Premalignant Lesions Skew Spleen Cell Responses to Immune Modulation by Adipocytes

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Abstract. *Obesity can promote a chronic inflammatory state and is associated with an increased risk for cancer. Since adipocytes can produce mediators that can regulate conventional immune cells, this study sought to determine if the presence of premalignant oral lesions would skew how immune cells respond to adipocyte-derived mediators to create an environment that may be more favorable for their progression toward cancer. While media conditioned by adipocytes stimulated normal spleen cell production of the T helper (Th) type-1 cytokines interleukin (IL)-2, interferon-γ (IFN-γ), IL-12 and granulocyte-monocyte colony-stimulating factor (GM CSF), media from premalignant lesion cells either blocked or had no added affect on the adipocyte-stimulated Th1 cytokine production. In contrast, media conditioned by premalignant lesion cells exacerbated adipocyte-stimulated spleen cell production of the Th2 cytokines IL-10 and IL-13, although it did not further enhance the adipocyte-stimulated spleen cell production of IL-4 and TGF-β. The premalignant lesion environment also heightened the adipocyte-stimulated spleen cell production of the inflammatory mediators IL 1α, IL-1β, IL-6 and IL-9, although it did not further increase the adipocyte-stimulated production of tumor necrosis factor-α (TNF-α). IL 17 production was unaffected by the adipocyte-derived mediators, but was synergistically triggered by adding media from premalignant lesion cells. These stimulatory effects on spleen cell production of Th2 and inflammatory mediators were not induced in the absence of media conditioned by adipocytes. In contrast, media conditioned by adipocytes did not stimulate production of predominantly monocyte-derived chemokine C-X-C motif ligand (CXCL)9, chemokine C-C motif ligand (CCL)3 or CCL4, although it stimulated production of CCL2 and the predominantly T cell-derived chemokine CCL5, which was the only chemokine whose production was further increased by media from premalignant lesions. These results suggest that the responsiveness of spleen cells to adipocyte-derived mediators is influenced by mediators from premalignant lesion cells to favor conventional immune cell production of a Th2 and inflammatory cytokines.*

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The prevalence of obesity worldwide is an increasing health problem due to its associated complications such as diabetes, cancer, poor wound healing and atherosclerosis, and it also carries a huge economic impact (1-6). Some of these obesity-associated complications can be attributed to the chronic inflammatory state that is promoted by adipose tissue, with an accumulation of macrophages and T-cells (7, 8). Adipocytes can contribute to this inflammatory state through their capacity to produce a multitude of mediators such as intercellular adhesion molecule-1 and vascular cell adhesion protein-1, chemokine C-C motif ligand (CCL)2, chemokine C-X-C motif ligand (CXCL)10, interleukin (IL)-6, IL-8, IL-10, tumor necrosis factor (TNF)-α and TNF-β (7, 9-11). In addition, levels of the pro-inflammatory and pro-angiogenic adipokine leptin are increased in obesity, while circulating levels of the adipokine adiponectin, having anti-inflammatory and anti-angiogenic properties, are reduced in obesity (12, 13). Animals fed a high-fat diet have early increases in plasma levels of the adipokines leptin, resistin and adipsin, with a later reduction in levels of adiponectin (14). Compared to levels prior to surgery, levels of leptin and adiponectin return to normal in patients that have undergone gastric bypass surgery (13).

Epidemiological studies showed an increased risk of liver cancer associated with obesity (15). Obesity has also been associated with increased breast cancer risk and a poorer prognosis (3, 16). The increased incidence of obesity correlates with an increased incidence of esophageal adenocarcinoma (17). The circulating levels of the adipokine adiponectin, whose levels decline with obesity, were inversely-

associated with the risk of multiple myeloma (18). Paradoxically, the risk of breast cancer risk was associated not only with obesity and increased circulating levels of leptin, but also with increased levels of adiponectin (19). In addition to associations between adipokines and cancer, the increased association between obesity and cancer may be mediated in part by pro-inflammatory and pro-angiogenic mediators such as vascular endothelial cell growth factor (VEGF), TNF-α, epidermal growth factor (EGF) and IL-6 that are produced by adipocytes (3, 16).

The mechanism by which immune and inflammatory mediators might promote tumor development is complex. To date, more than 150 cytokines have been identified and classified into several major categories, with the most common being considered as T-helper (Th)1 and Th2 type cytokines (20, 21). Th1-type cytokines drive cellular immunity against intracellular pathogens, including viruses, and protect against cancerous cells (21-23). Th2-type cytokines control humoral immunity by up-regulating antibody production to protect against extracellular pathogens and are involved in allergic reactions (21, 24, 25). There are also cytokines with dual functions and which are involved in inflammatory responses (26, 27). Inflammatory cytokines that are prominently overexpressed in obese mice include IL-6, TNFα and IL-1 (27). Leptin has been postulated to mediate inflammation, including that which occurs in inflamed dental pulp tissue, and can stimulate differentiation of human dendritic cells to enable their stimulation of Th1 reactions (28, 29). In a mouse model of sepsis, adiponectin reduced mortality through an anti inflammatory mechanism by which serum levels of TNF α and IL 6 were reduced (30).

Studies investigating premalignant oral lesions have shown that lesion tissue as well as the regional lymph nodes have a robust immune response with a greater percentage of conventional T-cells expressing markers for activation, memory, and exhaustion compared to levels seen in control mice (31). These cells produced increased levels of interferon (IFN) γ, IL 2, CCL3, CCL4 and CCL5 compared to cells from control mice. There was also an increase in the levels of Th17 cells and secreted IL-17 in both premalignant lesions and regional lymph nodes compared to levels in control animals. Since premalignant oral lesions induce an inflammatory state, not only within the lesion, but also within lymph nodes, it appears as though soluble mediators from the lesions contribute to the heightened immune and inflammatory state (31). Similarly, the immune modulatory effects of adipocytes occur through soluble mediators that they produce (7, 9-11). It is not known how the mediators from premalignant lesions impact on the inflammatory state that can be induced by mediators produced by adipocytes and, in turn, if this establishes an environment that could facilitate progression of a premalignant lesion to oral cancer.

As a result of the heightened level of inflammation in both adipose tissue and in premalignant lesion tissue, the present study examined the impact of the environment of the premalignant lesion on the responsiveness of immune cells to adipocyte-derived mediators.

Materials and Methods

Adipocyte differentiation from the mouse embryonic fibroblast cell line, 3T3-L1 (32). The mouse fibroblast cell line, 3T3-L1 (ATCC, Manassas, VA, USA), was maintained by culture in growth medium containing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.02 M HEPES buffer, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA). Fibroblasts were differentiated into adipocytes by supplementing the growth medium with 25 mM glucose, 0.5 mM of 3-Isobutyl-1-methylxantine (IBMX) and 1 μM of dexamethasone (Sigma-Aldrich). After 72 h of incubation, the medium was replaced with DMEM, culture medium containing 25 mM glucose plus 1.74 μM of insulin (Sigma-Aldrich) for 48 h. After differentiation, the insulin-containing medium was removed and adipocyte cultures were maintained in DMEM containing 25 mM glucose and 10% FBS for five to nine days. At days 5-9 following culture with insulin, the 3T3 L1 cultures are lipid-laden, contain larger lipid droplets, and produce more adipokines such as adiponectin and leptin than prior to that time. Cells are also markedly less adherent than the cultures at earlier time points, while remaining healthy under microscopic examination. Under these culture conditions, the 3T3 L1-derived adipocytes have previously been shown to be capable of producing inflammatory mediators (23). Conditioned medium was collected every other day and stored frozen until used in the experiments described below. Controls for the media used to generate the conditioned media contained the same concentrations of glucose and FBS as was present in the conditioned media.

Development of oral premalignant lesions and cell line. All animal procedures were conducted with Institutional Animal Care and Use Committee approval (ACORP #506). Nitroquinoline-1-oxide (4NQO) was mixed in propylene glycol and administered in drinking water bottles at a final 4NQO concentration of 50 μg/ml to 2-month-old (at start) female C57BL/6 mice (Charles Rivers Laboratory, Wilmington, MA, USA) until the development of premalignant oral lesions (6–8 weeks). The 4NQO-containing drinking water was replaced with fresh 4NQO-containing drinking water three times per week. Development of premalignant oral lesions in 4NQO-treated mice was monitored weekly by endoscopy using a Stryker 1.9 mm endoscope and a Stryker 1088 HD camera (Kalamazoo, MI, USA). For the endoscopic examination, mice were sedated with inhaled isoflurane (Piramal Healthcare, Boise, ID, USA). Control mice went untreated and were given fresh drinking water without 4NQO. The 4NQO model has all of the stages that are associated with development of oral cancer in humans, and has both the histological and molecular characteristics of human oral lesions and cancer (33).

To establish primary cultures of the tongue from control animals, fresh tongue tissue explants from normal C57BL/6 female mice were cleaned with multiple washes of antibiotic and antimycotic solutions containing 100 mg/ml of penicillin, 100 mg/ml of streptomycin, and 200 mg/ml of neomycin. Tissues were finely-minced and enzymatically-dissociated for 4 h in 1.2 U/ml Dispase II (Roche, Indianapolis, IN, USA) and 112 U/ml collagenase type-II (Worthington, Lakewood, NJ, USA). Cells were then washed and

seeded into culture in DMEM. Premalignant primary cell lines were derived from oral lesions located on the mouse tongues without enzymatic dissociation, and plated in growth medium for one week. To maintain an established premalignant lesion cell line, adherent cells were passaged by trypsinization, washing, and resuspension in fresh medium.

Harvest and treatment of splenocytes. Female C57BL/6 mice (Charles Rivers Laboratory) were humanely euthanized by CO_2 asphyxiation followed by cervical dislocation. Spleens from healthy mice were collected and used as a source of conventional immune cells. Whole spleens were homogenized with a glass homogenizer. Red blood cells were lysed by a 3 min incubation in ACK Lysing Buffer (Lonza, Walkersville, MD, USA). Cells were subsequently washed and suspended in DMEM. One million cells were plated into each well of 24 well plates containing immobilized antibody to CD3 antibody (2.5 μg/well) and 15 pg/ml IL-2 (R&D Systems, Minneapolis, MN, USA). Spleen cells were treated for 72 h with either media that had been conditioned independently by cells from premalignant lesions or fibroblast-derived adipocytes, or with conditioned media that were pooled from independently cultured premalignant lesion cells and independently cultured adipocytes. Culture medium was added as a control to spleen cells in lieu of conditioned media. All cultures contained uniform volumes and uniform amounts of FBS. After the incubation, supernatants from spleen cells were collected for measurement of levels of secreted immune mediators.

Measurement of levels of immune mediators. Following three days of spleen cell incubation with control medium, media conditioned independently by adipocytes or premalignant lesion cells, or a mixture of media conditioned independently by adipocytes or premalignant lesions, culture supernatants were collected and used to measure levels of soluble immune mediators. These analyses were conducted with reagents from BD Biosciences (San Jose, CA, USA). Using the manufacturer's instructions, levels of cytokines and chemokines in supernatants were measured with mouse Th1/Th2/Th17 cytometric bead array kits, while levels of chemokines and IL-13 in cell supernatants were measured with cytometric bead array flex sets for the individual mediators. Supernatants used for measurement of TGF-β1 levels were first acid-activated in accordance with the manufacturer's instructions. Relative amounts of each cytokine were analyzed using FCAP Array software (BD Biosciences).

Statistical analysis. Data were reported using the mean as a measure of central tendency±standard error of the mean (SEM). To compare one variable condition between groups, the two-tailed Student's *t*-test was used. Significance was reported with the 95% confidence interval.

Results

Impact of premalignant lesions on the immune-stimulatory immune-regulatory activity of adipocytes toward spleen cell production of Th1 cytokines. The present study showed that fibroblast-derived adipocytes not only produce inflammatory mediators, but can serve as immune-regulatory cells. This includes adipocyte stimulation of T-cell production of Th1 cytokines. In this study, it was also assessed if the environment of the premalignant oral lesions affects this Th1 response because the progression of premalignant lesions to oral cancer is associated with a shift from an inflammatory to an immune-inhibitory environment (31). Media conditioned by adipocytes, stimulated spleen cell production of the predominantly T cell-derived Th1-type cytokines IL-2, IFN-γ and GM-CSF (Figure 1). Addition of media conditioned by premalignant lesion cells blocked the adipocyte-stimulated spleen cell production of IL-2, but did not have a significant effect on the adipocyte-stimulated production of IFN-γ or GM-CSF. In the absence of adipocyte supernatant, spleen cell production of IFN-γ was slightly increased by the presence of medium conditioned from premalignant cells, but not to the extent that was stimulated by adipocytes. Production of IL-12 was low and was not affected by either media conditioned by adipocytes or premalignant lesion cells. Thus, mediators produced by cells from premalignant oral lesions have a minimal effect on the Th1-stimulatory response of spleen cells to adipocyte-derived mediators, with the exception of an inhibitory effect on adipocyte-stimulated IL-2 production.

Cells from premalignant lesions exacerbate adipocyte-stimulated spleen cell production of the Th2 cytokines IL-10 and IL-13. Addition of medium conditioned by adipocytes to normal spleen cells resulted in a marked stimulation of spleen cell production of the Th2 cytokines IL-10 and IL-13 (Figure 2). This stimulated Th2 response of spleen cells was further exacerbated by the addition of supernatants from premalignant lesion cells. The increase in spleen cell production of IL-10 and IL-13 in the presence of medium conditioned by cells from premalignant lesions was dependent on the presence of adipocyte-derived mediators, as spleen cells failed to produce significantly increased levels of these cytokines in the presence of supernatants from cultures of premalignant lesion cells alone (Figure 2).

Spleen cell production of the other Th2-type mediators measured, IL-4 and TGF-β, was also increased by adipocyte-conditioned medium, although a part of the increases could be attributed to additive effects of levels produced by the spleen cells-alone and what was in the adipocyte-conditioned medium. Addition of medium conditioned by premalignant lesion cells did not further increase spleen cell production of either IL-4 or TGF-β (Figure 2). Thus, the Th2 response of spleen cells to adipocyte-conditioned medium was further skewed toward the Th2 cytokine secretion pattern by supernatants from cultures of cells from premalignant lesions as seen by an increase in IL-10 and IL-13 production without a dampening of the adipocyte-stimulated IL-4 and TGF-β responses.

Cells from premalignant lesions accentuate the adipocyte-stimulated inflammatory cytokine responses of spleen cells. Adipocytes have been shown to have the capacity to produce inflammatory mediators (7). Likewise, our prior studies have

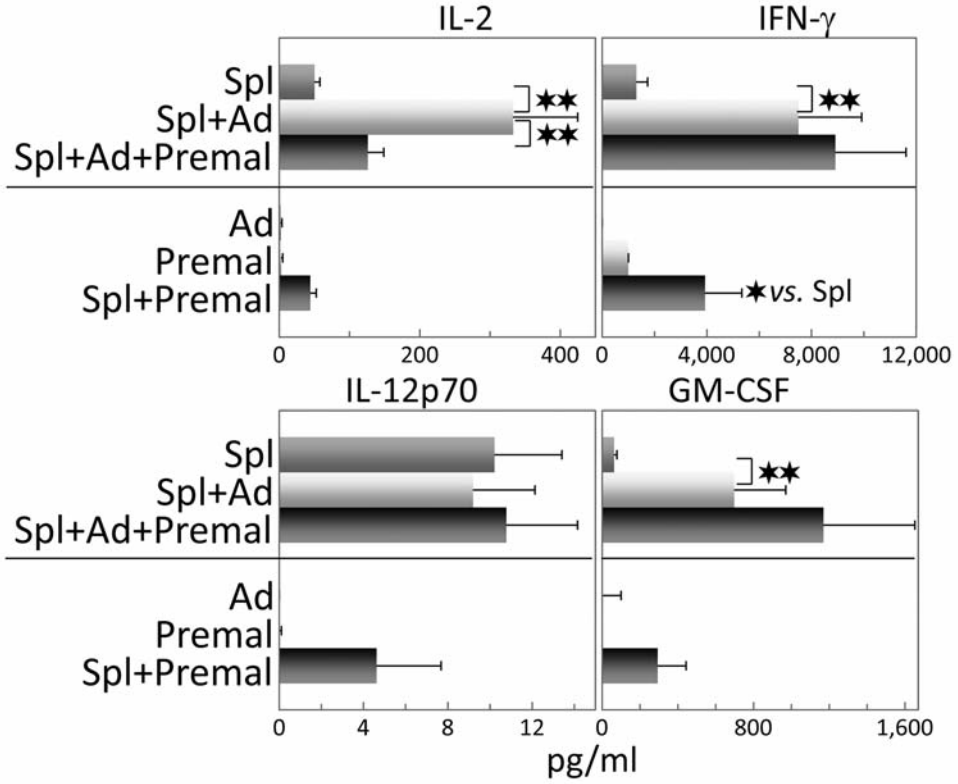

Figure 1. *Adipocyte-conditioned medium stimulates spleen cell production of predominantly T-cell-derived Th1-type cytokines, but this adipocyte-stimulated response is not further increased by premalignant lesion-derived mediators. Spleen cells (Spl) were incubated on anti-CD3-coated plates with 15 pg/ml IL-2 and media conditioned by adipocytes (Ad) or premalignant lesion cells (primal), or a pooled mixture of media conditioned independently by adipocytes or premalignant lesion cells. After three days, supernatants were collected and used to measure levels of the Th1 cytokines, interleukin (IL)-2, interferon-γ (IFN γ), IL-12 and granulocyte-macrophage colony-stimulating factor (GM CSF). Data shown are mean values±SEM. Significant differences are shown as **p<0.001.*

shown an inflammatory-skewed cytokine environment within premalignant oral lesions (31) and others have shown that inflammation contributes to cancer progression (34, 35). The presence of medium conditioned by adipocytes, stimulated spleen cell production of increased levels of the inflammatory mediators IL-1α, IL-1β, IL-6, IL-9 and TNF-α, although not of Th17 (Figure 3). With the exception of TNF-α, supernatant from cultures of premalignant lesions further enhanced spleen cells production of these inflammatory mediators. The increase in the production of inflammatory mediators after the addition of media conditioned by cells from premalignant lesions was dependent on the presence of the adipocyte-derived mediators. In control cultures, medium conditioned by cells from premalignant lesions stimulated spleen cell production of IL-1 and IL-6. However, the levels of these mediators induced by supernatants from cells from premalignant lesions alone were far less than those produced by spleen cells in the presence of adipocyte-conditioned medium. Levels in the culture media were also greater than the sum of the individual concentrations of these inflammatory mediators when spleen cell cultures were incubated with conditioned media from adipocytes or cells from premalignant lesions. It was also more than the additive levels of these inflammatory mediators in each of the conditioned media added to the spleen cell cultures.

While spleen cell production of IL-17 was not stimulated by adipocyte-conditioned medium, the addition of medium from cultures of cells from premalignant lesions triggered an increased spleen cell production of IL-17 (Figure 3). This IL 17 response of spleen cells was dependent on the presence of adipocyte-derived mediators as it was not stimulated by the supernatant of cells from premalignant lesions alone. These results show that the presence of the premalignant oral lesion further increases the inflammation-stimulatory effect of adipocytes toward spleen cells.

Spleen cell chemokine response to adipocyte mediators in the presence versus absence of media conditioned by cells from premalignant lesions. The CC chemokine family members CCL2 (MCP-1), CCL3 and CCL4 (MIP-1α and MIP-1β), and

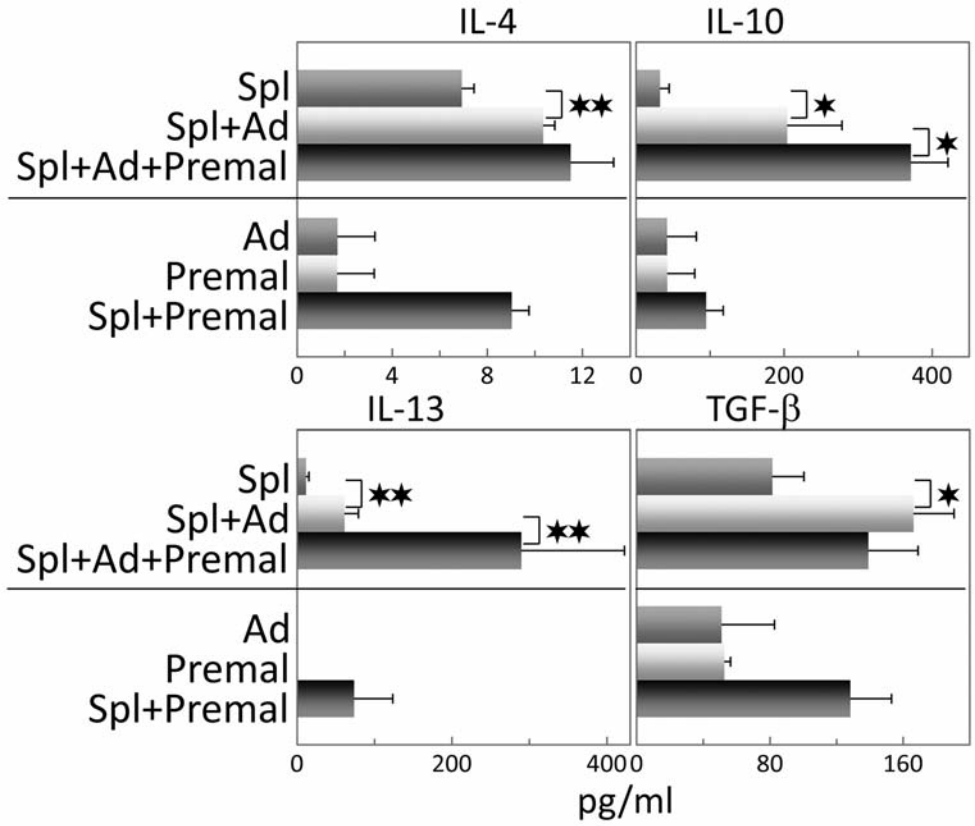

Figure 2. *Premalignant lesion cell-derived mediators further enhance the adipocyte-stimulated spleen cell production of the Th2-type cytokines interleukin (IL)-10 and IL-13. The same experimental design, as described in Figure 1 was used to measure the effects of premalignant lesion cell (Premal)-conditioned medium on adipocyte (Ad)-stimulated spleen cell (Spl) production of Th2-type cytokines. Significant differences are shown as *p<0.05 and **p<0.001.*

the CXC chemokine member CXCL9 (MIG) were originally shown to be monocyte products and are important in inflammatory cell recruitment (15, 36). Since the above results showed that the environment of a premalignant lesion exacerbates the adipocyte-stimulated spleen cell production of inflammatory mediators, spleen cell production of chemokines in the presence of media conditioned by adipocytes, and by cells from premalignant lesions was measured. The production of CCL3, CCL4 and CXCL9 was not stimulated by media conditioned by either adipocytes, premalignant lesion or the combination of media conditioned by adipocytes and by cells from premalignant lesions (Figure 4). In contrast to the secretion of the other CC chemokines, production of CCL2 was stimulated by adipocytes. However, this stimulation was not further enhanced by the premalignant lesion cell-conditioned medium. In fact, levels of CCL2 were lower in the cultures where spleen cells were exposed to adipocyte plus premalignant lesion supernatants than would be expected from the sum of the individual levels of CCL2 under each condition.

In contrast to the chemokines described above, which are predominantly macrophage-derived, production of the predominantly T-cell-derived chemokine, CCL5, was stimulated by adipocytes and further enhanced by the premalignant lesion cell supernatant (Figure 4). In fact, the production of CCL5 by spleen cells incubated with media conditioned only by supernatant from cells of premalignant lesions without the addition of adipocyte supernatant was significantly increased, but not to the level observed in cells incubated with adipocyte-derived mediators.

Influence of primary cultures of normal tongue tissues versus premalignant lesion tissues on the spleen cell-regulatory activities of adipocytes. The above studies showed the capacity of adipocytes to regulate conventional immune cell function and the impact of media conditioned by premalignant cells on this regulatory activity. These results prompted assessment of whether the *in vivo* environment of premalignant lesions similarly influences the responses of spleen cells to adipocyte-derived mediators. This was accomplished by determining

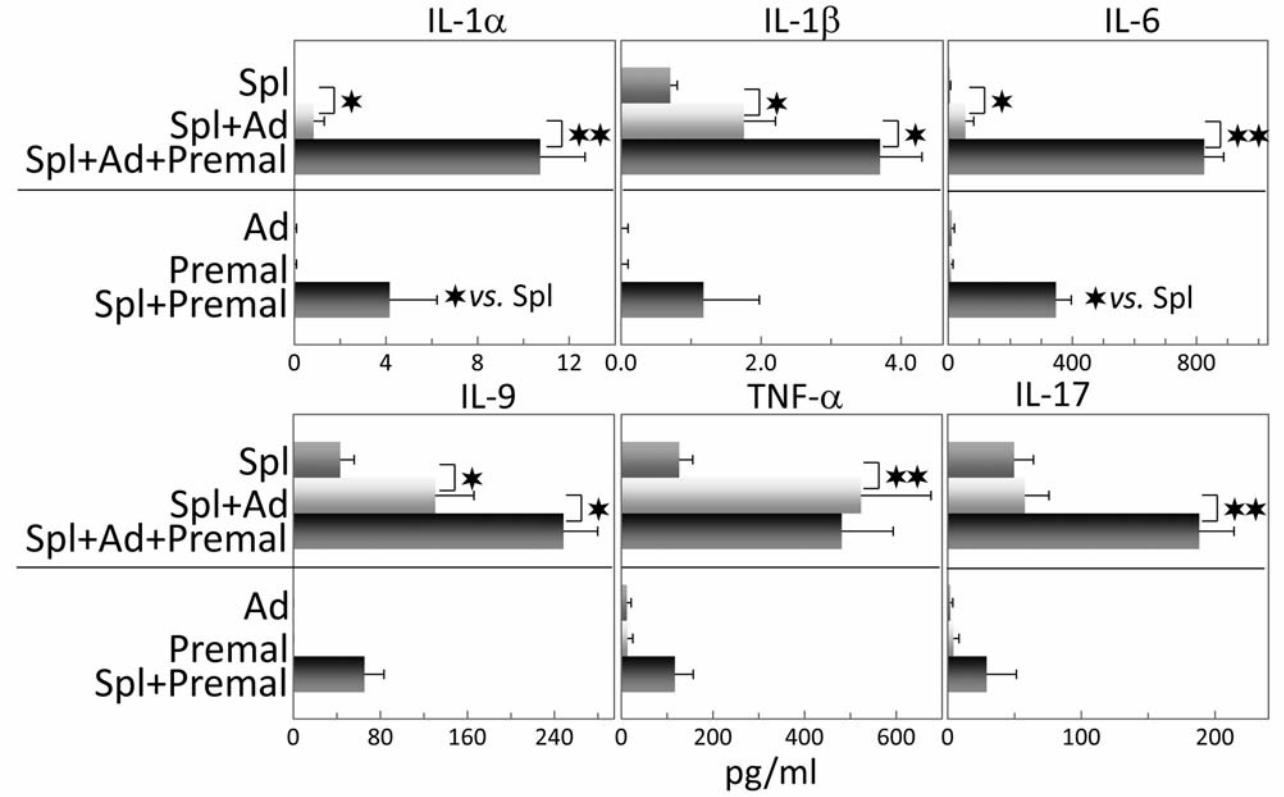

Figure 3. *Medium conditioned by cells from premalignant lesions exacerbates the adipocyte-stimulated spleen cell production of the inflammatory mediators interleukin (IL)-1α, IL-1β, IL-6 and IL-9, and synergistically triggers increased production of the inflammatory mediator IL-17. The same experimental design was used to measure the effects of premalignant lesion (Premal)-conditioned medium on adipocyte (Ad)-stimulated spleen cell (Spl) production of inflammatory mediators as was described for Figure 1. Significant differences are shown as *p<0.05 and **p<0.001.*

how media conditioned by cells from dissociated premalignant tissue and from normal tongue tissue impacted on the responses of spleen cells to adipocyte-derived mediators. One mediator for which the secretion was modulated by adipocytes and premalignant lesion cell supernatants was chosen from each of the categories of mediators: Th1, Th2, inflammatory and chemokine mediators. As shown above (Figures 1, 4), adipocyte-derived mediators stimulated spleen cells to produce IL-2, IL-10, IL-6 and CCL5 (Figure 5). The addition of media conditioned by cells from premalignant lesion tissue to spleen cell cultures resulted in a synergistic increase in production of IL-10, IL-6 and CCL5 (Figure 5). Media conditioned by normal tongue cells had no effect on the adipocyte-stimulated increased spleen cell production of IL-10 and CCL5. IL-6 secretion from spleen cells treated with conditioned medium from cells of normal tongue tissue was slightly increased, although not to levels observed when cells were incubated with conditioned media from adipocytes combined with that from cultures of premalignant lesions. Most notably, there was a striking increase in the adipocyte-stimulated spleen cell production of IL-10, IL-6 and CCL5 when cells were incubated with culture media conditioned by premalignant lesion tissue. The extent to which the premalignant lesion tissue environment enhanced adipocyte-stimulated production of these mediators (Figure 5) was significantly greater than when supernatants from established premalignant lesion cells were used instead (Figures 2, 4). This increased production of mediators in the presence of media conditioned by premalignant lesion tissue was dependent on the presence of adipocyte-derived mediators since culture of spleen cells with premalignant lesion tissue supernatant in the absence of media conditioned by adipocytes did not stimulate production of these mediators to the extent seen in the presence of adipocytes. In contrast to the stimulatory effect of the premalignant lesion environment on the adipocyte-stimulated spleen cell production of IL-10, IL-6 and CCL5, the effect on adipocyte-stimulated production of IL-2 was inhibitory (Figure 5). This was similar to that seen by the addition of media conditioned by established premalignant lesion cells (Figure 1). The magnitude of the inhibitory effect of supernatant from premalignant lesion tissue was also similar. The normal tongue environment had no effect on the adipocyte-stimulated spleen

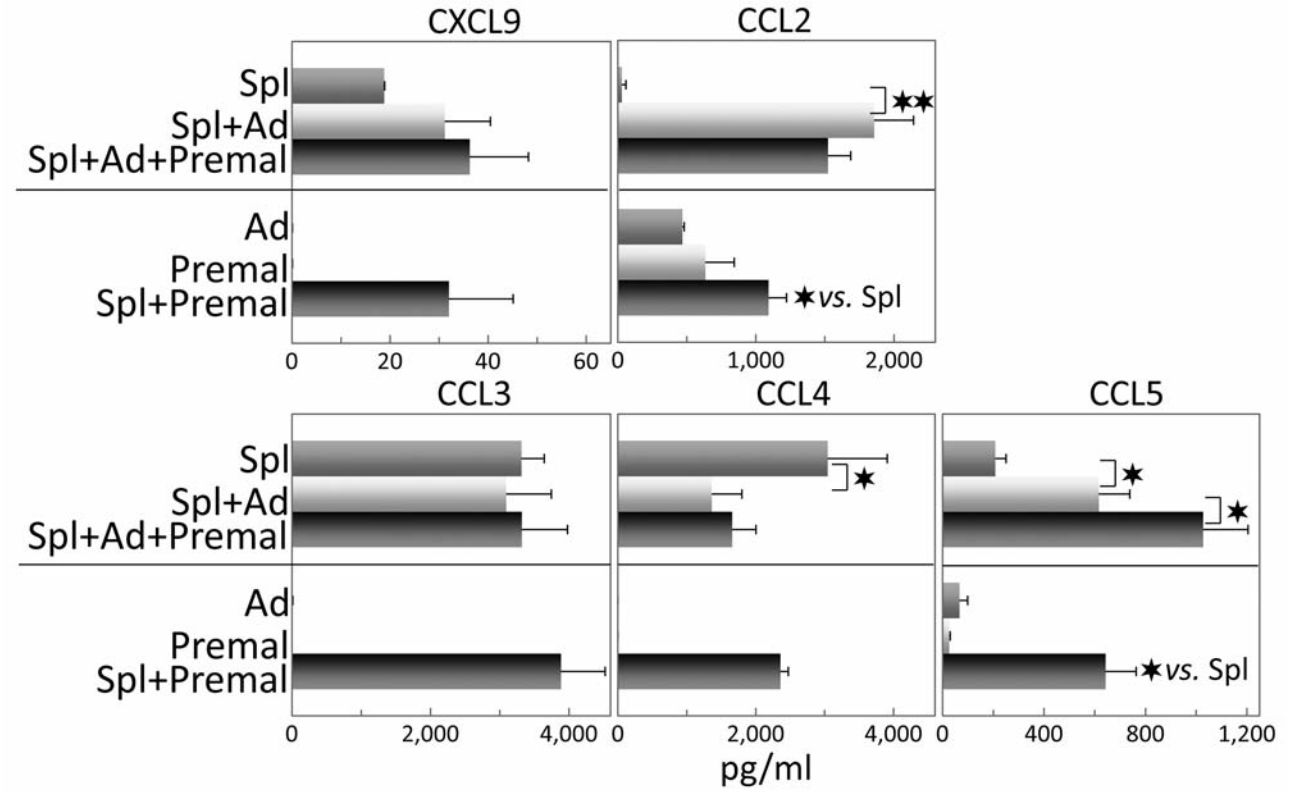

Figure 4. *Medium conditioned by cells from premalignant lesions synergizes with adipocyte-derived mediators to stimulate spleen cell production of the T-cell chemokine C-C motif ligand (CCL)5, but does not synergize to stimulate production of the predominantly macrophage-derived chemokine C-X-C motif ligand (CXCL)9, CCL2, CCL3 or CCL4. The same experimental design was used to measure the effects of media conditioned by cells from premalignant lesions (Premal) and adipocytes (Ad) on spleen cell (Spl) production of chemokines, as was described for Figure 1. Significant differences are shown as *p<0.05 and **p<0.001.*

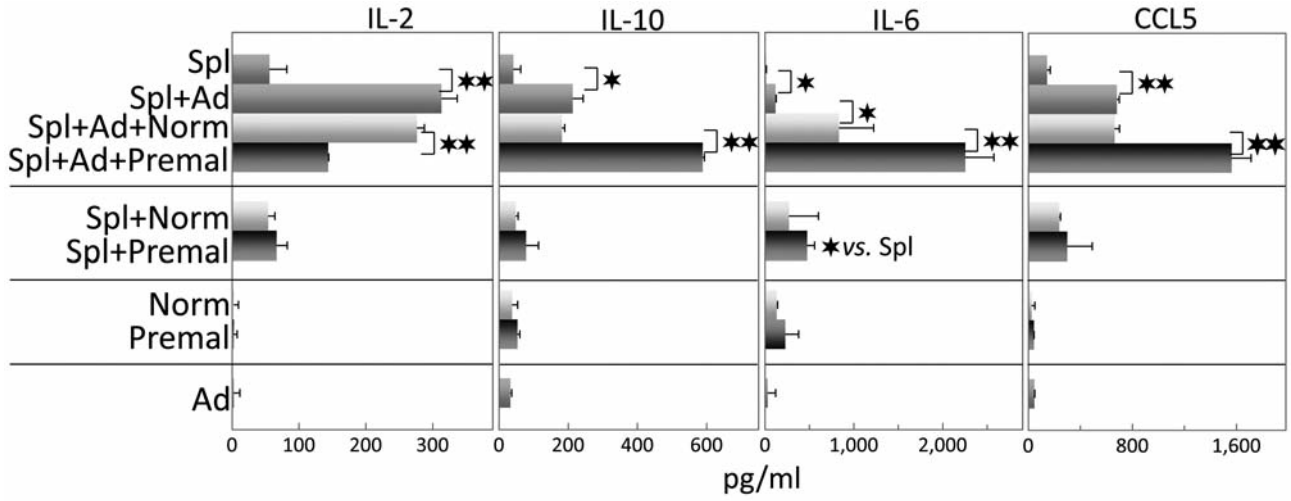

Figure 5. *Medium conditioned by primary cell cultures from premalignant lesion tissues more prominently enhances adipocyte-stimulated spleen cell production of interleukin (IL)-10, IL-6 and chemokine C-C motif ligand (CCL)5 than does medium from primary normal tongue tissue cells. The same experimental design as described in Figure 1 was used to measure the effects of media conditioned by primary cultures from premalignant lesions (Premal) or normal tongue (Norm) on adipocyte (Ad)-stimulated spleen cell (Spl) production of representative Th1 and Th2 cytokines, inflammatory mediators and chemokines. Significant differences are shown as *p<0.05 and **p<0.001.*

cell production of IL-2. These results extend those found when cells were incubated with media conditioned by established premalignant lesion cells by showing that the premalignant lesion environment can influence immune responsiveness to adipocyte-derived mediators.

Discussion

Adipose tissue has been shown to be in a chronically-inflamed state in obese patients (7, 8). While adipocytes produce a spectrum of adipokines that can either promote or diminish the state of inflammation, pro-inflammatory mediators such as leptin become more prominently produced in obesity, and production of anti-inflammatory mediators such as adiponectin declines (13, 14, 29). Cancer-related inflammation is an essential process in malignant disease, with common and defined players at different stages of progression (35, 36). Therefore, a chronic inflammatory state induced by adipocytes may eventually trigger the inflammation associated with human cancer (9, 37).

Despite the fact that adipocytes can produce inflammatory mediators, their capacity to serve as immune-regulatory cells by modulating conventional immune cells has not been studied, to our knowledge. Our prior studies showing a skewing toward an inflammatory environment within premalignant oral lesions (31), together with studies showing that inflammation can promote cancer development (27, 38), prompted us to determine if the environment of premalignant lesions influences the immune-regulatory activity of adipocytes, which themselves can stimulate immune cell production of Th1, Th2 and inflammatory mediators. The model used for this assessment was to culture normal spleen cells with media conditioned by cells from premalignant lesions or adipocytes differentiated from 3T3 L1 fibroblasts, or by culture of spleen cells with a mixture of these two conditioned media. Under the conditions used to differentiate the fibroblasts into adipocytes, they have previously been shown to be capable of producing inflammatory mediators, which is compatible with the increased production of inflammatory mediators in obese tissues (14).

The results of the present study show that the presence of the premalignant lesion further increases adipocyte-stimulated spleen cell production of Th2 and inflammatory mediators, although it had either no effect or an inhibitory effect on adipocyte stimulation of spleen cell Th1 cytokine production. The premalignant lesion-conditioned medium also directly stimulated spleen cell production of some immune mediators, in particular inhibitory mediators such as TGF-β and IL-4. To a lesser extent, this was also seen for the inflammatory mediators, but the effect was more prominent in the presence of adipocyte-conditioned media. It seems paradoxical that the combination of media conditioned independently by adipocytes and premalignant lesion cells stimulated spleen cell production of IL-10 and IL-13, both of which have anti-inflammatory properties. This paradox may be due to a spleen cell response attempting to temper the inflammatory environment or, since IL-10 and IL-13 are Th2-type cytokines, it may be consistent with a skewing from a Th1- to a Th2-type phenotype. The stimulation of chemokine production varied, but secretion of the predominantly macrophage-derived chemokines CCL3, CCL4 and CXCL9 was not stimulated by adipocyte-conditioned medium neither alone nor when combined with premalignant lesion supernatants. The exception to this was CCL2, whose production was stimulated by adipocytes, but not further stimulated by also adding premalignant lesion cell supernatant. Of interest was the more prominent enhancing effect of media conditioned by primary cells from premalignant lesion tissues, as opposed to established premalignant cell line on adipocyte-stimulated spleen cell production of inflammatory and inhibitory mediators. Since the media conditioned by premalignant lesion tissues would have mediators not only produced by premalignant lesion cells, but also by infiltrating cells, it is not possible to fully dissect the origin of the mediators that influenced spleen cell responsiveness to adipocyte-derived mediators. However, since the only difference in spleen cell responses to adipocyte-derived mediators in the presence of media conditioned by established premalignant lesion cells *versus* by premalignant lesion tissue is quantitative, it suggests that the environment of the premalignant lesion, rather than its infiltrating cells, impacts on the spleen cells.

Since spleen cell production of a number of inflammatory mediators was stimulated by adipocyte-derived mediators, the absence of a stimulatory effect by adipocytes on levels of IL-17 was unexpected as it too is an inflammatory mediator. However, IL-17 production was triggered by the addition of premalignant lesion supernatant. This triggering was dependent on the presence of adipocyte-conditioned medium as premalignant lesion supernatants were not sufficient to stimulate spleen cell production of IL-17 in the absence of adipocyte-conditioned medium. These results are consistent with our previous studies which demonstrated that there was a marked increase in Th17 cell levels and IL-17 release in premalignant tongue lesions and regional lymph nodes (31).

The mechanisms by which adipocytes modulate conventional immune cell production of cytokines or chemokines has not yet been determined. Prominent adipocyte-derived mediators include the anti-inflammatory mediator adiponectin, whose levels decrease in obesity, and the pro-inflammatory mediator leptin, whose levels increase in obesity (13, 39). The immune-regulatory capacity of leptin has previously been suggested by the expression of leptin receptors on lymphocytes, and by immune dysfunction in leptin knock-out and leptin receptor knock out animals (40-42). In addition, adipocytes can be activated to produce a host

of mediators that could influence conventional immune cell functions. These include CCL2, IL-6, IL-8, TNF-α, TNF-β and leukotrienes (7, 38). Whether any of these mediators or combinations of mediators provide adipocytes with their immune-regulatory capacity is yet to be determined. The mechanisms by which premalignant lesions, with their inflammatory environment, impact on the immune-modulatory effects of adipocyte-derived mediators have also not been determined. There are also multi-faceted interplays to consider, for example, the contribution of soluble adipocyte mediators in inducing immune cell production of inflammatory cytokines such as CCL2 to drive the recruitment of macrophages and other inflammatory cells. Their response to inhibitory and inflammatory cytokines whose production is escalated by cytokines from premalignant lesions, can lead to the acquisition of macrophage M2 properties or T-cell Th2 properties, promoting tumor proliferation and progression, stromal deposition and remodeling, and inhibiting protective immunoreactivity (43).

The design of the present studies used media conditioned separately by adipocytes, cells from premalignant lesions or from dissociated normal and premalignant lesion tongue tissue to minimize the complexity of the cross-talk that can occur in co-cultures of spleen cells plus either adipocytes and/or cells from premalignant lesions. In reality, the *in vivo* environment is expected to have the added complexity of this cross-talk. The influence of obesity on progression of oral cancer can be tested by applying the 4NQO carcinogen-induced lesion model to either diet-induced obese mice, or to leptin- or leptin receptor-knockout mice and then determining the impact of obesity on progression of premalignant oral lesions to cancer and the role of leptin in this progression. Nevertheless, the results of this study are consistent with prior studies showing skewing toward an inflammatory state in the presence of a premalignant lesion (31) and the inflammatory state that is associated with obesity (7, 8). Whether the present results demonstrating that the enhanced Th2 and inflammatory state that is compounded by both premalignant oral lesions and adipocytes translates into a similar state in obese patients having premalignant lesions, and how that impacts on the progression of premalignant lesions to oral cancer is yet to be explored. Such possibilities are, however, consistent with studies showing an increased risk of premalignant lesions and cancer in obese patients. For example, obese patients have a higher probability of developing hepatic cancer (15). In addition, the risk of developing premalignant or malignant endometrial polyps is increased in obese women (44). Studies in carcinogen-induced mouse models have shown increased development of premalignant colonic lesions in obese mice (45). While obesity has been associated with an increased risk for a variety of cancer types, studies have not been conducted to determine if obesity is a risk factor for oral premalignant lesions and oral cancer. How obesity and obesity-associated inflammation, together with the inflammatory environment of premalignant oral lesions, impacts on the progression of premalignant oral lesions to cancer is yet to be determined.

Conflicts of Interest

None of the Authors have any conflict of interests to declare.

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