaluate pro-tumorigenic DC cytokine production via Multiplex.

These experiments were not performed due to financial constraints. Additionally, due to the failure of any other experiments in this Aim to show reproducible, statistical differences between DIO and NW mice, we felt it was unlikely that this set of experiments would yield meaningful results.

D. Determine the effects of adipocyte-derived cytokines on DC phenotype and function.

Visceral adipocytes undergo functional changes with the onset of obesity, and increase production of pro-inflammatory cytokines such as leptin and IL-6. The consequences of high local levels of adipocyte-derived cytokines on immune cell function are unknown. DC maturation and migration are greatly influenced by cytokines. For example, *in vitro* differentiation of DC precursors in the presence of IL-10 creates tolerogenic DC that secrete VEGF, and DC matured in the presence of TNF are tolerogenic and promote tumor metastasis. As the amount of visceral adipose tissue is increased in obese mice, high local levels of adipokines will likely alter DC migration and maturation.

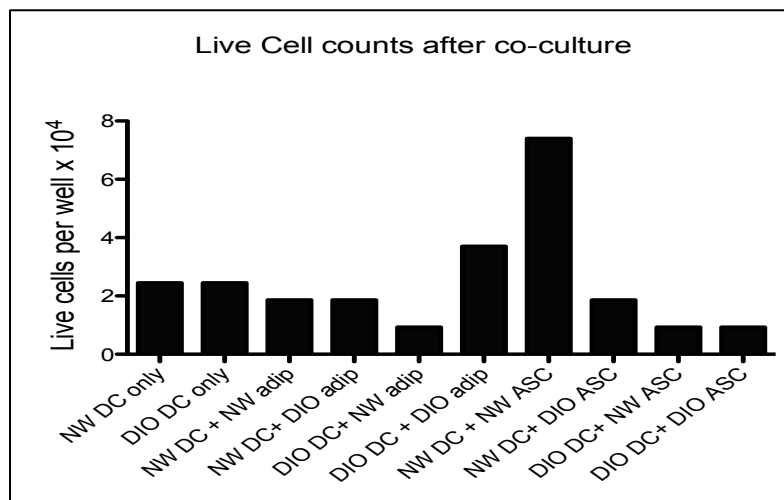
We performed a series of experiments in which DC (defined as CD11c⁺/ I-Ad⁺) from 3-5 pooled DIO or NW mice were sort-purified and placed into culture with adipocytes or adipose stromal cells (ASC) isolated from the fat pads of either DIO or NW mice. For these experiments, purified DC were placed in the lower chamber of transwell plates, and equal numbers of enriched adipocytes or ASCs were placed into the upper chamber. DCs were cultured in this manner for 48 hours. At this time, DC from the lower chamber were harvested, counted, and stained for

phenotyping.

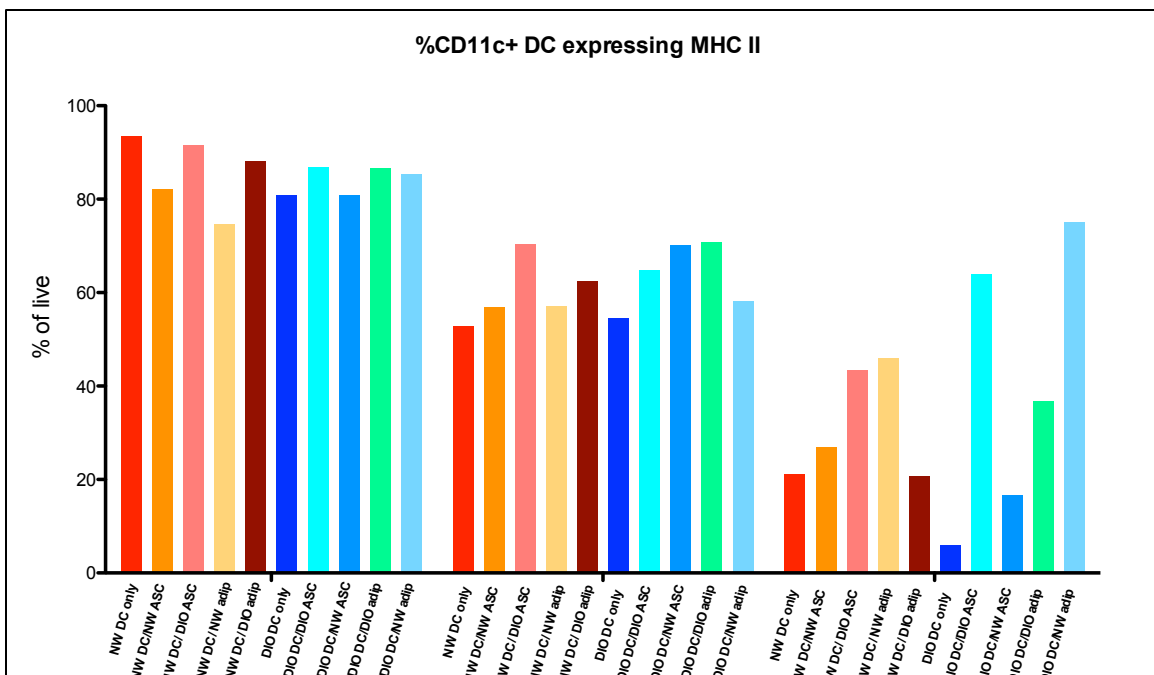
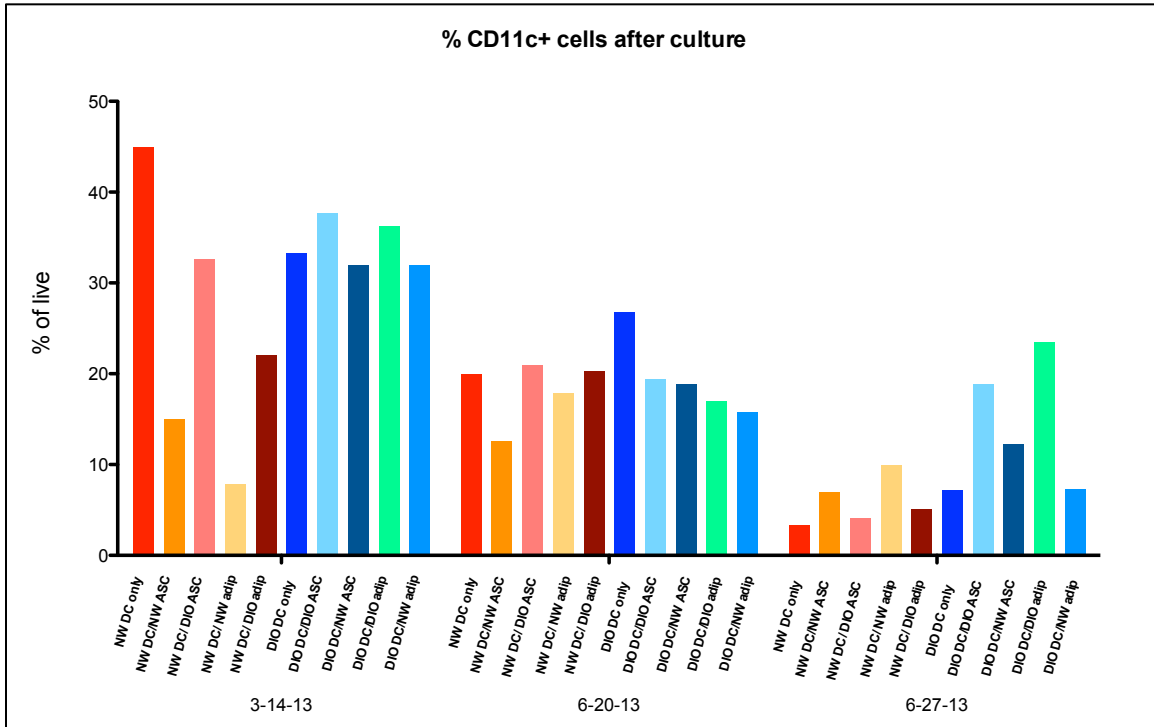
Example experimental setup:	NW DC	DIO DC
Cultured alone	X	X
+ NW ASC	X	X
+ DIO ASC	X	X
+ NW adipocytes	X	X
+ DIO adipocytes	X	X

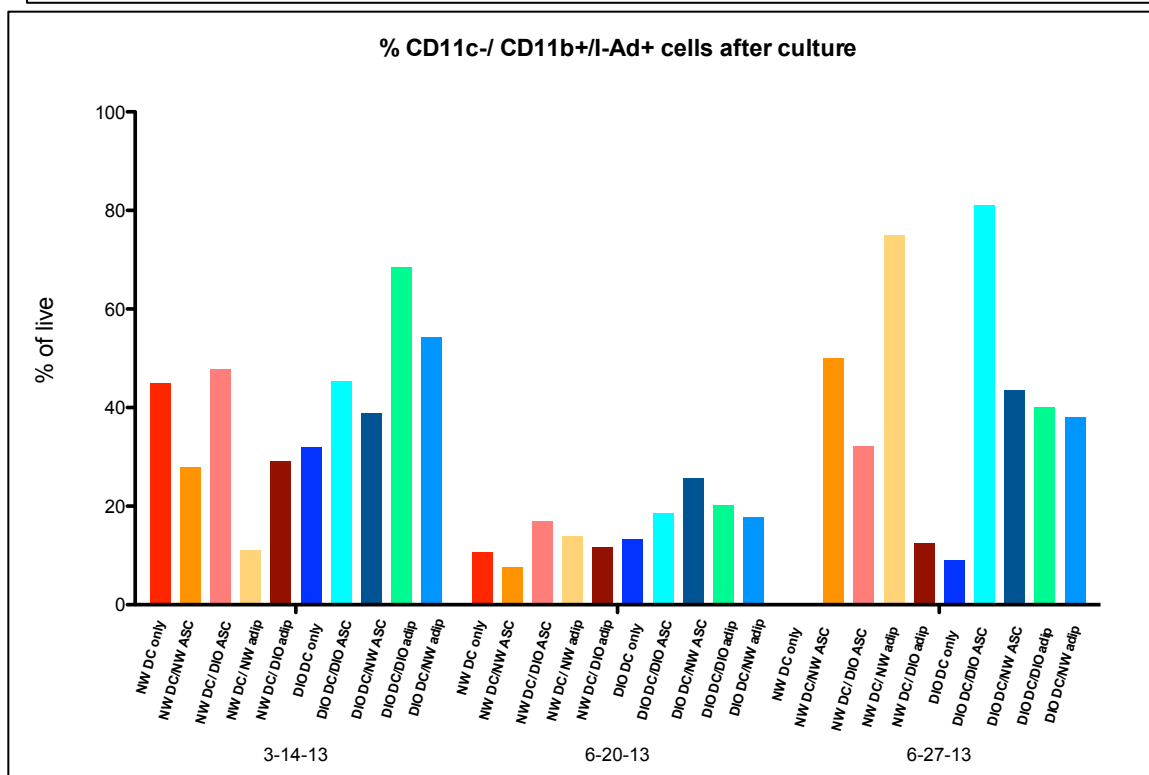
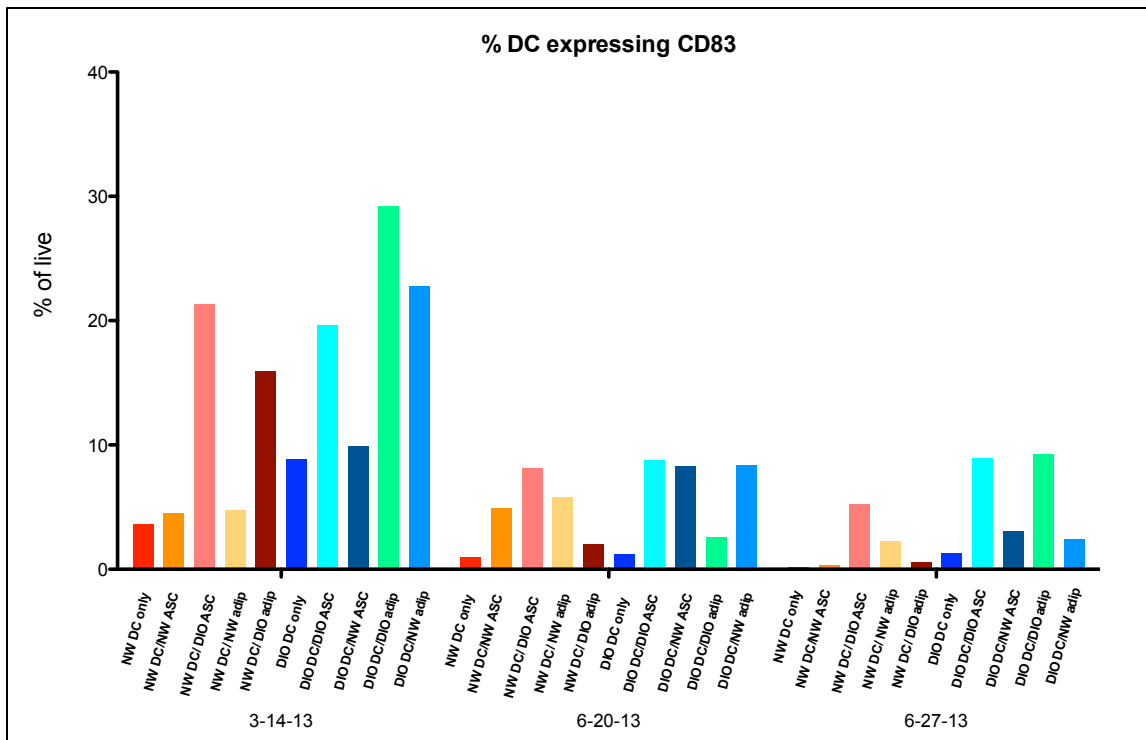
Several practice experiments were run to ensure the DC sort-purification, adipocyte, and ASC protocols were working properly.

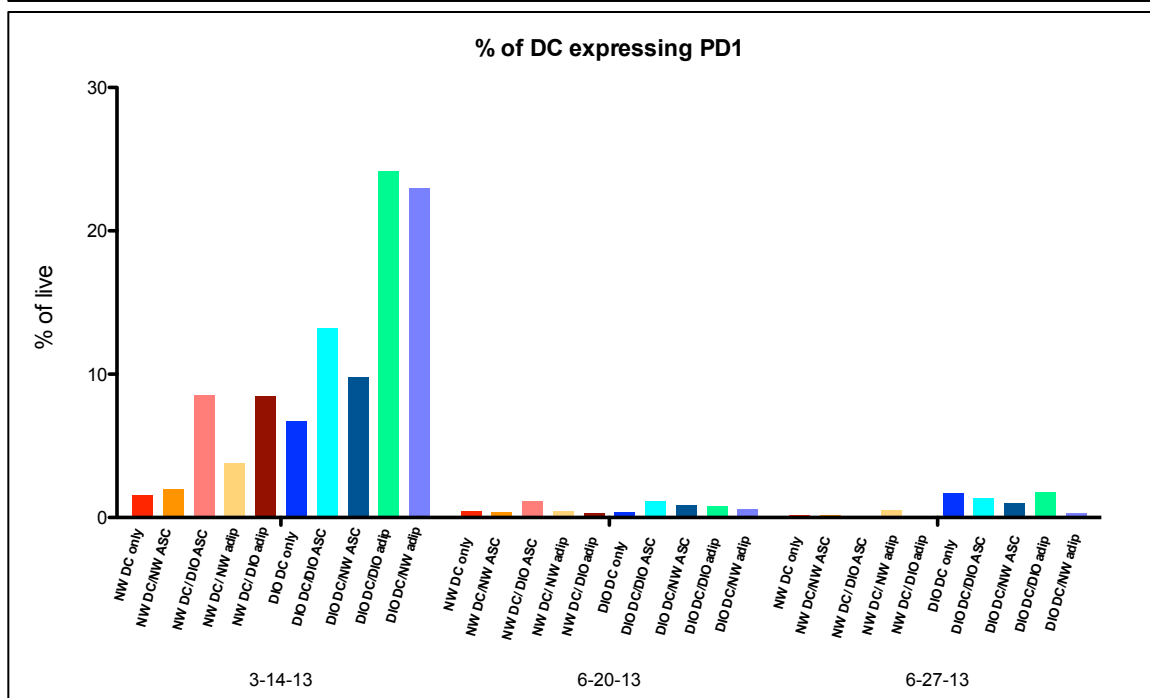
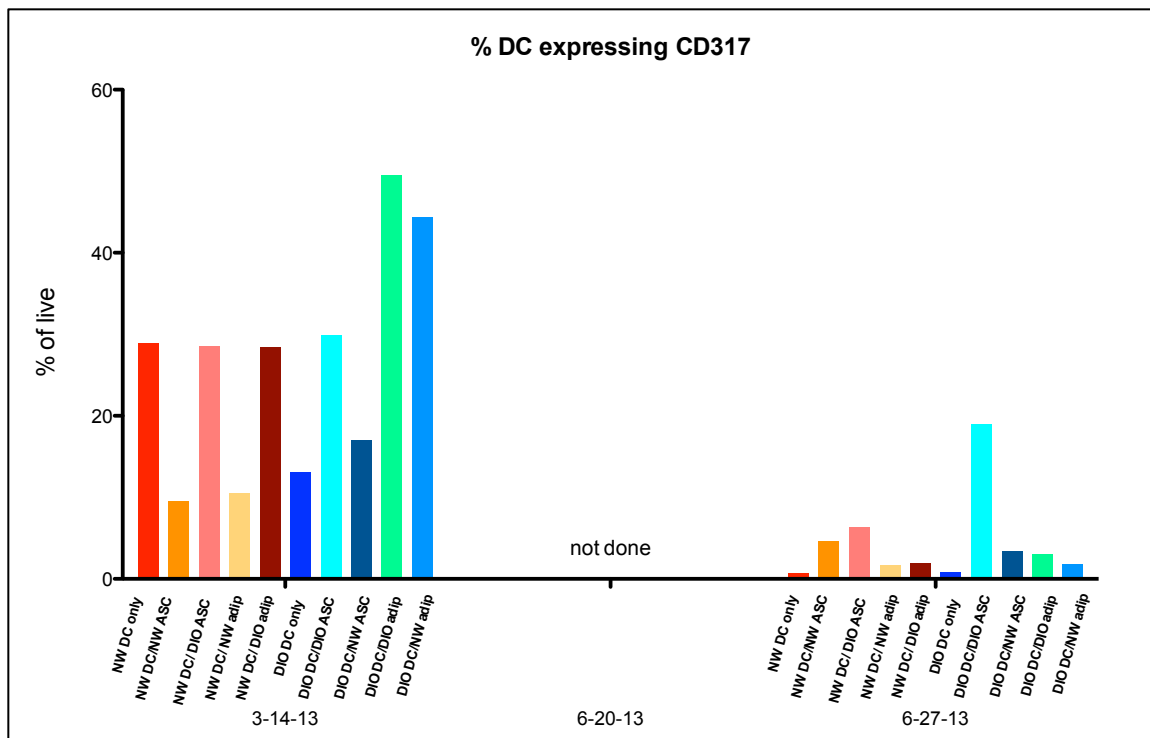
We asked whether DC percentage, number or phenotype were differentially altered by a 48-hour culture with adipocytes or ASC from either NW or DIO mice. Due to experiment-to-experiment variation in cell recovery, results from one experiment are shown below. In this experiment, it appeared that DIO and NW DC had similar viability when cultured alone. NW DC cultured with either NW or DIO adipocytes had similar viability. DIO DC cultured with DIO adipocytes had increased viability as compared to culture with NW adipocytes. Cell recovery was highest for NW DC cultured with NW ASC, and this was increased over NW DC culture with DIO ASC. DIO DC cultured with either NW or DIO ASC had equivalent cell recovery.



To allow for comparison between experiments, and to illustrate the large amount of variability observed in these experiments, the figures that follow show data from three individual experiments (dates provided, below). As is obvious from these figures, we found no reproducible trends in DC survival or phenotype after culturing with DIO or NW adipocytes or ASC.







Conclusions from Aim 2 experiments:

1. Primary tumor outgrowth and metastasis are equivalent in DIO and NW BALB/c mice after 20 weeks on high fat diet.
2. The combination of obesity and 4T1 tumor challenge has minimal impact on the DC response in primary tumors or lungs, the site of spontaneous metastases in this model. There is also minimal impact on DC responses in the spleen or tumor-draining lymph node.
3. A simultaneous evaluation showed little to no impact of obesity and combined tumor growth on MDSC accumulation in spleens, tumors, lungs, or lymph nodes.
4. The combination of obesity and tumor growth does not appear to impact DC function *in vivo*, as evidenced by largely equivalent CD4 and CD8 T cell responses (percentages over time and phenotype).
5. Obesity does not appear to affect the ability of adipocytes or ASC to influence DC viability or phenotype during *in vitro* co-culture for 48 hours. Both adipocytes and ASC had minimal reproducible effects on DC viability or phenotype during co-culture. The large amount of variability in results precluded us from making generalizable conclusions about the effects of adipocyte- or ASC-derived soluble factors on DC function.

Key Research Accomplishments

- Establishment of diet-induced obesity model
- Tumor outgrowth is not altered in lean vs. obese mice
- 4T1 tumor progression is suppressed in lean mice after Ad5-TRAIL/CpG therapy, but not in obese mice

Conclusions

Our data suggests Ad5-TRAIL/CpG therapy is an effective treatment against the 4T1 breast tumor model in lean mice. In contrast, this therapy is ineffective in obese mice. While, preliminary data suggested DC function was impaired in DIO mice compared to lean (NW) mice, subsequent studies could not identify a mechanism for the difference. Regardless, the demonstration that the therapy was effective in lean tumor-bearing mice and not obese mice has significant implication with regard to the potential success of tumor immunotherapy. This observation has the potential to significantly impact the future development of immunotherapy protocols for breast cancer that would be used in obese patients. Understanding the defects in the DIO setting will be essential in identifying additional agents to the immunotherapy protocol to overcome the immune system defects present in the face of obesity.

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