

Role of the adipocyte-specific NF- κ B activity in the regulation of IP-10 and T cell migration

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Krinninger P, Brunner C, Ruiz PA, Schneider E, Marx N, Foryst-Ludwig A, Kintscher U, Haller D, Laumen H, Hauner H. Role of the adipocyte-specific NF- κ B activity in the regulation of IP-10 and T cell migration. *Am J Physiol Endocrinol Metab* 300: E304–E311, 2011. First published November 9, 2010; doi:10.1152/ajpendo.00143.2010.—Infiltration of immune cells into adipose tissue plays a central role in the pathophysiology of obesity-associated low-grade inflammation. The aim of this study was to analyze the role of adipocyte NF- κ B signaling in the regulation of the chemokine/adipokine interferon- γ -induced protein 10 kDa (IP-10) and adipocyte-mediated T cell migration. Therefore, the regulation of IP-10 was investigated in adipose tissue of male C57BL/6J mice, primary human and 3T3-L1 preadipocytes/adipocytes. To specifically block the NF- κ B pathway, 3T3-L1 cells stably overexpressing a transdominant mutant of I κ B α were generated, and the chemical NF- κ B inhibitor Bay117082 was used. Adipocyte-mediated T cell migration was assessed by a migration assay. It could be shown that IP-10 expression was higher in mature adipocytes compared with preadipocytes. Induced IP-10 expression and secretion were completely blocked by an NF- κ B inhibitor in 3T3-L1 and primary human adipocytes. Stable overexpression of a transdominant mutant of I κ B α in 3T3-L1 adipocytes led to an inhibition of basal and stimulated IP-10 expression and secretion. T cell migration was induced by 3T3-L1 adipocyte-conditioned medium, and both basal and induced T cell migration was strongly inhibited by stable overexpression of a transdominant I κ B α mutant. In addition, with the use of an anti-IP-10 antibody, a significant decrease of adipocyte-induced T cell migration was shown. In conclusion, in this study, we could demonstrate that the NF- κ B pathway is essential for the regulation of IP-10 in 3T3-L1 and primary human adipocytes. Adipocytes rather than preadipocytes contribute to NF- κ B-dependent IP-10 expression and secretion. Furthermore, NF- κ B-dependent factors and especially IP-10 represent novel signals from adipocytes to induce T cell migration.

obesity; inflammation; signaling; adipokine; nuclear factor- κ B; interferon- γ -induced protein 10 kiloDalton

OBESITY IS CHARACTERIZED AS a state of chronic low-grade inflammation with elevated plasma levels of proinflammatory adipokines (7). Chemokines came into the focus of diabetes research, since studies in mouse models and in humans have shown that obesity is associated with an enhanced infiltration of macrophages (4, 20) and T cells, especially Th1 (T helper) polarized T cells, into adipose tissue (AT) (6, 11, 22). Recent research revealed a functional link between specific T cell

subpopulations and obesity-associated insulin resistance (5, 13, 21). However, the molecular mechanism involved in T cell infiltration in AT is still poorly understood.

There is growing evidence that local chemokine secretion promotes lymphocyte infiltration into AT. The chemokines regulated upon activation, normal T cell expressed and secreted (RANTES)/chemokine CC motif ligand 5, stromal cell-derived factor 1 α /chemokine CXC motif ligand 12, and macrophage inflammatory protein 3 α /chemokine CC motif ligand 20 (CCL20) seem to play a role in T cell recruitment into AT (5, 11, 22). Yet, except for CCL20, their cellular origin is unknown. One further potent chemoattractant for T cells, particularly Th1 cells, is the CXC chemokine interferon- γ -induced protein 10 kDa (IP-10). The serum concentrations of IP-10 correlate positively with the incidence of type 2 diabetes (9). In addition, it is produced by freshly isolated mature human adipocytes from various depots with a positive correlation to body mass index (BMI) (10). Thus it may be hypothesized that IP-10 is also involved in the chronic inflammatory process observed in obesity and type 2 diabetes. In different cell types, IP-10 was shown to be regulated mainly by NF- κ B and/or Jak-Stat activation in a highly cell type-specific manner (14, 18, 23). NF- κ B is a major regulator of inflammatory processes with an important function in the pathogenesis of obesity-related insulin resistance (24). However, its specific function for the accumulation of immune cells in AT remains elusive.

The aim of the present study was 1) to investigate whether NF- κ B is involved in the regulation of IP-10 in preadipocytes and adipocytes and 2) to characterize the potential role of NF- κ B activity and IP-10 for T cell infiltration into AT.

MATERIALS AND METHODS

Subjects. For the isolation of preadipocytes, adipocytes, and stromal vascular fraction (SVF), human subcutaneous AT was obtained from healthy women and men undergoing elective abdominal surgery; no selection was made for BMI, age, or gender. Patients with diabetes, cancer, serious diseases, acute infection, malignancies, or any other consuming disease were excluded. Informed consent was obtained from all subjects. The study was approved by the ethical committee of the Technische Universität München, Germany.

Mice. For all experiments, male C57BL/6J mice were housed in a temperature-controlled (25°C) facility with a 12:12-h light-dark cycle. Mice were fed a standard chow D12450B (%kcal: 20% protein, 70% carbohydrate, and 10% fat) or a high-fat diet D12492 (%kcal: 20% protein, 20% carbohydrate, and 60% fat) from Sniff (Soest, Germany). Experimental feeding was started at the age of 10 wk and then for 16 wk (Fig. 1A) or for 10 wk (Fig. 1B). Besides these experiments,

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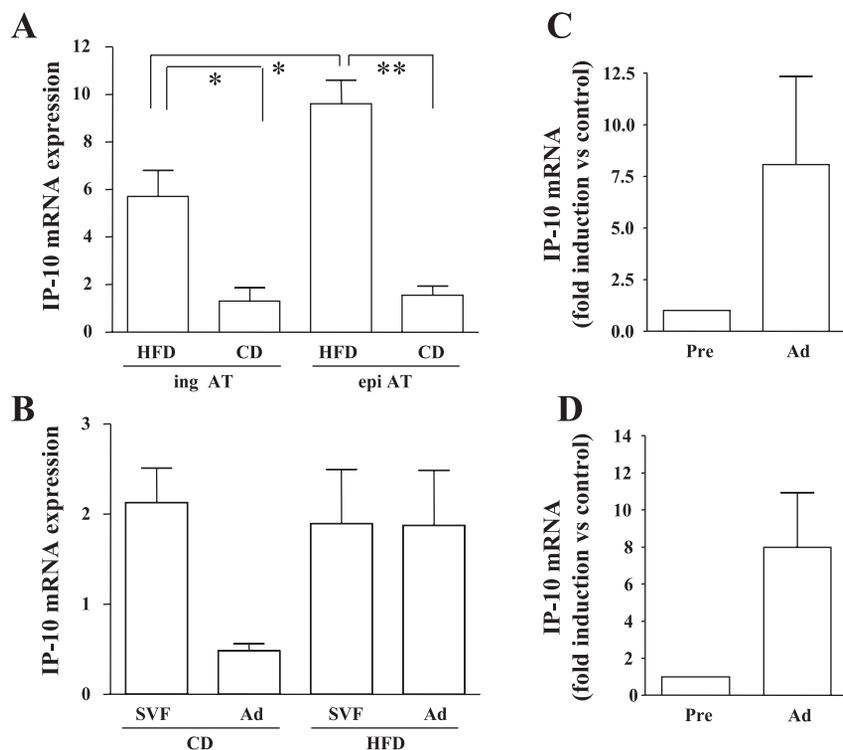


Fig. 1. Interferon γ -induced protein 10 kDa (IP-10) expression in mouse and human adipose tissue and adipose tissue cell types. Total mRNA was isolated from inguinal (ing) and epididymal (epi) AT of 4 male C57BL/6 mice fed a standard chow compared with mice fed a high-fat diet for 16 wk (A); from primary stromal vascular fraction (SVF) and adipocytes (Ad), freshly isolated from adipose tissue of 6 male mice/group either fed a high-fat diet (HFD) or control mice fed a standard chow diet (CD) for 10 wk (B); from freshly isolated primary human preadipocytes (Pre) and mature human adipocytes (Ad) of adipose tissue from subjects (C); and from 3T3-L1 preadipocytes and adipocytes ($n = 3$) (D). mRNA was reverse transcribed, and IP-10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was determined by qRT-PCR. The expression level of one sample CD/ingAT (A) and of quantitative RT-PCR (qRT-PCR) one sample CD/SVF (B) was set to one. Results are means \pm SE. * $P < 0.05$ and ** $P < 0.01$.

we obtained cDNA from mouse experiments published earlier (11) for additional analysis of gene expression. Male C57BL/6J mice were fed a high-fat diet for 10 wk vs. a control diet, and AT samples were collected after 0, 5, and 10 wk. All animal procedures were in accordance with institutional guidelines with national law and were approved.

Cell culture. Freshly isolated primary mature adipocytes and preadipocytes/SVF were isolated and cultured as previously described (10). Isolation of SVF and adipocytes from murine gonadal fat tissue was performed according to the protocol published by Weisberg et al. (19). 3T3-L1 cells (Xantos Biomedicine, Munich, Germany) and the retroviral packaging cell line Phoenix-Eco provided by B. Baumann (University of Ulm, Ulm, Germany) were cultured in basal medium containing DMEM (Invitrogen, Karlsruhe, Germany), 10% FCS (Invitrogen), and 50 μ g/ml gentamycin (Roth, Karlsruhe, Germany) at 37°C, 5% CO₂. Differentiation of 3T3-L1 cells was performed as described (12). Cells were incubated in the presence or absence of the following agents: IL-1 β (R&D, Wiesbaden, Germany), lipopolysaccharide (LPS; Sigma-Aldrich, Munich, Germany), Bay117082 (NF- κ B inhibitor; Biomol, Hamburg, Germany), AG-490 (Jak-inhibitor; Sigma-Aldrich), U-0126 [mitogen-activated protein kinase kinase 1 (MAP2K1) inhibitor; Promega], LY-294002 [phosphoinositide 3-kinase (PI 3-kinase) inhibitor; Sigma-Aldrich], goat anti-IP-10 antibody (R&D), or the respective control goat IgG (R&D). All agents were not cytotoxic at the concentrations used and as demonstrated in a cell viability assay (data not shown).

Transfection of cells. 3T3-L1 cells were transfected on various days of differentiation using Lipofectamine 2000 transfection reagent (Invitrogen). DNA (1.5 μ g) and 3 μ l of reagent were mixed and added to the cells for 4 h. After transfection (24 h), luciferase activity was measured using the dual-luciferase assay (Promega, Mannheim, Germany). In all transfections, 0.3 μ g of ubiquitin-promoter Renilla luciferase vector was cotransfected to normalize for transfection efficiency. Ubiquitin-promoter Renilla luciferase vector and 6xNF- κ B-luciferase vector were provided by B. Baumann (University of Ulm) and IP-10 promoter-luciferase vector by D. Haller (Technische Universität München, Munich, Germany).

Retroviral infection of 3T3-L1. To inhibit NF- κ B in 3T3-L1 cells, a dominant-interfering I κ B α mutant protein (I κ B α -mut, serine 32/36 mutated to alanine, resulting in a nondegradable repressor) or an empty vector as control (mock) was stably integrated into 3T3-L1 cells using retroviral gene transfer. For virus production, the pCFG5-I κ B α -mut retroviral vector (provided by B. Baumann, University of Ulm) was transfected using calcium phosphate transfection in the ecotrophic Phoenix virus-producer cell line. Supernatant containing the retrovirus was collected 48 and 72 h after transfection and used to infect 3T3-L1 preadipocytes in the presence of 8 μ g/ml diethylaminoethyl. After a second infection (4 h), the selection with zeocin was started for 5 days until all cells were 100% positive for green fluorescent protein.

ELISA. 3T3-L1 cells were stimulated with IL-1 β or LPS for 20 h. The supernatant was immediately frozen at -80° C. IP-10 protein level was determined by a mouse-specific DuoSetELISA assay, according to the manufacturer's instructions (R&D).

Quantitative RT-PCR. Total RNA was isolated using the NucleoSpinRNAII kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using the cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). PCR for detection of human/mouse IP-10, chemokine CXC motif receptor 3 (CXCR3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mouse adiponectin were performed using qPCR-SYBR-Green (ABgene; Epsom) or for primary mouse AT using the universal probe library (Roche, Germany). Primers ordered from MWG (Ebersberg, Germany) were designed using PrimerExpress-Software (Applied Biosystems); the sequences are given in Supplemental Table 1 (Supplemental data for this article may be found on the *American Journal of Physiology: Endocrinology and Metabolism* website.). Quantification of gene regulation was performed by the $\Delta\Delta C_t$ method; results were corrected for GAPDH expression.

Western blot and electrophoretic mobility shift assay. For Western blot analysis, 50 μ g of total protein extracts were separated on 12.5% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany). Membranes were blocked (PBS, 0.1% Tween, and 2% Amersham blocking reagent), stained

with anti-I κ B α (Santa Cruz Biotechnology, Heidelberg, Germany) followed by incubation with a horseradish secondary antibody (Dianova, Hamburg, Germany), and visualized by enhanced chemiluminescence (Amersham Bioscience, Freiburg, Germany). As a loading control, stripped membrane was incubated with anti- β -actin (Santa Cruz Biotechnology). Electrophoretic mobility shift assays were performed as previously described (3).

Migration assay. For the analysis of the ability of splenocytes to migrate toward a chemokine gradient established by adipocytes, 3T3-L1 cells were differentiated as described. Adipocytes were stimulated as indicated in DMEM medium containing 2% heat-inactivated FCS. After 18 h, the supernatant was taken and filled in the bottom chambers of Costar Transwells (Costar, Cambridge, MA). Lympholyte-M (Cedarlane Laboratories, Hornby, ON, Canada) purified splenocytes (10⁶/sample in DMEM medium containing 2% FCS) were assayed for transmigration with 5- μ m-pore-size Costar Transwell culture inserts. The migration occurred at 37°C in a humidified atmosphere with 5% CO₂ over 3 h. After incubation, cells that migrated to the lower chamber were harvested, stained with anti-CD8-FITC (eBioscience, Frankfurt, Germany) and anti-CD4-PerCP (Peridin Chlorophyll Complex Protein; BD Biosciences, Heidelberg, Germany), counted by Becton-Dickinson FACS-LSRII (inhibitory antibody experiments) or Beckton-Dickinson FACS-CantoII (all other experiments), and analyzed by FACS-Diva software 6.1.

Statistical analysis. Values for all experiments are expressed as means \pm SD or means \pm SE of a minimum of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's test, Student's *t*-test, or one-sample *t*-test, with *P* < 0.05 and *P* < 0.01 indicated.

RESULTS

High-fat diet-induced weight gain increases IP-10 mRNA expression levels in epididymal and inguinal white AT of C57BL/6J mice. To investigate IP-10 expression in AT under a high-fat diet, we performed a first set of experiments with male mice fed a high-fat diet for 16 wk. These mice showed a significant increase in body weight and inguinal/epididymal fat pad weight (Supplemental Fig. 1, A and B). Real-time PCR analysis revealed a 5.7- and 9.6-fold increase in IP-10 mRNA expression in inguinal (subcutaneous) and epididymal (visceral) AT, respectively, in mice fed the high-fat diet compared with control mice fed a standard chow (Fig. 1A). This upregulation was significantly higher in epididymal compared with inguinal AT.

IP-10 is differentially expressed in different AT cell types in mice and humans. AT is composed of adipocytes and the so called "SVF" that in turn includes preadipocytes, but also macrophages and T cells. To further elucidate the origin of AT IP-10, we isolated SVF and adipocytes from lean mice as well as from high-fat diet-fed mice. In lean mice, SVF expressed 2.5-fold higher levels of IP-10 mRNA (*P* < 0.05) than adipocytes, whereas, in overweight mice (high-fat diet fed), no difference was detectable because of the increase of IP-10 expression in adipocytes (Fig. 1B). Hence, upon weight gain, adipocytes are the cell type that contributes to the higher IP-10 mRNA expression in AT. Similar to overweight mice, in samples derived from overweight human subjects, a comparable expression level of IP-10 in isolated SVF and adipocytes was observed (Supplemental Fig. 2).

Primary human and 3T3-L1 adipocytes express higher levels of IP-10 mRNA than preadipocytes. Next, the cellular origin of IP-10 synthesis in AT was investigated in more detail. Human subcutaneous AT samples were used for the isolation of pre-

adipocytes and adipocytes. mRNA expression of IP-10 was considerably higher in adipocytes compared with undifferentiated preadipocytes (Fig. 1C). The same results were obtained using mouse 3T3-L1 adipocytes compared with undifferentiated preadipocytes (Fig. 1D). On average, adipocytes expressed eightfold more IP-10 mRNA than preadipocytes in both models.

IL-1 β and LPS induce IP-10 protein secretion in 3T3-L1 cells. Next, we asked if IP-10 secretion by adipocytes is further enhanced by stimuli that mimic an inflammatory status. 3T3-L1 adipocytes were exposed to IL-1 β or LPS. Both stimuli are known to mediate their inflammatory response through NF- κ B. An increase in IP-10 release was observed at concentrations of 10 ng/ml IL-1 β or 1 μ g/ml LPS (Supplemental Fig. 3).

IP-10 expression/secretion and promoter activity increase during adipogenesis. To further explore the mode of IP-10 regulation in adipocytes, IP-10 mRNA expression/secretion and IP-10 promoter activity were analyzed during 3T3-L1 adipogenesis. IP-10 mRNA expression was initially low in 3T3-L1 cells (on days 1-6 after induction of adipogenesis) but increased steadily during adipogenesis and reached a maximal eightfold increase in freshly isolated mature adipocytes on day 14 of adipocyte differentiation compared with day 0 preadipocytes (Fig. 2A). To verify whether the increase of IP-10 mRNA expression during differentiation is accompanied by an enhanced protein secretion, IP-10 protein release was analyzed during differentiation. IP-10 protein secretion was detectable exclusively in fully differentiated adipocytes, whereas, in preadipocytes, no protein release could be observed (Fig. 2B).

In parallel, we assessed the mRNA expression level of adiponectin and glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity as markers of adipogenesis (Supplemental Fig. 4, A and B). These data indicate that IP-10 expression increases with the level of adipocyte differentiation to mature adipocytes.

To study the molecular mechanisms involved in the expression of IP-10 in adipocytes, a reporter containing 673 bp of the proximal murine IP-10 promoter (containing 2 κ B sites) in front of the luciferase gene was transfected into 3T3-L1 cells. In parallel, an NF- κ B-responsive luciferase-reporter construct containing six κ B consensus sites was transfected to determine NF- κ B activity. The activities of both the IP-10 promoter (Fig. 2C) and the synthetic NF- κ B-responsive reporter (Fig. 2D) behave exactly the same, with a slight decrease after induction of differentiation and an overall increase in luciferase activity over the 14-day differentiation period.

NF- κ B binds to IP-10 promoter κ B sites and inhibition of NF- κ B decreases IP-10 expression in primary human adipocytes and secretion in 3T3-L1 adipocytes. Because IP-10 is known to be regulated by two κ B sites in its promoter, we analyzed NF- κ B DNA-binding activity to these sites. Electrophoretic mobility shift assays revealed an increased NF- κ B binding activity to the IP-10 promoter κ B sites upon IL-1 β stimulation in 3T3-L1 adipocytes (Fig. 3). To further investigate the role of NF- κ B for the expression of IP-10 in AT, we used an NF- κ B inhibitor (Bay117082). Mouse 3T3-L1 adipocytes and freshly isolated mature human adipocytes were preincubated for 1 h with or without Bay117082 (1 and 5 μ M), followed by incubation with IL-1 β (10 ng/ml) for 20 h. Bay117082 (1 and 5 μ M) significantly prevented IL-1 β stimula-

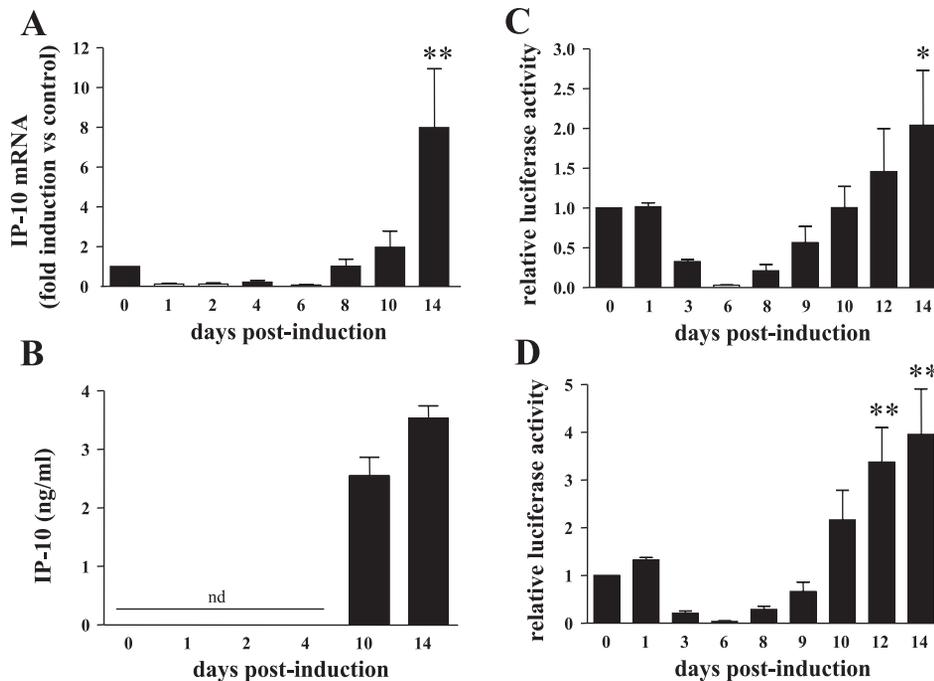


Fig. 2. Evolution of IP-10 gene mRNA expression/protein secretion and IP-10 promoter and 6xNF- κ B reporter activity during adipogenesis. 3T3-L1 preadipocytes were harvested on several days after induction of differentiation. mRNA was reverse transcribed, and IP-10 and GAPDH mRNA expression was determined by qRT-PCR (A). IP-10 protein secretion in supernatant was measured by enzyme-linked immunosorbent assay (ELISA, B). 3T3-L1 cells were transiently transfected with an IP-10 promoter luciferase reporter (C) or a 6xNF- κ B-luciferase reporter (D). Luciferase activities of extracts were determined 24 h after transfection and normalized based on Renilla luciferase activity. Results are means \pm SE ($n \geq 3$). * $P < 0.05$ and ** $P < 0.01$.

tion of IP-10 protein release from 3T3-L1 adipocytes (Fig. 4A) and of IP-10 mRNA expression in freshly isolated mature human adipocytes (Fig. 4B) in a dose-dependent manner. The Jak/Stat inhibitor AG-490 reduced protein release in 3T3-L1 cells significantly by twofold only at a high dose. MAP2K1 (U-O126) and PI 3-kinase inhibitor (LY-294002) reduced IL-1 β -induced IP-10 protein release in 3T3-L1 adipocytes; however, these effects were not statistically significant (Supplemental Fig. 5). Treatment of cells with IL-1 β stimulation or Bay117082 inhibitor did not affect cell viability (Supplemental Fig. 6).

Stable overexpression of a dominant-negative I κ B α mutant inhibits NF- κ B activity in 3T3-L1 cells. To block the NF- κ B pathway more specifically and to further elucidate the contribution of NF- κ B signaling to IP-10 regulation in 3T3-L1

preadipocytes and adipocytes, we used retroviral gene transfer to express a dominant-negative interfering I κ B α -mut or an empty vector as control (mock). I κ B α -mut overexpression was confirmed by Western blot (Fig. 5A). In control cells (mock), IL-1 β -induced I κ B α degradation started after 10 min (Fig. 5B); however, no degradation occurred in cells expressing the mutant protein (Fig. 5C). Adipogenic differentiation capacity was monitored morphologically and by measuring the specific activity of GPDH, a lipogenic marker enzyme. 3T3-L1-I κ B α -mut cells showed no changes in GPDH activity (data not shown) and Oil red O staining vs. the control cells (Supplemental Fig. 7).

Inhibition of NF- κ B signaling in preadipocytes and adipocytes leads to a significant decrease in basal and stimulated IP-10 expression and secretion. IP-10 expression and secretion by preadipocytes and adipocytes were measured depending on the overexpression of a dominant-negative mutant of I κ B α . Basal and stimulated IP-10 mRNA expression (Fig. 5D) and protein secretion (Fig. 5E) were higher in mock adipocytes compared with mock preadipocytes. This reduced level of IP-10 mRNA expression correlates with the observation that almost no IP-10 protein is released by preadipocytes. Moreover, in adipocytes, the overexpression of I κ B α -mut significantly reduced basal and stimulated IP-10 mRNA expression and protein release compared with control cells. These results demonstrate the central role of NF- κ B in the regulation of IP-10 in 3T3-L1 adipocytes rather than in preadipocytes in which the reduction of IP-10 expression and secretion in cells overexpressing I κ B α -mut was less evident.

IL-1 β - and LPS-induced T cell migration is inhibited by overexpression of I κ B α -mut. NF- κ B regulates the expression of several chemokines in many cell types. To elucidate the relevance of the NF- κ B pathway for adipocyte-induced T cell migration, we performed migration experiments using conditioned medium from the here-established cell lines. In a migration assay using isolated mouse spleen leukocytes, minimal

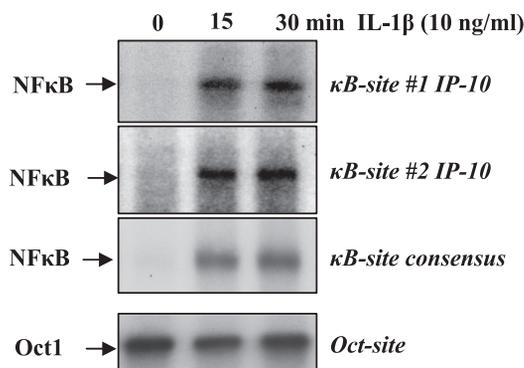


Fig. 3. IL-1 β induces NF- κ B-DNA binding to IP-10 promoter κ B sites. Protein of 3T3-L1 cells induced with IL-1 β for the indicated times was harvested, and NF- κ B binding activity was analyzed by electrophoretic mobility shift assay (EMSA). The radioactive labeled DNA probes used were: κ B-site#1 IP-10, first binding site for NF- κ B in the IP-10 promoter; κ B-site#2 IP-10, second binding site for NF- κ B in the IP-10 promoter; κ B-site consensus, consensus κ B site; Oct1 site, octamer binding site here showing Oct1 binding as loading control.

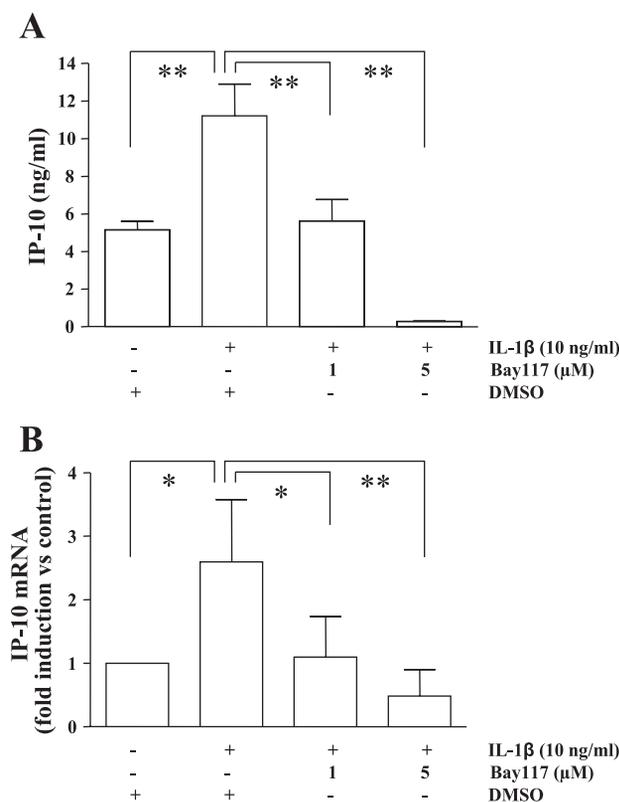


Fig. 4. An NF- κ B inhibitor decreases IP-10 expression in primary human adipocytes and protein secretion in 3T3-L1 adipocytes. 3T3-L1 adipocytes on *day 14* of differentiation (A) and freshly isolated primary human mature adipocytes (B) were preincubated for 1 h with or without the NF- κ B inhibitor Bay117082 as indicated, followed by incubation with IL-1 β (10 ng/ml) for 20 h. Medium with vehicle (DMSO) was used as control. IP-10 protein concentration was measured using ELISA (A); mRNA was reverse transcribed and IP-10 and GAPDH mRNA expression was determined by qRT-PCR (B). Results are means \pm SD ($n \geq 4$). * $P < 0.05$ and ** $P < 0.01$.

induction of T cell migration was observed in response to supernatants from preadipocytes stimulated with either IL-1 β or LPS (Fig. 6, A and B). However, in response to supernatants from stimulated adipocytes, CD4⁺ T cells exhibited a significant increased migratory activity compared with supernatants from unstimulated adipocytes (Fig. 6, C and D). This activation of migration was significantly abrogated using media from 3T3-L1 cells overexpressing I κ B α -mut. Similar results were obtained analyzing the migration of CD8⁺ T cells.

Adipocyte-induced T cell migration is inhibited by neutralization of IP-10. To answer the question whether the ability of 3T3-L1 adipocyte supernatants to induce T cell migration was mediated by the chemokine IP-10, a specific neutralizing antibody was added to the supernatants. Addition of this antibody to conditioned 3T3-L1 adipocyte media inhibited basal CD4⁺ T cell migration (Fig. 7A) and CD8⁺ T cell migration (Fig. 7B) in a dose-dependent manner, whereas addition of an isotype control did not alter migration of T cells.

Expression of IP-10 and its receptor CXCR3 is upregulated in AT during progression of weight gain. The progression of leukocyte migration in AT in obese humans or mice raises the question whether the expression of responsible chemokines in AT is upregulated during the time course of weight gain. We reanalyzed samples from a recently published study (11) show-

ing T lymphocyte infiltration in visceral AT as a primary event in AT inflammation. In AT from mice fed a high-fat diet for 0, 5, or 10 wk or a chow control diet for 10 wk, a slight upregulation of IP-10 mRNA level after 5 wk and a significant threefold upregulation after 10 wk of a high-fat diet compared with initial levels (*week 0*) was observed (Fig. 8A). In the same tissues, expression of the IP-10 receptor CXCR3 was significantly upregulated at *week 5* (1.7-fold) and *week 10* (2.5-fold) (Fig. 8B). Both IP-10 and CXCR3 expression increased in parallel to T cell infiltration shown recently (11). These data give further evidence that IP-10 secreted from AT plays a role in the recruitment of T cells in obese AT.

DISCUSSION

In the present study, we could demonstrate that IP-10 is upregulated in AT of a diet-induced obese mouse model. In addition, adipocytes rather than SVF and preadipocytes significantly contribute to elevated IP-10 levels in obesity. Our data clearly show that the NF- κ B pathway plays a central role for the regulation of IP-10 in 3T3-L1 and primary human adipocytes. These results also provide evidence that NF- κ B target genes, especially IP-10, are major activators of AT CD4⁺ and CD8⁺ T cell migration.

Upon feeding a high-fat diet, IP-10 expression was significantly stronger upregulated in epididymal (visceral) AT compared with subcutaneous (inguinal) AT. This observation is in line with the hypothesis that visceral AT contributes to a larger extent to obesity-associated inflammation compared with subcutaneous AT. In humans, a weak correlation of IP-10 secretion from adipocytes with BMI was reported (10), sustaining the function of IP-10 in the pathophysiology of obesity.

Adipokines are expressed by several different cell types in AT (7). It has been postulated that AT stromal vascular cells, especially preadipocytes, are the main source of proinflammatory adipokines. However, some data also indicate an active role of freshly isolated mature adipocytes in obesity-related inflammation. Compared with preadipocytes, IP-10 was predominantly expressed and secreted in fully differentiated adipocytes in parallel with the late differentiation marker adiponectin. The preadipocyte/adipocytes difference in IP-10 mRNA expression was more pronounced in the stably infected vs. naïve 3T3-L1 cells, indicating an influence of infection on IP-10 expression. However, the amount of secreted IP-10 protein was similar comparing infected vs. naïve 3T3-L1. Therefore, we can assume that infection and selection of cells influence the expression of IP-10, but only marginal its protein secretion. The here-observed predominant expression and secretion of IP-10 in adipocytes and its upregulation in obesity provides evidence that adipokines released from freshly isolated mature adipocytes contribute to local inflammation in obesity.

Furthermore, besides adipocytes, SVF cell types other than preadipocytes strongly contribute to IP-10 expression. The higher IP-10 expression in SVF of lean mice is turned to an equal expression level when comparing SVF vs. adipocytes in obese mice. This shows the strong contribution of adipocytes to the obesity-dependent increased expression of IP-10. However, the molecular mechanisms regulating its expression and their contribution to the mild inflammation observed in obese AT are still topics that require further investigations.

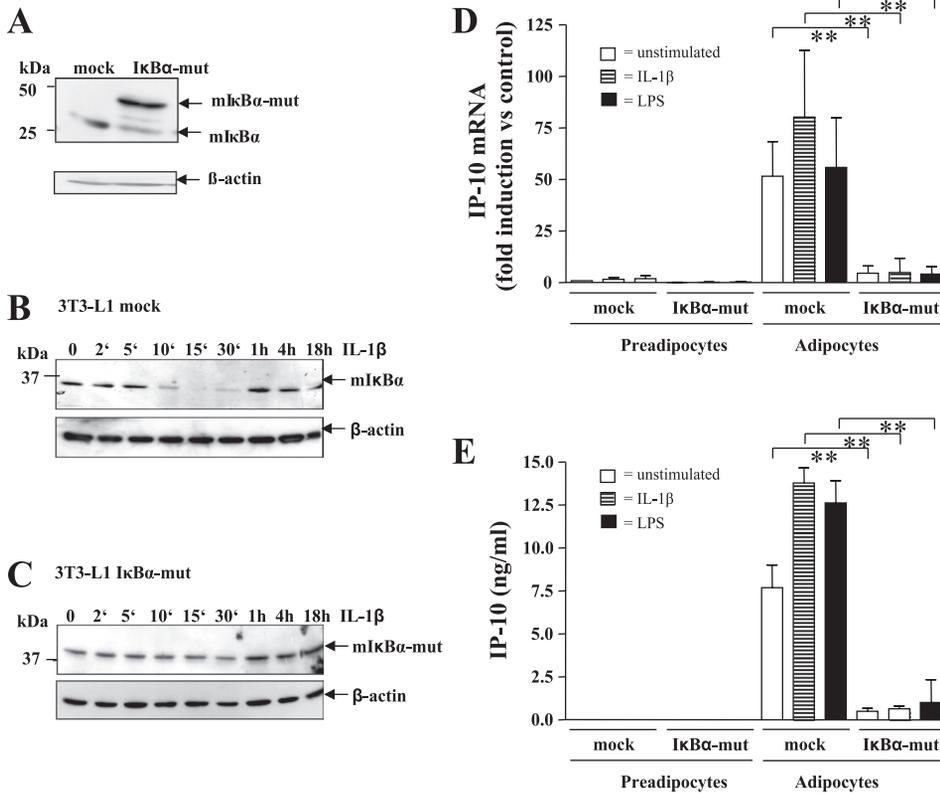


Fig. 5. Stable overexpression of IκBα mutant protein (IκBα-mut) in 3T3-L1 cells inhibits NF-κB activity and IP-10 expression/secretion. Protein from 3T3-L1 cells was harvested, and 50 μg protein were used for Western blot with anti-IκBα antibody. β-Actin was assayed as an internal loading control (A). 3T3-L1-mock (B) and 3T3-L1-IκBα-mut (C) adipocytes were incubated with IL-1β (10 ng/ml). Protein of cells was harvested at the indicated time points, and Western blot was performed as described above. RNA and supernatant from stably infected 3T3-L1 preadipocytes and adipocytes were harvested 20 h after adding fresh media with or without IL-1β (10 ng/ml) or lipopolysaccharide (LPS, 1 μg/ml) stimulation. mRNA was reverse transcribed, and IP-10 and GAPDH mRNA expression was determined by qRT-PCR (D). IP-10 protein concentration was measured from supernatants using ELISA (E). Results are means ± SD (D and E) (n ≥ 5). **P < 0.01.

It was shown that the secretion of IP-10 by adipocytes is enhanced by stimulation with interferon γ (IFNγ) (10). Our experiments reveal that proinflammatory stimuli like IL-1β and LPS, known activators of the NF-κB pathway, are also able to induce IP-10 secretion by adipocytes. In cell types other than adipocytes, IP-10 gene expression is controlled in a complex manner with interrelated roles of NF-κB or interferon regulatory factor, and several studies suggested that the regulation of

IP-10 by immunological stimuli is highly cell type-specific (14, 18, 23). In our experiments, the Jak-Stat pathway showed only a weak influence on IP-10 secretion from adipocytes. However, IP-10 regulation was largely dependent on NF-κB activity in 3T3-L1 and primary human adipocytes. Both basal and stimulated IP-10 expression and secretion were reduced in 3T3-L1 cells stably overexpressing a transdominant IκBα protein. However, no changes could be observed in preadipocytes,

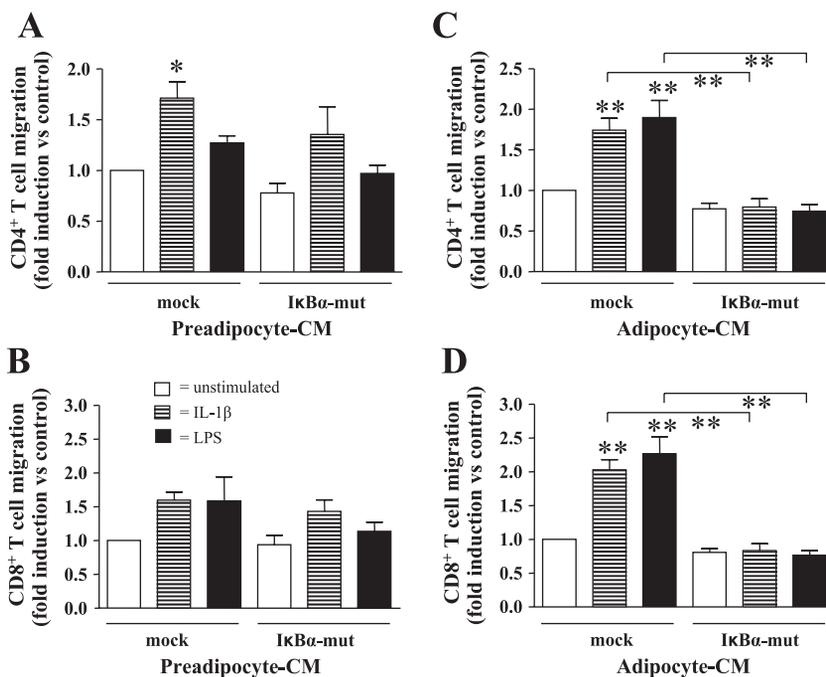


Fig. 6. Migration of T cells in response to 3T3-L1 adipocyte-conditioned medium is inhibited by overexpression of IκBα-mut. Stably infected 3T3-L1 preadipocytes (A and B) and adipocytes (C and D) were induced for 20 h by adding fresh media with or without IL-1β (10 ng/ml, striped bar) or LPS (1 μg/ml, black bar). Conditioned media (CM) were harvested and transferred to the lower chamber of Costar Transwells. Freshly isolated mouse splenocytes were placed in Costar Transwell permeable supports. Migration of CD4⁺ T cells (A and C) and CD8⁺ T cells (B and D) toward conditioned media was assayed by fluorescence-activated cell sorter (FACS) analysis. Results are means ± SE (n ≥ 9). *P < 0.05 and ***P < 0.01.

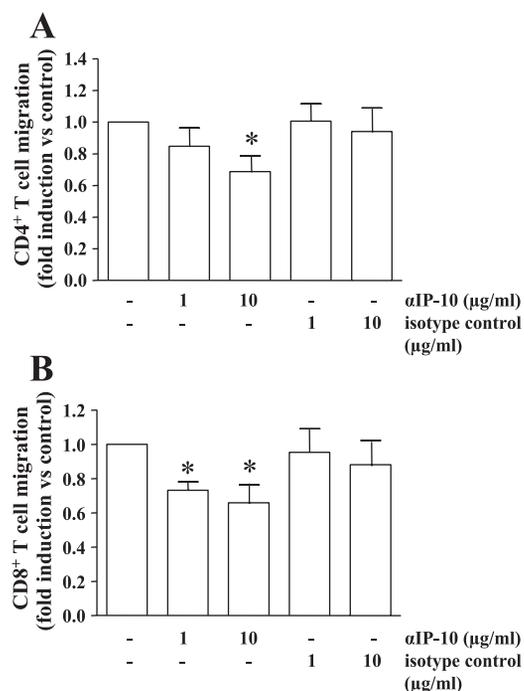


Fig. 7. Migration of T cells in response to 3T3-L1 adipocyte-conditioned medium is inhibited by a neutralizing IP-10 antibody. 3T3-L1 adipocytes were cultivated for 20 h in fresh medium, and conditioned medium was transferred to the lower chamber of Costar Transwells. A neutralizing anti-IP-10 antibody (α IP-10) or as isotype control an anti-IgG antibody was added 30 min before addition of splenocytes. Freshly isolated mouse splenocytes were placed in Costar Transwell permeable supports. Migration of CD4⁺ T cells (A) and CD8⁺ T cells (B) toward conditioned media was assayed by FACS. Results are means \pm SE ($n \geq 4$). * $P < 0.05$.

since they produce almost no detectable amounts of IP-10 at all. Thus NF- κ B-dependent IP-10 expression and secretion seem to play a prominent role in adipocytes. These results are also consistent with previous work that demonstrated an overall increase in constitutive NF- κ B activity, including changes in NF- κ B subunit expression, during differentiation (1). Based on these data, and on the growing evidence implicating NF- κ B as an important factor for IP-10 regulation, we further characterized NF- κ B-dependent IP-10 regulation during adipocyte differentiation. The parallel luciferase activity driven by an IP-10 promoter reporter and a synthetic NF- κ B-dependent reporter with an increased IP-10 expression during adipogenesis additionally supports the essential role of NF- κ B for IP-10 regulation. Taken together, we conclude that IP-10 gene regulation is strongly dependent on the κ B-binding sites present in its promoter.

In light of an increased constitutive NF- κ B activity and IP-10 expression in obesity, we further analyzed the physiological relevance of IP-10 for obesity-associated chronic inflammation. A number of studies demonstrated that obesity is associated with substantial macrophage (4, 20) and T cell accumulation in AT of obese mice and humans (5, 11, 22). Proinflammatory T lymphocytes are present in visceral AT and may contribute to the local inflammatory cell activation even before appearance of macrophages, suggesting that T cells play an important role in the initiation and perpetuation of AT inflammation. Recently, the different subsets of AT T cells and their alterations in obesity have been characterized (5, 6, 13,

21). In obese AT, a shift toward an inflammatory phenotype of T cells was observed, with an increase in CD8⁺ and Th1 polarized T cells and a decrease in regulatory T cells. Furthermore, AT T cells from obese mice produce large amounts of the Th1 cytokine IFN γ (5, 15). As discussed by Duffaut et al. (5), the molecular mechanisms leading to the trafficking of T cells into AT are so far poorly understood. We could show that conditioned media from 3T3-L1 adipocytes can induce CD4⁺ and CD8⁺ T cell migration when cells were stimulated by LPS or IL-1 β in an NF- κ B-dependent manner. This finding was substantiated by the observation that T cell migration was reduced to basal levels by overexpression of dominant-negative I κ B α in 3T3-L1 adipocytes, clearly indicating the importance of the pathway for the migration process. We can conclude that NF- κ B-regulated adipokines are of central importance for the recruitment of T cells. Indeed, for most of the chemokines secreted by AT (CCL20, RANTES, and IP-10), a contribution of NF- κ B to their promoter activity could be observed (2). Our data support the strong molecular link of NF- κ B to inflammation, metabolic dysregulation in adipocytes, and finally diabetes, which has been shown by analyzing inhibitor of nuclear factor κ B kinase β -subunit and salicylate-treated mice (24). Even more, in a recent work, an activation of NF- κ B in primary human AT of obese subjects has been observed (5a).

By specifically inhibiting IP-10 activity with an anti-IP-10 antibody, the migration of CD4⁺ and CD8⁺ T cells toward the media from 3T3-L1 adipocytes was reduced. These data suggest that IP-10 is an important factor for recruitment of T cells in AT. Moreover, we could show that expression of both IP-10

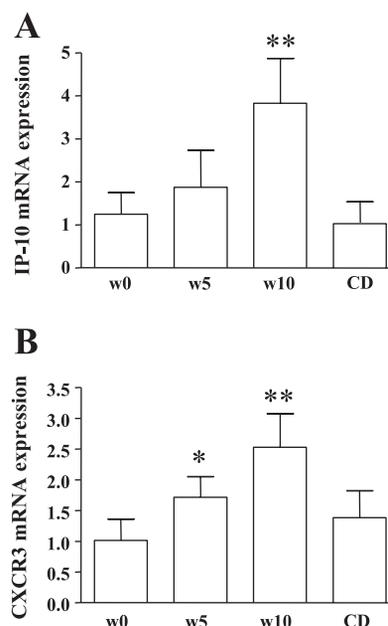


Fig. 8. Expression of AT IP-10 and chemokine CXC motif receptor 3 (CXCR3) mRNA increases during progression of weight gain. Total mRNA was isolated from visceral/epididymal AT of male C57BL/6J mice at baseline (w0) compared with mice fed a high-fat diet for 5 (w5) or 10 (w10) wk and with mice fed a chow diet (CD) for 10 wk. mRNA was reverse transcribed, and IP-10 (A) and CXCR3 (B) expression was determined by qRT-PCR; GAPDH mRNA was measured as control. Expression level of one sample (w0) was set to one. Results are means \pm SD ($n \geq 5$). * $P < 0.05$ and ** $P < 0.01$ compared with w0.

and its receptor CXCR3 increases during weight gain in mouse AT in parallel to T cell infiltration shown recently (11). Notably, CXCR3 is highly expressed on activated T cells (16), mainly on Th1 cells, and recent publications indicate that AT infiltrating T cells represent Th1 cells (5, 6, 15, 21).

Taken together, these observations highlight the role of IP-10 secreted from adipocytes as a new candidate for the recruitment of T cells in AT. CXCR3 is also expressed on myeloid cells; however, the inflammatory process leading to an accumulation of monocytes/macrophages is characterized by low CXCR3 and high chemokine receptor 2 levels, which is mainly responsible for the capacity of monocytes/macrophages to migrate into inflamed sites (17). Therefore, it is rather likely that IP-10 produced by adipocytes mainly recruits Th1-primed T cells into AT. As recently shown, the balance between regulatory and effector CD4⁺ T cell subsets is a crucial factor of obesity-associated insulin resistance. Moreover, in atherogenesis, it was shown that IP-10 modulates the balance of effector and regulatory T cells. In IP-10-deficient mice, diminished effector T cell trafficking switches the lesional balance to a regulatory phenotype (8). Because atherosclerosis and obesity share common pathophysiological features, a similar functional role for IP-10 to modulate the local balance of effector and regulatory T cells in obese AT can be postulated. An IP-10 knockout model could provide additional insight into the physiological relevance of IP-10 in the context of AT inflammation.

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DISCLOSURES

The authors declare that there is no duality of interest associated with this manuscript.

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