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Skewing of immune cell cytokine production by mediators from adipocytes and endothelial cells

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Since adipose tissue is composed of adipocytes as well as other cell types including endothelial cells, this study sought to determine how mediators from adipocytes and from endothelial cells impact on immune cell production of cytokines. A minimalistic design was used in which media conditioned by adipocytes or by endothelial cells were added individually or as a mixture to normal spleen cells. Media from adipocytes or endothelial cells each stimulated spleen cell production of Th1 cytokines, Th2 cytokines, most of the measured inflammatory cytokines, and some chemokines. However, a mixture of media conditioned by adipocytes and by endothelial cells inhibited production of Th1 cytokines and skewed reactivity toward a Th2 and inflammatory phenotype. Adiponectin, but not leptin, was shown to contribute to the skewing of immune responsiveness to endothelial cell-derived mediators.

Introduction

Obesity is rapidly becoming not only a national healthcare crisis, but also a significant contributor to the economic problems in the US as well as worldwide.[1,2] Accompanying obesity are increases in diabetes and diabetes-associated illnesses, cardiovascular diseases, and the incidence of cancer.[1-6] Adipose tissue is comprised of multiple cell types in addition to adipocytes. This includes fibroblasts, mesenchymal stem cells, endothelial cells, and infiltrating inflammatory cells,[7,8] although the characteristics of cells populating adipose tissue can vary depending on the site and microenvironment.[9] The maintenance of adipose tissue relies on a well-formed vasculature. This vascularization is dependent on VEGF, without which adipose tissue has reduced vascular density and is hypoxic.[10] Obesity is often considered to be a chronic inflammatory state. Consequently, adipose tissue is also populated with inflammatory cells such as lymphocytes and macrophages, which themselves can additionally contribute to the inflammatory condition.[11,12]

The mechanism(s) that contribute to the inflammatory state of adipose tissue can have multiple sources and, most likely, result from of a combination of factors. Adipocytes themselves can produce adipokines such as leptin and VEGF, which are pro-inflammatory and pro-angiogenic.[13,14] They can also produce IL-6, prostaglandins, and a variety of chemokine.[11] Similarly, endothelial cells are well equipped to produce immune-stimulatory mediators and to promote inflammation. This includes their capacity to produce inflammatory mediators such as IL-6, PGE_2, and TNF-α.[15,16] Adipose tissue expresses increased levels of chemokines, which can contribute to the recruitment of immune cells such as T cells and macrophages.[12] In terms of the biological importance of adipokines, adiponectin has anti-diabetic, anti-atherosclerotic, and anti-inflammatory properties and, thus, may influence cardiovascular disease and some types of cancer.[17-22] Leptin, an adipokine that can also be produced by activated endothelial cells, regulates food intake, energy expenditure, and has immune regulatory functions.[23] However, in obesity, levels of adiponectin decline and levels of leptin increase.[24,25]

The pro-inflammatory potential of endothelial cells, especially following stimulation, has been reasonably well characterized.[15] The location of vascular endothelial cells establishes an intimate relationship with immune cells and facilitates recruitment of the immune infiltrate. While adipocytes have pro-inflammatory capabilities,[26] their interaction with endothelial cells can further heighten the pro-inflammatory environment. For example, mediators from adipocytes can upregulate endothelial cell adhesion molecules to enhance the transmigration of monocytes from the vasculature into adipose tissue.[27] Less clear is how adipocytes and endothelial cells might each function in a regulatory capacity toward conventional immune cells. Since the adipose tissue environment contains mediators from both endothelial cells and adipocytes, the present study assessed the responsiveness of splenic immune cells to media conditioned individually by adipocytes or by endothelial cells, and the combination of mediators from both adipocytes and from endothelial cells. This study design allows the dissection of how select populations of cells that comprise adipose tissue can impact on normal immune function, realizing that there are yet other cells types within adipose

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tissue. Our results show that media conditioned individually by adipocytes and endothelial cells each stimulated conventional immune cell production of Th1, Th2, most inflammatory, and some chemokine mediators. However, exposing splenocytes to the mixture of mediators secreted from adipocytes and endothelial cells inhibited their Th1 profile and skewed the immune phenotype toward sustainment of a Th2-type inhibitory and inflammatory-type of phenotype.

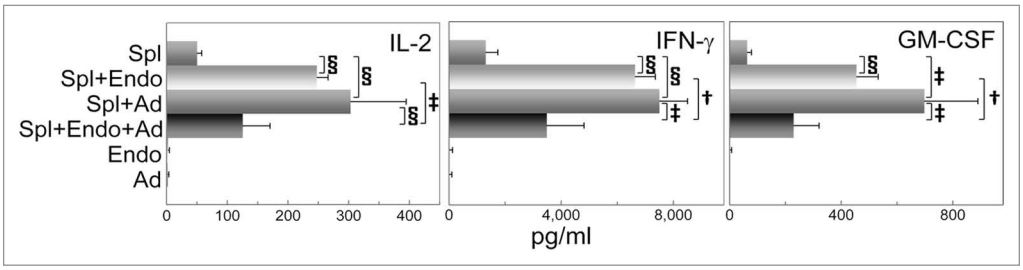

Figure 1. Stimulated Th1 responses are inhibited by combining media from adipocytes and from endothelial cells. Spleen cells (Spl) were incubated on anti-CD3-coated plates with media conditioned by adipocytes (Ad) or endothelial cells (Endo), or a pooled mixture of media conditioned independently by adipocytes or endothelial cells. After 3 d, supernatants were collected and used to measure levels of the Th1 cytokines, IL-2, IFN-γ, and GM-CSF. Data shown are mean values ± SEM with significance of differences being indicated as $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.02$, or $^{\S}P < 0.01$.

Results

Although both adipocytes and endothelial cells can produce a variety of inflammatory mediators,[11,15,24,28-31] the impact of adipocytes and endothelial cells as regulators of conventional immune cell activity is less well defined. As shown by the data presented in **Figure 1**, normal spleen cells that were incubated with media conditioned only by endothelial cells were stimulated to produce increased levels of the Th1-type cytokines IL-2, IFN-γ, and GM-CSF. Media conditioned only by adipocytes also stimulated production of these cytokines. In contrast, addition of a mixture of media conditioned by endothelial cells and by adipocytes diminished the levels of Th1 cytokines that were produced by spleen cells compared with the stimulated levels produced in response to media conditioned by either endothelial cells or adipocytes alone. These results show that while endothelial cells and adipocytes each stimulate spleen cell production of Th1 cytokines, the combination of the media inhibits the stimulatory effect of mediators from the individual cell populations.

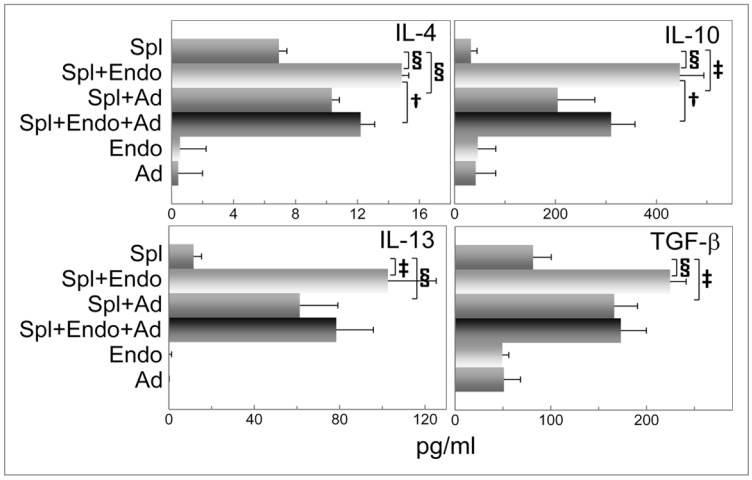

Figure 2. Endothelial cell- and adipocyte-derived mediators stimulate spleen cell production of Th2-type inhibitory cytokines. The same experimental design as described in **Figure 1** was used to measure the effects of media conditioned by adipocytes or endothelial cells, or a mixture of media from each of these cell populations on spleen cell production of Th2-type cytokines. Data shown are mean values ± SEM with significance of differences being indicated as $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.02$, or $^{\S}P < 0.01$.

Spleen cell production of the inhibitory Th2-type mediators IL-4, IL-10, IL-13, and TGF-β were prominently stimulated by media conditioned by endothelial cells and, to a lesser extent, media from adipocytes (**Fig. 2**). Also, endothelial cell-derived mediators prominently stimulated spleen cell production of the pro-inflammatory cytokines IL-6, IL-9, IL-17, and TNF-α, production of the predominantly T-cell-derived chemokine CCL5, and production of several predominantly monocyte-derived chemokines to include CXCL9 and CCL2 (**Figs. 3 and 4**). Adipocyte-conditioned media also stimulated spleen cell production of TNF-α, IL-9, and CCL5 to levels that were comparable to those induced by endothelial cell-derived mediators, but stimulated production of IL-6, IL-17, and monokines to a lesser extent. Production of the monokine CCL4 was inhibited by media from endothelial cells or from adipocytes. When media conditioned by adipocytes and from endothelial cells were mixed and added to the spleen cells, production of Th2-type inhibitory cytokines and inflammatory mediators was either slightly reduced or comparable to the endothelial cell-stimulated levels (**Figs. 2 and 3**). The mixture of endothelial- and adipocyte-derived mediators sustained the increased spleen cell production of CXCL9 and CCL2, and restored the basal level of CCL4 production (**Fig. 4**). Of note is that production of Th2 cytokines, inflammatory mediators, and chemokines was not increased to a level that was additive for endothelial cell- or adipocyte-stimulated production of mediators, but was not inhibited by the mixture of conditioned media from adipocytes and from endothelial cells as was seen for Th1 cytokine production.

Studies were initiated to identify adipokines that might skew the immune responsiveness to endothelial cell mediators. Two adipokines that are prominently produced by adipocytes are adiponectin, which tends to have anti-inflammatory properties, and leptin, which tends to have pro-inflammatory properties.[32,33] Therefore, we conducted pilot studies to determine if either adipokine might mediate the adipocyte modulation of

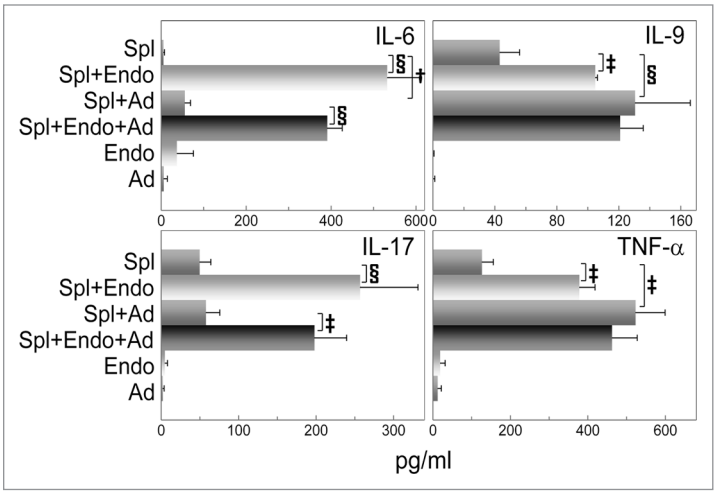

Figure 3. Media derived from endothelial cells and from adipocytes stimulate spleen cell production of inflammatory mediators. The same experimental design as described in **Figure 1** was used to measure the effects of media conditioned by adipocytes or endothelial cells, or a mixture of media from each of these cell populations on spleen cell production of inflammatory mediators. Data shown are mean values ± SEM with significance of differences being indicated as $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.02$, or $^{\S}P < 0.01$.

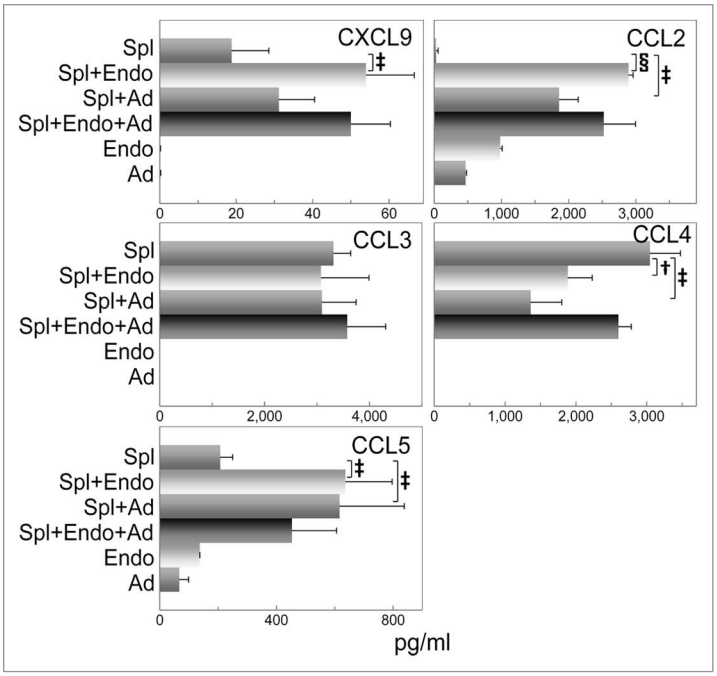

Figure 4. Stimulation of select chemokines by media conditioned by adipocytes or by endothelial cells. The same experimental design as described in **Figure 1** was used to measure the effects of media conditioned by adipocytes or endothelial cells, or a mixture of media from each of these cell populations on spleen cell production of chemokines. Data shown are mean values ± SEM with significance of differences being indicated as $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.02$, or $^{\S}P < 0.01$.

spleen cell cytokine production and or contribute to the altered response of spleen cells to the cytokine-stimulatory effects of endothelial cells. Recombinant globular adiponectin (gArcp30) and leptin were added to spleen cells in lieu of adipocyte-conditioned media at levels that were comparable to those measured in adipocyte-conditioned media, which approximated 10 μg/ml and 30 ng/ml, respectively. Spleen cell production of representative Th1, inhibitory and inflammatory cytokines that were shown above to be modulated by endothelial cell-conditioned media was then measured and the results are summarized in **Figure 5**. Addition of leptin to spleen cells in the absence of endothelial cell-conditioned media inhibited spleen cell production of IFN-γ ($P < 0.05$), but had no effect on spleen cell production of the other mediators measured, regardless of whether they were from Th1, Th2 inhibitory, or inflammatory categories. Adiponectin tended to inhibit spleen cell production of IFN-γ but this inhibitory trend was not statistically significant. Adiponectin had no effect on spleen cell production of other mediators. When added to spleen cells in the presence of endothelial cell-conditioned media, leptin had no effect on endothelial cell-stimulated spleen cell production of the measured cytokines. However, adiponectin inhibited the spleen cell activation to produce the Th1-type cytokines IL-2 and IFN-γ in response to endothelial cell-conditioned media. Adiponectin had a far lesser level of inhibitory activity toward endothelial cell-stimulated spleen cell production of the Th2-type cytokine IL-4 and the inflammatory mediator TNF-α, and it had no inhibitory activity toward endothelial cell-stimulated production of the other Th2 and inflammatory mediators. These results suggest that adiponectin may contribute to the skewing of the spleen cell responsiveness to endothelial cell-derived mediators away from a Th1 profile while only slightly limiting the endothelial cell-stimulated spleen cell production of Th2 and inflammatory mediators.

Discussion

Obesity is considered to be a chronic state of inflammation.[12,26,34] In addition to an infiltrate consisting of T cells and macrophages, adipose tissue contains other cell types such as fibroblasts, mesenchymal stromal cells, and endothelial cells. The maintenance of adipose tissue is dependent on vascularization.[10] Both adipocytes and endothelial cells can produce a variety of inflammatory mediators.[11,35] Less well studied is their capacity to serve as immune regulatory cells. To enable dissection of the contribution of adipocytes and endothelial cells on immune activity, our study determined how mediators produced by each of these cell types or the combination of mediators from these cells impact on immune cell production of cytokines and chemokines.

Our results showed that, in general, endothelial cells and adipocytes each stimulated immune production of Th1 cytokines, Th2 inhibitory cytokines, most pro-inflammatory cytokines, and some select chemokines. Perplexing was the consequence of exposing spleen cells to a mixture of mediators from endothelial cells and from adipocytes, which skewed the

immune phenotype away from Th1 cytokines toward sustainment of a Th2 and inflammatory phenotype.

The above results raise several questions, including the mechanism by which media conditioned by adipocytes or endothelial cells stimulate spleen cell cytokine production. There is a multitude of candidates. Prominent adipokines are adiponectin and leptin.[25,34-36] In some disease states, endothelial cells can also upregulate expression of leptin.[37] Adipocytes can also produce cytokines including IL-6, TNF-α, PAI, IL-10, and VEGF.[13,17-19] Upon stimulation, endothelial cells can produce IL-1, IL-6, IL-7, IL-8, IL-10, PGE_2, and various chemokines.[15,38,39] Which among the many potential mediators produced by endothelial cells or adipocytes contribute to the stimulation of spleen cell production of cytokines and chemokines is yet to be determined. Studies were initiated to identify the mediators secreted by adipocytes that influence spleen cell responsiveness to endothelial cell-derived mediators. Neither leptin nor adiponectin stimulated spleen cell production of Th1, inhibitory, or inflammatory mediators. Also, leptin did not affect spleen cell responsiveness to mediators from endothelial cells. However, adiponectin was inhibitory to spleen cell activation to produce Th1-type cytokines in response to endothelial cell-derived mediators, but did not impact on production of Th2 or inflammatory mediators. It is important to note that the present study used the globular form of adiponectin (glycosylated homotrimer). Thus, further studies need to assess the impact of the form of adiponectin, such as the forms resulting from association into high molecular weight oligomeric complexes, in regulating immune cell cytokine responsiveness to endothelial cell-derived mediators.

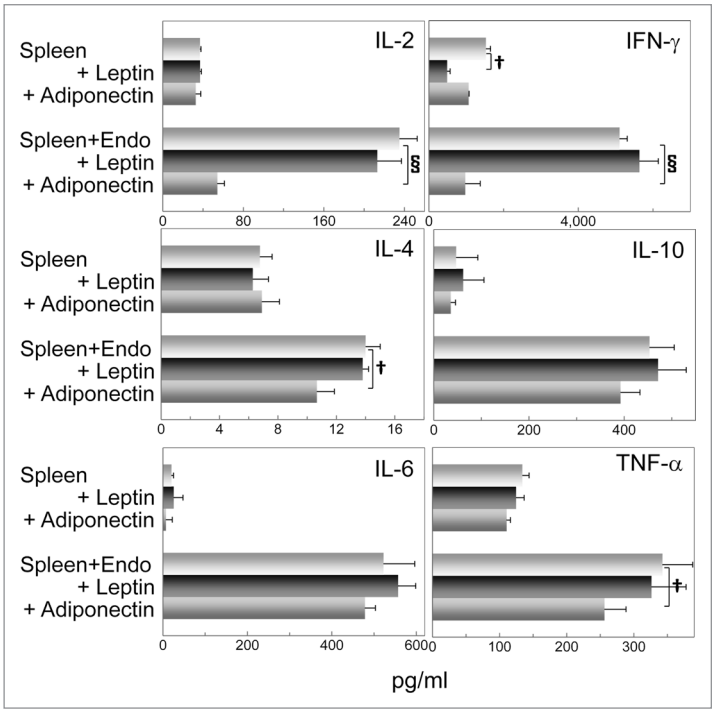

Figure 5. Adiponectin inhibits endothelial cell-stimulated spleen cell production of Th1 cytokines. Either adiponectin (10 μg/ml) or leptin (30 ng/ml) were added to spleen cells in the presence or absence of endothelial cell-conditioned media. After 3 d, supernatants were collected and used to measure levels of the Th1, Th2 inhibitory and inflammatory mediators. Data shown are mean values ± SEM with significance of differences being indicated as $^{\dagger}P < 0.05$, or $^{\S}P < 0.01$.

While the stimulatory effect toward Th2 or inflammatory cytokines seen with media conditioned by endothelial cells or adipocytes is sustained when media from each of these cells are combined, it is puzzling that there isn't an additive effect. It is possible that there is overlap in the signaling pathways that are stimulated in spleen cells by media conditioned by adipocytes and endothelial cells. Overlapping candidates could include the JNK and p38 MAPK pathways.[40,41] In such a scenario, combining the media would not further increase production of Th2 cytokines or inflammatory mediators.

The skewing of cytokines toward a Th2 and inflammatory phenotype by the combination of mediators from endothelial cells and from adipocytes is reminiscent of skewing that has been shown to occur in multiple cancer types. For example, studies with head and neck cancer from patients and a murine lung cancer model showed that the cancer cells skew the immune regulatory activity of endothelial cells such that they inhibit T-cell production of Th1 cytokines and stimulate their production of Th2 cytokines.[39,42,43] The mechanisms by which endothelial cells are induced by tumor to skew T-cell reactivity toward the Th2 phenotype is by tumor cell production of VEGF.[44] Adipocytes are also capable of producing VEGF, with the health of adipose tissue being dependent on VEGF-dependent maintenance of a strong vasculature.[13] This raises the possibility that, like tumor cells, adipocytes have the capacity to influence endothelial cells to skew T-cell reactivity toward a Th2 phenotype. This possibility has yet to be explored in vivo.

The minimalistic design of our studies first assessed spleen cell responsiveness to adipocyte-derived or endothelial cell-derived mediators, and then looked at responsiveness to a mixture of the conditioned media. This design avoided the added complexities of interactions between adipocytes and endothelial cells that would occur if they were co-cultured. Future studies will need to be expanded to co-culture spleen cells with endothelial cells and adipocytes to better approximate the intercellular interactions that occur in vivo. These in vitro co-culture analyses will also need to be validated by assessment of the immune regulatory capacity of adipocytes and endothelial cells isolated from lean or obese mice.

These studies showed that the admixture of mediators from endothelial cells and adipocytes regulate immune cell cytokine phenotypes in a manner that is somewhat unanticipated. However, they are consistent with the inflammatory state that is attributed to obesity. Our results suggest that the combination of adipocytes and endothelial cells could orchestrate a Th2 and inflamed state in adipose tissue. These immune regulatory interactions in turn are likely to be contributors to the obesity-associated complications of inflammation such as increased incidence of asthma and cancer risk.[34]

Materials and Methods

So as to maintain uniformity among experiments, the mouse 3T3-L1 fibroblast cell line (ATCC) was used for the present studies as opposed to freshly isolated adipocytes, which have a higher likelihood of variability. The 3T3-L1 cells were grown in DMEM (Invitrogen) culture medium containing 10% 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. A medium to differentiate confluent cultures of fibroblasts into adipocytes was additionally supplemented with 25 mM glucose, 0.5 mM 3-isobutyl-1-methylxantine (IBMX, Sigma-Aldrich), and 1 μM dexamethasone (Sigma-Aldrich). After 72 h, the medium was replaced with DMEM culture medium containing 25 mM glucose plus 1.74 μM insulin (Sigma-Aldrich) for 48 h. After differentiation, the insulin was removed and adipocytes were maintained in DMEM culture medium containing 25 mM glucose and 10% FBS. After 5 d, the spent medium was removed and replaced with fresh DMEM culture medium without added glucose. Twenty-four hours later, the conditioned medium was collected and used for spleen cell cultures.

The bEnd.3 cells (ATCC), were grown in the same DMEM culture medium as was described above for culture of 3T3-L1 fibroblasts. Once the endothelial cell cultures approached confluence, the culture medium was removed and replaced with fresh DMEM culture medium and then collected after 24 h for addition to spleen cell cultures.

To assess the immune regulatory capacity of adipocytes and endothelial cells, studies analyzed cytokine production by spleen cells after incubation with media conditioned by 3T3 adipocytes, by bEnd.3 endothelial cells, or mixture of media conditioned individually by adipocytes or endothelial cells. Unfractionated spleen cells from healthy 8–12 wk old female C57BL/6 mice (Charles Rivers Laboratories) were used as a source of immune cells so as to better capture the array of cytokine mediators that would be produced in the in vivo setting. The spleens from multiple mice were homogenized using a Stomacher™80 homogenizer (Seward Limited) and then pooled. They were washed and suspended in the DMEM culture medium described above for growth of 3T3-L1 fibroblasts and 1×10^6 cells were plated into 24-well plates coated with 2.5 μg/well immobilized anti-CD3 antibody. The spleen cells were not fractionated so that T cells as well as antigen-presenting cells were present in the cultures. Control cultures contained DMEM culture medium alone that had been treated in the same manner as conditioned media. Experimental cultures contained 1:2 diluted medium conditioned by adipocytes or endothelial cells admixed with fresh culture medium to yield a 25% solution of conditioned media, or a mixture of an equal volume of media conditioned individually by adipocytes or endothelial cells diluted 1:2 with fresh culture medium to yield a 25% solution of each of the conditioned media. In several studies, recombinant globular adiponectin (gArcp30) or leptin (R&D Systems) were added to spleen cells in lieu of adipocyte-conditioned media at levels that were comparable to those measured in media from adipocytes, which approximated 10 μg/ml and 30 ng/ml, respectively. For each of the analyses, the total volume per well was 2 ml. Spleen cells were incubated with experimental media for 72 h prior to collection of supernatants for cytokine measurements. During this incubation time, cells were microscopically observed to assure that any impact of the conditioned media on the immune cells was due to their cytokine production as opposed to toxicity or cell death. Also, viability of the cells following culture was examined. There was no impact of the experimental media on viability of spleen cells as judged by trypan blue exclusion.

Levels of immune mediators in supernatants from spleen cell cultures were conducted with reagents from BD Biosciences using their instructions. Cytokine levels were measured with the mouse Th1/Th2/Th17 cytometric bead array kits, while levels of chemokines and IL-13 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 to the manufacturer's instructions. Relative amounts of each cytokine were analyzed using FCAP Array software (BD Biosciences).

Data were reported using the mean ± standard error of the mean (SEM). Differences among levels of cytokines produced in response to the experimental media were compared by the one-way ANOVA. To compare one variable condition between two groups, the 2-tailed Student *t* test was used. Studies with endothelial- or adipocyte-conditioned media, or with adiponectin or leptin were conducted as 4 independent experiments.

Disclosure of Potential Conflict of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

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