Adiposity induces lethal cytokine storm after systemic administration of stimulatory immunotherapy regimens in aged mice

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Aging is a contributing factor in cancer occurrence. We recently demonstrated that systemic immunotherapy (IT) administration in aged, but not young, mice resulted in induction of rapid and lethal cytokine storm. We found that aging was accompanied by increases in visceral fat similar to that seen in young obese (ob/ob or diet-induced obese [DIO]) mice. Yet, the effects of aging on inflammatory responses to immunotherapeutics are not well defined. We determine the effects of adiposity on systemic IT tolerance in aged compared with young obese mice. Both young ob/ob- and DIO-generated proinflammatory cytokine levels and organ pathologies are comparable to those in aged ad libitum mice after IT, culminating in lethality. Young obese mice exhibited greater ratios of M1/M2 macrophages within the peritoneal and visceral adipose tissues and higher percentages of TNFα macrophages in response to αCD40/IL-2 as compared with young lean mice. Macrophage depletion or TNF blockade in conjunction with αCD40/IL-2 prevented cytokine storms in young obese mice and protected from lethality. Calorie-restricted aged mice contain less visceral fat and displayed reduced cytokine levels, protection from organ pathology, and protection from lethality upon αCD40/IL-2 administration. Our data demonstrate that adiposity is a critical factor in the age-associated pathological responses to systemic anti-cancer IT.

The use of immunotherapy (IT) in cancer has recently resulted in impressive responses. Yet, the usages of IT regimens, especially those involving cytokine therapies, have also resulted in the induction of severe systemic toxicities often not previously characterized in their preclinical animal studies. Because such toxicities often require intensive care management of patients, the causes for the discrepancies in observations between clinical and preclinical toxicity merit further study.

Cancer has been considered a disease of aging, with persons over 65 accounting for over 60% of newly diagnosed malignancies (Balducci and Ershler, 2005). Consequences of aging include gradual decreases in immunological function, thymic involution leading to decreased naive T cell output, restriction within T cell repertoire...
diversity, increases in genomic instability, and increases in cellular senescence (Campisi, 2013). However, despite the majority of patients being of this demographic, the overwhelming majority of preclinical studies are performed in young inbred mice. We recently demonstrated that treatment of young mice with a combination IT regimen consisting of agonistic monoclonal antibody to CD40 (αCD40) given in conjunction with IL–2 resulted in synergistic anti-tumor effects and was well tolerated (Murphy et al., 2003). Yet, administration of the same regimen in an aged cohort resulted in 100% lethality within two days of treatment (Bouchlaka et al., 2013). Importantly, the mortality in the aged was perpetuated by an aberrant cytokine storm mirroring a systemic inflammatory response syndrome (SIRS), which resulted in multi-organ damage (Bouchlaka et al., 2013). These systemic toxicities were closely representative of those previously noted clinically with FDA-approved immunostimulatory IT therapeutics such as high-dose IL–2 and IFN-α (Lee and Margolin, 2011).

Aging is associated with a gradual decline in immunological function but is also accompanied by the coincidence of a systemic, chronic, and low-grade proinflammatory response termed inflammaging that leads to increased systemic cytokine levels, namely IL–1β, IL–6, and TNF (Franceschi, 2007). Emerging data suggests that inflammaging may be influenced by age-associated changes in body mass composition such as decreased lean muscle mass, increased adiposity, and increases in overall body mass index (BMI; Franceschi et al., 2000). Whether adiposity plays a role in the generation of systemic toxicities or whether it is primarily an age-associated occurrence remains unknown.

Like aging, hallmark to the obese microenvironment is the development of a low-grade chronic "meta-inflammatory" state. Excess adiposity is associated with increased infiltration of macrophages and proinflammatory mediators such as dendritic cells, NK cells, and T cells into fat depots that ultimately results in environmental remodeling. Consequences include increased circulating levels of C-reactive protein (CRP), IL–6, leptin, and TNF (Ronti et al., 2006; Vázquez-Vela et al., 2008). Importantly, the immunomodulatory effects of increased adipose tissue extends through the ability for both adipocytes and the stromal vascular fraction to express a variety of immunologically important surface receptors, such as the leptin receptor, IL–6 receptor, and both p55 and p75 TNF receptors (Ailhaud, 2000). Systemic consequences of increased adiposity are also seen at distance sites through premature induction of aging-associated changes such as thymic involution, which leads to the restriction of naive T cell output and ultimately restriction of the T cell repertoire (Yang et al., 2009).

Collectively, the field of IT has made great progress in the last decade toward the development of therapeutic candidates against cancer. Yet, these candidates have been met in the clinic with the induction of severe, often limiting, systemic toxicities that hinder their usage. Additionally, given the rise of obesity within society, as well as the estimation that cancer is a disease primarily of the aged, using lean and young models may not accurately reflect patient outcomes. Therefore, this study seeks to establish the consequences of increased adipose tissue accumulation throughout aging upon IT-induced toxicities. Here, we find that adiposity results in a skewing toward increased levels of proinflammatory M1 macrophages within the peritoneal cavity and visceral adipose tissues that ultimately result in heightened production of proinflammatory cytokines, namely TNF, during strong immune stimulation with IT. Importantly, aged ad libitum (AL)-fed mice and young obese mice succumb to TNF-mediated pathological responses culminating in rapid lethality, which are prevented through caloric restriction in the aged or through either macrophage depletion or TNF blockade.

RESULTS

IT administration in the aged results in rapid lethal cytokine storm and is associated with increased accumulation of visceral adipose tissue

The combination IT regimen consisting of αCD40 and IL–2 (αCD40/IL–2) has previously shown synergistic anti-tumor effects resulting in the regression of metastatic tumors in young (<6 mo) mouse models (Murphy et al., 2003). Recently, we demonstrated that administration of the therapeutic dose (high dose [HD]) of αCD40/IL–2 into aged mice (≥15 mo) resulted in a heightened cytokine storm that prompted multi-organ damage and resulted in 100% lethality by day 2 of treatment (Bouchlaka et al., 2013). To address whether the increased lethality was specific to this therapeutic dosage in the aged, we administered αCD40/IL–2 at a lower dose (LD), less than half, into aged AL-fed mice. Consistent with our previous findings, treatment of aged mice with LD IT resulted in 100% mortality by day 2 of treatment (Fig. 1 A) and demonstrated increased serum levels of proinflammatory cytokines TNF, IL–6, and IFN–γ, indicative of a cytokine storm (Fig. 1 B). In addition, to ensure that the lethality observed in the aged mice was not IT regimen–specific, we administered an IT combination of CpG and IL–2 (CpG/IL–2) into young WT and aged AL mice. Confirming our previous findings, aged mice treated with CpG/IL–2 therapy incurred a similar cytokine storm that culminated in rapid lethality by day 3 (Fig. 1, C–E), in contrast to young mice which exhibited lower levels of cytokines and demonstrated complete treatment tolerance (Fig. 1, C–E).

Normal aging is inherently associated with changes in body mass composition—specifically with decreases in muscle mass and increases in intra-abdominal visceral adiposity (Harris, 2002). Equally, within our animal studies an observable difference between aged and young mice is noted in overall size, in particular increased waist circumference (Fig. 1 F). On average, aged mice weigh twice as much as young (Fig. 1 G). To determine whether the difference in size is due to increases in fat deposits, we sought to quantify differences in body mass and the presence of visceral adiposity. Young (<6 mo) and aged (≥15 mo) C57BL/6 mice were analyzed through magnetic resonance imaging (MRI) to quantify the presence of visceral adiposity. Aged mice exhibited markedly increased body mass indices and total visceral fat volumes in contrast with young
were similar to young mice (Fig. 1, G–I). MRI scans of young (4 mo), middle-aged (9 mo), and aged (15 mo) mice that were either AL-fed or CR throughout life showed significant differences in adipose accumulation over time (Fig. 1 I). We found that with increasing age, AL-fed mice have a proportional increase in body fat content, whereas mice placed on a CR diet lack increased fat accumulation regardless of age. Aged CR mice show no significant difference in fat volume ratios to their middle-age and young counterparts, and remain similar to young AL mice (Fig. 1 I).

Caloric restriction in aged mice results in decreased accumulation of adiposity and protection from IT-induced toxicity

To examine the effect of adiposity and aging on the development of systemic toxicities after IT, aged AL, aged CR, and young AL mice were administered IT or a control therapy.
Obesity induces immunostimulatory therapy toxicity | Mirsoian et al.

...however, 100% of aged CR mice placed on the HD amount of IT demonstrated complete protection from \( \alpha_{CD40/IL-2} \)-mediated lethality (Fig. 2 G). Collectively, these data suggest that increased body fat plays a critical role in the induction of systemic toxicities by IT. Increased adiposity played a critical role in the age-associated cytokine storm and subsequent organ pathologies after IT and calorie restriction diminished the development of such toxicities conferring a protective effect that allowed for increased survival in the aged.

Increased adipose tissue accumulation through obesity, in the absence of age, results in IT-induced lethal cytokine storm

To further dissect the role of adipose tissue in the IT-associated inflammatory responses seen in aged mice, we next sought to determine the impact of adiposity, in the absence of age, as a possible cofactor. Young \( \text{ob/ob} \) mice (B6.Cg-Lep\(^{ob/ob}\)) are leptin-deficient and therefore start exhibiting obesity by 3 wk of age. In comparison to age-matched young WT (2 mo) control mice placed on an AL diet (young AL), \( \text{ob/ob} \) mice have significantly higher body masses and weigh, on average, \( \sim 2.5 \times \) greater (Fig. 3 A). MRI analysis of body fat content demonstrated (rIgG/PBS). IT resulted in increased lymphocytic infiltrates to the liver of aged CR and aged AL mice, in contrast to age-matched control-treated mice (Fig. 2, A–C). To assess whether IT resulted in organ damage, livers and intestines were collected on day 2 of treatment, the day of mortality in aged AL mice, and assessed for pathological changes. Importantly, livers and intestines from aged CR mice demonstrated significantly less inflammation and less necrosis in comparison with aged AL mice (Fig. 2, A and C). Serum analysis of alanine aminotransferase (ALT) enzyme levels, a correlate of liver damage, showed a significant reduction in ALT levels in aged CR mice in comparison with aged AL mice (Fig. 2 B). Additionally, serum proinflammatory cytokine analysis of TNF (Fig. 2 D), IL-6 (Fig. 2 E), and IFN-\( \gamma \) (Fig. 2 F) resulted in all cytokines being significantly decreased in aged CR mice in comparison with the aged AL mice. These decreases in cytokine values were to levels that were either lower-than or as-low-as those in young AL mice that received IT (Fig. 2, D–F). To determine if these lower cytokine values during IT therapy would confer protection against IT-induced mortality, aged AL mice and aged CR mice were administered either LD IT or HD IT and monitored for mortality. Independent of treatment dose, aged AL mice all succumb to mortality on day 2 of treatment (Fig. 2 G). However, 100% of aged CR mice placed on the HD amount of IT demonstrated complete protection from \( \alpha_{CD40/IL-2} \)-mediated lethality (Fig. 2 G). Collectively, these data suggest that increased body fat plays a critical role in the induction of systemic toxicities by IT. Increased adiposity played a critical role in the age-associated cytokine storm and subsequent organ pathologies after IT and calorie restriction diminished the development of such toxicities conferring a protective effect that allowed for increased survival in the aged.

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that young ob/ob mice have higher total body fat content that is similar in accumulation to the aged mice, being deposited largely in the intra-abdominal cavity, in comparison with young AL mice (Fig. 3 B).

To determine if adiposity could solely induce toxic consequences in aged AL, young ob/ob and age-matched young AL mice were treated with HD IT and at day 2 of treatment analyzed for the presence of immune-mediated organ pathological changes within the liver and intestines (Fig. 3, C and D). IT resulted in mild inflammatory infiltrates in young AL mice but resulted in moderate to severe inflammation in the ob/ob mice, which was comparable to the aged within the intestines (Fig. 3 D). Yet, analysis of IT-induced liver pathology was hindered by the presence of extensive steatosis in ob/ob mice, resulting in a paradoxical interference in determining the intensity of immune-mediated inflammatory liver damage (Fig. 3 C). IT resulted in both the intestines and liver displaying the most immune-mediated inflammatory damage within the ob/ob that was comparable to the aged AL mice and significantly greater than the young AL (Fig. 3, C and D).

Because HD IT resulted in significant changes within the obese ob/ob mice, we next administered the lower dose (LD) of IT to young ob/ob, young age-matched AL mice, aged AL, and aged CR mice. Administration of LD IT still resulted in significant increases in TNF and IL-6 proinflammatory cytokine levels within the young ob/ob that were not significantly different to the aged AL mice (Fig. 3, E). Yet, analysis of IT-induced liver pathology was hindered by the presence of extensive steatosis in ob/ob mice, resulting in a paradoxical interference in determining the intensity of immune-mediated inflammatory liver damage (Fig. 3 C).

Figure 3. Increased adiposity independently of age results in systemic release of proinflammatory cytokines and organ pathology after IT in young ob/ob (obese) and aged AL mice. (A) Body weights of naive young (2 mo) WT and young ob/ob C57BL/6 mice, n = 6 mice/group. Representative pictures of young-AL WT mice and obese ob/ob naive mice are shown. (B) Total fat content in naive young WT (2 mo), young ob/ob (2 mo), and aged (18 mo) C57BL/6 mice was visualized by MRI. Fat is bright. (C and D) Young AL (2 mo), young ob/ob (2 mo), and aged AL (18 mo) mice were treated with HD αCD40/IL-2 and liver (C) and gut (D) were stained with HE and scored for histopathology on day 2. Bars, 200 µm. Arrows indicate areas of steatosis and/or immune cell infiltration and asterisks indicate patchy necrosis and/or fibrosis. (E–G) Young AL (2 mo), young ob/ob (2 mo), aged AL (18 mo), and aged CR (18 mo) were treated with LD αCD40/IL-2 and serum was collected day 2 of treatment and analyzed by CBA for levels of: TNF (E), IL-6 (F), and IFN-γ (G), n = 3 mice/group. (H and I) Young AL (2 mo), young ob/ob (2 mo), aged AL (18 mo), and aged CR (18 mo) were treated with LD αCD40/IL-2 (H) or CpG/IL-2 (I) or rIgG/PBS control and survival was monitored. (J) Serum levels of TNF were measured on day 2 of CpG/IL-2 therapy by CBA. Data in all panels are representative of one of at least three experiments with similar results. MRI scans are representative of at least three mice imaged per group with similar results. Survival data were plotted using the Kaplan-Meier method and statistical differences were determined with the log-rank test. Bar graph (mean value ± SEM) statistics performed using Student’s t test or one-way ANOVA with Bonferroni post-hoc tests. *** P < 0.001; ** P < 0.01; * P < 0.05.
This data further supports that increased adiposity plays a crucial role in the induction of systemic toxicities after IT. These data are suggestive that not only does an aging environment result in lethal toxicities but toxicity may also be dependent on the increase in fat accumulation as the young ob/ob mice succumb to cytokine storm induction and mortality after IT, similar to that observed in the aged mice.

**IT toxicity is TNF- and M1 macrophage–dependent within the peritoneum and visceral adipose tissues**

A hallmark of obesity is the increasing infiltration of macrophages into adipose tissues (ATMs) through the secretion of MCP-1 by adipocytes. Phenotypically, these macrophages are

Young WT cohort (Fig. 3 H). 100% of aged AL mice yielded to lethality from the IT by day 2 of treatment, whereas 100% of aged CR mice survived the entire regimen (Fig. 3 H).

The pathological and lethal response observed within the young ob/ob mice was not αCD40/IL-2 IT administration specific. Young AL and age-matched young ob/ob mice were administered CpG in combination with HD IL-2 and, likewise, resulted in lethality of 75% of young ob/ob mice by day 5 of treatment but resulted in complete tolerance by young AL mice (Fig. 3 I). CpG/IL-2 therapy led to significantly increased levels of serum proinflammatory cytokine TNF in an analogous trend as αCD40/IL-2 administration on day 2 of therapy (Fig. 3 J).
predominately classically activated M1 cells that also express proinflammatory mediators IL-1β, TNF, and IL-6 (Lumeng et al., 2007; Fujisaka et al., 2009). As such, we next sought to determine if increases in M1 macrophages within the peritoneal cavity and visceral adipose tissues could account for the pathological increases in systemic TNF during IT administration. Young AL and age-matched young ob/ob mice were administered LD αCD40/IL-2. At day 2 of treatment, visceral adipose tissues and peritoneal lavages were phenotypically assessed by flow cytometry for the presence of M1 (CD206−) macrophages, M2 (CD206+) macrophages, and percentages of TNF-expressing macrophages. Consistently, young ob/ob mice demonstrated significant increases in M1 macrophage proportions during IT administration, whereas young AL mice were able to maintain consistent M1-to-M2 ratios (Fig. 4 A). Additionally, both the visceral adipose tissues and peritoneal lavages of ob/ob mice experienced significant increases in percentages of TNF-expressing macrophages as opposed to young AL mice (Fig. 4 B–D). Significant changes were not observed in the spleen of either young ob/ob or AL mice (unpublished data). This data, consistent with obesity literature, suggests that increases in adipose tissue accumulation within the visceral cavity may impact regulation of M1 and M2 macrophage ratios that ultimately may affect TNF responses to immune stimulation with immunotherapeutic regimens.

Given the increases in M1 macrophages within the obese mice and dysregulation observed with IT, young ob/ob and young AL mice were treated with LD αCD40/IL-2 in conjunction with either liposomal clodronate for macrophage depletion (Fig. 4, E–H) or etanercept (Enbrel) for TNF blockade (Fig. 4, I–L). Both macrophage depletion and co-treatment with etanercept during LD αCD40/IL-2 resulted in complete protection of young ob/ob mice from lethality (Fig. 4, E and I). As expected, treatment with liposomal clodronate led to significantly decreased levels of TNF-producing macrophages within both the visceral fat and peritoneal lavages (Fig. 4, F and G), which significantly reduced systemic circulating serum levels of proinflammatory cytokine TNF (Fig. 4 H). Additionally, co-administration of LD αCD40/IL-2 with etanercept did not reduce the percentages of TNF-expressing macrophages within the visceral fat or peritoneal cavity (Fig. 4, J and K) but mediated complete protection through an overall reduction in systemic TNF levels (Fig. 4 L).

Together, these data indicate that proinflammatory M1 macrophages are the cellular mediator in the induction of IT toxicity, particularly through increased presence within the peritoneal and visceral adipose tissues. Importantly, these effects are TNF-dependent, as blocking TNF levels either through etanercept administration or macrophage depletion resulted in complete protection of young ob/ob mice from lethality.

Diet-induced obese (DIO) mice succumb to cytokine storm, organ pathology, and lethality similar to ob/ob and aged AL mice after IT

ob/ob mice are congenic for the spontaneous mutation in the OB gene, resulting in leptin hormone deficiency. To extend our findings, we generated young DIO mice. Mice were placed on a purified 60% fat diet to induce obesity beginning at 3 wk of age and were determined to be obese when weighing >40 g (DIO). Age-matched counterparts were placed on a matching...
purified diet that was 10% fat and served as lean controls. Increases in body mass were validated in DIO mice before IT administration (Fig. 5, A–C). DIO mice displayed increases in overall body weight and size (Fig. 5, A and B). MRI analysis of body fat content showed increased total body fat accumulation, largely as increased visceral fat, in young DIO mice that was similar to the aged AL mice (Fig. 5 C).

Confirming our previous findings, DIO mice treated with HD αCD40/IL-2 displayed increased serum TNF, IL-6, and IFN-γ levels compared with age-matched young mice on a 10% fat diet after IT administration (Fig. 5, D–F). The increases in proinflammatory cytokines in DIO mice were lower, yet not significantly so, than aged mice after IT (Fig. 4, C–E). Young DIO mice started succumbing to IT lethality at day 3 of treatment (>60%), and <40% were still alive by day 4 (Fig. 5 G).

Together, these results suggest that adipose tissue is a critical component to the development of systemic cytokine storm and lethality after IT. Yet, due to the observation that treatment of DIO mice did not result in 100% of mice succumbing to lethality, aging itself may also likely contribute to the overall heightened inflammatory responses incurred by IT.

**DISCUSSION**

After initiation of strong immune stimulation with IT, we show that both young obese and aged AL mice demonstrate increased levels of adiposity, which led to aberrant increases in both TNF and IL-6 levels that ultimately resulted in multi-organ damage. Similar to aging, obesity has been shown to be immunomodulatory, where the cross talk between adipocytes and macrophages is thought to lead toward the development and perpetuation of a meta-inflammatory state through continual NF-κB activation, which is currently hypothesized to be responsible for the induction of metabolic diseases. A study conducted by Spaulding et al. (1997) examined the effects of age and calorie restriction on the production of TNF and IL-6 in resting mice and demonstrated that serum levels of both cytokines were significantly higher in aged mice in comparison to young, and yet aged mice subjected to long-term calorie restriction resulted in TNF and IL-6 serum levels that were comparable to the young mice. These findings lend support to the protective effects seen within our studies in the aged CR cohort administered IT.

Importantly, the data presented here demonstrate the need for using age and body fat content as variables in preclinical assessment of therapeutics and modeling diseases, such as cancer. However, the vast majority of inbred mouse studies are housed in specific pathogen-free colonies where normal aging may result in higher body fat content and yet less immune challenge from pathogens making them more susceptible to proinflammatory responses and toxicities after immune therapies. It will therefore be important that future studies ascertain responses in mice that have been exposed to pathogens and immune challenges as they age.

Furthermore, our data demonstrate that an obese phenotype resulted in lethal consequences that were ameliorated through calorie restriction in the aged. Also, differences in lethality were observed between young obese and aged AL mice, where aged mice die by day 2 but young obese mice die at day 4 of treatment. Therefore, although our data strongly demonstrates that adiposity plays a central role in the induction of toxicities, aging is also a likely key factor. Therefore, our findings highlight the importance in considering both the status of aging and obesity of patients receiving systemic administration of stimulatory IT regimens. It will be important to conduct studies dissociating the consequences of aging upon increased cytokine responsiveness, possibly through either investigating epigenetic modifications induced by the meta-inflammatory and/or inflammaging states associated with increased adipose accumulation or whether aging is accompanied by changes in expression levels of various receptors that allow for a quicker response.

The development of IT regimens continues on a positive trajectory with increasing successes within a variety of cancers. Active IT has taken a greater comprehensive form focusing on activating humoral and cell-mediated immunity, and these strategies have included antigen-specific vaccines (i.e., MART-1 vaccines), DC-based vaccines (i.e., Sipuleucel-T), inhibitors of checkpoint blockade (i.e., αCTLA-4 and αPD-1), and systemic stimulatory regimens through cytokine based therapies and application of agonistic antibodies (i.e., αCD40, IFN-γ, and IL-2). Our data presented herein demonstrates that both young obese and aged AL mice succumb to rapid lethality during systemic administration of stimulatory therapies. Our data strongly suggests that excess adiposity may be a prognostic factor in determining patient outcome and treatment tolerance. However, additional studies need to be conducted examining the effects of adiposity on the development of adverse reactions within other classes of IT regimens, such as checkpoint inhibitors.

Clinically, we hypothesize that taking into account patient BMI, adipose accumulation, and age may better modulate treatment type choices as well as the inclusion of supportive therapeutics such as TNF blockade in conjunction with anti-cancer therapies that, overall, may increase patient survival and lead to greater anti-cancer responses. Follow-up investigations in tumor-bearing models merit further study.

**MATERIALS AND METHODS**

**Animals and diets.** Female young (2–5 mo old), middle-aged (9–12 mo old), and aged (15–22 mo old) C57BL/6 mice were purchased from Charles River or from the animal production area at the National Cancer Institute. Young female B6.Cg-Lep^{db/+} and young age-matched female WT control C57BL/6 mice were purchased from The Jackson Laboratory at 8–12 wk of age.

DIO obese and control lean mice were generated from WT C57BL/6 mice purchased at 3 wk of age from Charles River or from the animal production area at the National Cancer Institute. Mice were placed on an open-source purified diet consisting of either 60% high-fat (D12492) or 10% low-fat (D12458) constitution (Research Diets, Inc.). Age-matched mice were placed on either a high-fat or low-fat diet beginning at 3 wk of age for 16–20 wk and weighed weekly. Mice were deemed obese upon reaching a minimum of 40 g of weight.

For studies comparing aged AL and CR dietary effects, both female aged CR and age-matched female AL-littermates (15–22 mo old) were purchased from the CR, rodent colony at the National Institutes on Aging, National Institutes of Health (NIH). In brief, mice were either raised through free access, AL, feeding on the NIH31 regular chow, or placed on a limited daily feeding.
schedule with the NIH31-fortified chow. CR is initiated at 14 wk of age at 10% restriction, increased to 25% restriction at 15 wk, and to 40% restriction at 16 wk of age where it is maintained throughout the life of the animal. Information on survival curves, food intake curves, and body weights of the NIA strains has been previously published (Turturro et al., 1999).

All experimental mice were housed in the Animal Facilities at the University of California, Davis under specific pathogen-free (SPF) conditions. For the comparison in fat volume ratios, young, middle-aged, and aged AL and CR mice were included and fed to the National Institute on Aging for MRI analysis and also housed under SPF conditions. All of the animal protocols were approved by the Institutional Animal Care and Use Committees of both institutions. Body weights were also measured several times at different ages and on dietary regimens. Mice were euthanized by CO2 asphyxiation and the visceral fat pads (mesenteric, gonadal, and renal) were collected and weighed together for each mouse to determine the visceral fat weight.

Reagents. The agonistic anti-mouse CD40 antibody (clone FGK11B3) was generated via ascites production in our laboratory as previously described (Murphy et al., 2003; Bouchlaka et al., 2013). The endotoxin level of the CD40 was <1 endotoxin unit/mg of antibody as determined by a quantitation (Murphy et al., 2003; Bouchlaka et al., 2013). The endotoxin level of the endotoxin was generated via ascites production in our laboratory as previously described (Murphy et al., 2003; Bouchlaka et al., 2013). The endotoxin level of the endotoxin was generated via ascites production in our laboratory as previously described (Murphy et al., 2003; Bouchlaka et al., 2013).

Schedule of αCD40/IL-2 and CpG/IL-2 IT treatments. Mice were treated with agonist anti-CD40 antibody and rhIL2 as previously described (Murphy et al., 2003; Bouchlaka et al., 2013). αCD40 or CpG was administered daily for a total of 5 consecutive days (days: 0, 1, 2, 3, and 4) and IL-2 was administered twice a day for a total of 4 d (days: 1, 4, 8, and 12). Control mice received rat IgG (IgG; Jackson ImmunoResearch Laboratories, Inc.) and PBS (Cellgro). Survival was monitored daily. An HD or LD of αCD40/IL-2, or HD CpG/IL-2, was administered as follows on the same scheduling pattern as previously described (Bouchlaka et al., 2013): HD αCD40/IL-2, mice received 80 µg of agonist αCD40 and 10^6 IU of IL-2 in 0.2 ml PBS i.p.; control mice received 80 µg rIgG in PBS; LD αCD40/IL-2, mice received 40 µg of agonist αCD40 and 4 × 10^5 IU of IL-2 in 0.2 ml PBS i.p.; control mice received 40 µg rIgG in PBS; CpG/HD IL-2, mice received 100 µg CpG ODN 1826 (InvivoGen) and 10^6 IU of IL-2 in 0.2 ml PBS i.p.

Macrophage depletion and TNF blockade with etanercept (Enbrel). In some experiments, mice were in vivo depleted of macrophages using liposomal clodronate or control-loaded liposomes (Encapsula NanoScience) before and during IT administration, as previously described (Bouchlaka et al., 2013). For experiments where mice were euthanized by day 2 of treatment, 0.2 ml per dose was injected i.p. of either clodronate or control liposomes on days −2 and 0. For all survival experiments, clodronate or control liposomes were administered on days −2, 0, 2, and 4.

TNF blockade was performed through in vivo subcutaneous injection of 1.5 mg/0.1 ml etanercept (Enbrel) on days −1 and 1 for experiments where mice were euthanized on day 2 of IT therapy. For all survival experiments, etanercept was administered on days −1, 1, 3, 7, and 9.

Murine macrophage studies. On day 2 of treatment, murine macrophages were isolated from either the peritoneal cavity or visceral adipose tissue. For peritoneal lavages, 4 ml of cold PBS was injected into the peritoneal cavity of each mouse and then aspirated. Lavages were spun down at 1,200 rpm for 5 min and the resulting pellet was resuspended in RF10c media and plated for flow cytometry staining.

The stromal vascular fraction of visceral adipose tissues was collected day 2 of treatment. Adipose tissues from the visceral cavity (gonadal) were collected and then incubated for 60 min in a digestion solution consisting of 2 mg/ml type IV collagenase (Sigma-Aldrich) dissolved fresh in a 2% BSA in PBS solution. After incubation samples were centrifuged at 1,200 rpm for 5 min for layer separation of the stromal vascular fraction from adipocytes. The resulting stromal vascular fraction was then removed, resuspended into a single cell suspension, counted, and plated for flow cytometry staining.

Flow cytometry analysis of macrophages. Single cell suspensions from the peritoneal lavages and visceral adipose tissues were plated in RF10c media with the addition of GolgiPlug (containing Brefeldin A) and GolgStop (containing Monomen) Protein Transport Inhibitors (BD) according to manufacturer's protocol. Plates were incubated for 9 h at 37°C and 5% CO2.

After incubation, cells were washed with PBS and stained for flow analysis. Single cell suspensions were labeled with Fc block (purified anti-mouse CD16/32; BD) for 10 min, followed by labeling with antibodies for 20 min at 4°C. Cells were washed twice with a staining buffer consisting of 5% FBS (Gemini Bio-products) in DPBS (Corning). For intracellular staining, the Cytofix/Cytoperm kit (BD) was used per manufacturer's protocols. After staining, cells were analyzed using a custom configured Fortessa cytometer, and by using FACSDiva software (BD). Data were analyzed using FlowJo software vX (Tree Star). Antibodies used to distinguish macrophages were: PB-conjugated anti-mouse CD45, BV711-conjugated anti-mouse CD11b, BV605- and PE-conjugated anti-mouse CD206, BV785-conjugated anti-mouse CD19, APC-conjugated anti-mouse F4/80, and PE-Cy7-conjugated anti–mouse TNF (BioLegend).

Serum cytokine bead array. Serum levels of TNF, IFN-γ, and IL-6 were quantified by multiplex measurement using the Cytometric Bead Array (CBA; BD) kit as described previously (Bouchlaka et al., 2013). In brief, serum samples and standards were incubated for 1 h at 25°C with a bead mixture containing bound antibodies to TNF, IFN-γ, and IL-6 according to manufacturer's instructions. Samples and standards were resuspended in wash buffer and analyzed by flow cytometry. Data were acquired on a custom-configured Fortessa cell analyzer using FACSDiva software (BD) and analyzed using FlowJo software (Tree Star). Each sample was analyzed in triplicate. Upon analysis of raw data, the mean fluorescent intensities (MFIs) of each bead cluster were quantified. Where indicated in the figures, protein concentrations were extrapolated relative to a standard curve created by serial dilution of the mouse positive control cytokine.

Colorimetric liver enzyme assay. Serum ALT was quantified using the ID Labs ALT Enzymatic Assay kit (ID Labs Biotechnology Inc.) as previously described (Bouchlaka et al., 2013). Colorimetric determination of ALT levels was performed by reading the absorbance of each well at 340 nm on a plate reader (VERSAmax tunable plate reader). The concentration of ALT (U/liter) in the samples was calculated from a standard curve generated by using the kit's provided standards.
in each sample was then directly determined from the change in absorbance within 5 min time. Dilutions of the Pyruvate Control, included in the kit, were used to construct a standard curve to calibrate the assay. Every serum sample was assayed in triplicate.

<table>
<thead>
<tr>
<th>Table 2. Grading of acute intestinal damage</th>
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<tr>
<td><strong>Global assessment</strong></td>
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<tr>
<td>0: None</td>
</tr>
<tr>
<td>I: Minimal/indeterminate</td>
</tr>
<tr>
<td>II: Mild</td>
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<tr>
<td>III: Moderate</td>
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<tr>
<td>IV: Severe</td>
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MRI. To quantify abdominal fat, MR images were acquired in collaboration with the National Institute on Aging. Images were acquired using a Biospec 7 Tesla 30-cm MRI scanner (Bruker Biospin) with a 72-mm diameter transmit-receive birdcage coil. In each experiment, two mice were inserted into the scanner, side by side, in feet-first prone orientation immediately after sacrifice. During scanning, the mice were cooled with a stream of cold air from a vortex tube (Exair, Inc.) to minimize decomposition. Two different pulse sequences were used: a heavily T1-weighted fast spin echo (RARE) sequence yielding bright-fat (water-suppressed [WS]) images and a fat-suppressed proton density weighted fast gradient echo (FLASH) sequence yielding fat-suppressed (FS) images. 3D datasets were acquired in axial orientation with a field of view of 72 × 36 × 80 mm (left-right × anterior-posterior × head-foot) and matrix size 256 × 128 × 128. The voxel size (spatial resolution) was 0.3 × 0.3 × 0.625 µm. The spectral bandwidth in both sequences was 100 kHz (391 Hz/pixel) and two averages were acquired for improved signal-to-noise ratio. For the RARE sequence, the RARE factor (i.e., number of spin echoes per shot or number of k-space lines per segment) was 8, the repetition time (TR) was 125 ms, the actual echo time (TE) was 11.4 ms, and the effective echo time (T Eff) was 46.3 ms. Each RARE scan took 8 min and 32 s to acquire. For the FLASH sequence, the repetition time was 25 ms and the echo time was 3.2 ms. The excitation pulse had a flip angle of 30°. Each FLASH scan took 13 min 39 s to acquire. Image processing was performed in ImageJ 1.45 (NIH). The statistical analyses were performed using one-way ANOVA and the Newman-Keuls post-hoc test. P < 0.05 was considered to be significant.

To visualize fat distribution and content imaging of young ob/ob, DIO, and lean age-matched controls were conducted in collaboration with the Center for Molecular and Genomic Imaging (CMGI) Facility at the University of California, Davis. Mice were anesthetized before imaging using 2% isoflurane in an induction chamber and then maintained on 1–2% isoflurane throughout imaging via a nose cone fitted for anesthetic inhalation. Mice were imaged using a BioSpec 70/30 7T (Bruker Biospin) horizontal bore system designed specifically for small animal imaging. Animals were kept at normal body temperature using warm air. Mice were inserted into the scanner in a head-first prone orientation. Spin-echo T1-weighted images were obtained through the mouse body (neck-to-base of the tail) using a 72-mm linear volume coil. Scan sequence parameters were the following: TR, 1,000 ms, TE 15 ms, and 2 averages. The field of view was 7.7 × 3.85 × 2.0 cm, with a matrix size of 256 × 128 × 40. The corresponding voxel size was 0.3 × 0.3 × 0.5 mm. Images were acquired using ParaVision 5.0 software. After acquisition, images were transferred into Invenon Research Workplace 4.0 software (Siemens Preclinical) allowing fat to be displayed with high intensity (white).

Statistical analysis. Statistical analyses were performed using Prism software (GraphPad Software Inc.). Data were expressed as mean ± SEM. For analysis of three or more groups, a nonparametric ANOVA test was performed with a Bonferroni post-hoc test. Analysis of differences between two normally distributed test groups was performed using the Student’s t test. Non-parametric groups were analyzed with the Mann-Whitney test. Welch’s correction was applied to datasets with significant differences in variance before Student’s t test.

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