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INVESTIGATIVE REPORT

Insulin Resistance May Contribute to Upregulation of Adhesion Molecules on Endothelial Cells in Psoriatic Plaques

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Psoriasis primarily affects the skin, but also has a systemic dimension and is associated with severe comorbidities. Since endothelial cells play an important role in psoriasis as well as in the development of cardiovascular comorbidities, we investigated whether a common mechanism, namely cytokine-induced insulin resistance, underlies both pathologies. Activation of the insulin pathway was studied in psoriatic skin and dermal endothelial cells. Expression of adhesion molecules was assessed by flow cytometry, as well as their biological function in flow chamber experiments. The phosphorylation status of Akt, a central kinase in the insulin pathway, suggests that endothelial cells within psoriatic plaques are rendered insulin resistant by pro-inflammatory cytokines. Insulin counteracts the expression of adhesion molecules, but has limited effects on interactions between T cells and endothelial cells. Pro-inflammatory cytokines induce insulin resistance in endothelial cells, which may contribute to the development of the inflammatory infiltrate in psoriasis. *Key words: cytokines; endothelial cells; insulin resistance; psoriasis; T cells*.

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Psoriasis, a chronic inflammatory skin disease, not only shows a cutaneous phenotype in the form of sharply demarcated, red scaly plaques, but also has a systemic dimension, as it is associated with comorbidities such as the metabolic syndrome, diabetes, and cardiovascular disease (1). Endothelial cells play an important role in this skin disease and its comorbidities, as they are implicated in the extravasation of leukocytes (2). This process is mediated by microvascular endothelial cells and comprises 4 steps: (*i*) rolling and tethering of leukocytes on the endothelial cell surface; (*ii*) chemokine sensing; (*iii*) arrest and firm adhesion of leukocytes; and (*iv*) migration into the tissue, where they contribute to dermal inflammation or the formation of atherosclerotic plaques (3). Adhesion molecules, such as selectins and immunoglobulin superfamily members, mediate these steps. E-selectin is expressed on activated endothelial cells and mediates the initial recruitment of leukocytes to the endothelial cells (4). Intercellular adhesion molecule (ICAM)-1, a member of the immunoglobulin superfamily, is implicated in steps 3 and 4 of the extravasation process (5). The expression of both adhesion molecules can be induced upon inflammatory stimuli like tumour necrosis factor (TNF)-α (5).

Interestingly, psoriatic and atherosclerotic plaques show mechanistic and histological similarities (6). In both plaques inflammation plays a major role and the same cytokines and cells are implicated in the pathogenesis (6, 7). It is hypothesized that a common molecular mechanism, namely cytokine-induced insulin resistance, contributes to the development and maintenance of psoriatic and atherosclerotic plaques (8, 9). This is supported by the fact that patients with moderate-to-severe psoriasis show signs of reduced insulin sensitivity (10, 11). Insulin resistance is a pathophysiological state in which the balance between the insulin-dependent activation of the anti-atherogenic phosphatidylinositol 3-kinase (PI3-K)/Akt and pro-atherogenic mitogen-activated protein kinase (MAPK) pathway is shifted towards the latter (12). During systemic inflammation, as in psoriasis, pro-inflammatory cytokines activate stress kinases, such as c-Jun N-terminal kinase (JNK) or p38MAPK, which in turn negatively regulate the insulin receptor substrate-1 (IRS-1) and thereby block PI3-K/Akt signalling (13). At the same time insulin-dependent activation of the MAPK pathway persists, leading through the enhanced expression of adhesion molecules and decreased nitric oxide (NO) production to a pro-inflammatory and pro-atherogenic milieu, resulting in endothelial dysfunction and atherosclerosis (14, 15).

The objective of this study was to investigate whether insulin resistance in dermal endothelial cells is a pathomechanism that contributes to the pathogenesis of psoriasis. We provide evidence that skin biopsies of psoriatic patients show signs of insulin resistance in the dermal endothelium. A mix of pro-inflammatory cytokines involved in psoriasis was able to induce molecular insulin resistance in microvascular endothelial cells *in vitro*. Furthermore, the functional effects of insulin resistance were examined by investigating the expression of adhesion molecules of endothelial cells and the extravasation process of leukocytes.

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METHODS

Chemicals and antibodies

All chemicals were purchased from Sigma (Steinheim, Germany). Cytokines were from Peprotech (Hamburg, Germany) except for interleukin (IL)-23, which was from R&D Systems (Wiesbaden, Germany). Phospho-specific (#4060) and corresponding pan antibody (#4691) for Akt, ICAM-1 (#4915) and β-tubulin (#2128) antibodies were from Cell Signaling Technology (Frankfurt, Germany). E-Selectin antibody was from R&D Systems (Wiesbaden, Germany) and CD31 antibody was from Dako (Hamburg, Germany). Flow cytometry and isotype control antibodies were from BD (Heidelberg, Germany).

Cell culture and conditions

Human dermal blood endothelial cells (HDBEC) (Promocell, Heidelberg, Germany) were cultured in Endothelial Cell Media MV (Promocell, Heidelberg, Germany) with additional 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C in 5% CO_2 atmosphere.

Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 1 mM phenylmethanesulfonylfluoride (PMSF)), normalized, subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes. After blocking in 5% milk in TBS-T (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20), membranes were probed with the indicated antibodies and visualized with horse-radish peroxidase (HRP)-conjugated secondary antibodies using LumiGlo reagent (Cell Signaling Technology, Frankfurt, Germany). ImageJ software (NIH Image, Bethesda, MD, USA) was used to analyse band intensities densitometrically. Intensity of phosphorylated protein bands was normalized to the intensity of the total Akt signals.

Flow cytometry analysis

Cells were detached with accutase, washed and stained with the appropriate antibodies for 30 min at 4°C. All samples were analysed using BD FACS Calibur and BD CellQuest Pro 4.0.2 software.

Isolation of human T cells

PBMCs were isolated from leukocyte-rich buffy coats (BSD, Hessen, Germany) with a Ficoll gradient, followed by T-cell isolation using the human Pan T-cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Cell number and purity of enriched T cells was determined by flow cytometry analysis. T cells were cultivated in RPMI medium (Invitrogen, Karlsruhe, Germany) with additional 5% foetal calf serum and 1% penicillin/streptomycin overnight at 37°C in 5% CO_2 atmosphere and used the next day for flow chamber experiments.

Flow chamber assays

De-adhesion assay. Ibidi µ-slide VI 0.4 ibiTreat chambers (Munich, Germany) were coated with 0.1% gelatine solution. The day before treatment with cytokines 5×10^4 endothelial cells were seeded in these chambers. After 48 h of cytokine treatment 1×10^5 T cells were allowed to attach on the endothelium for 3 min. Non-adherent T cells were flushed away and shear stress was increased stepwise from 0.35 to 4, 6, 8 and 10 dyn/cm^2 for 30 s each. During the experiment photographic images were taken every 30 s, with a charge-coupled device (CCD) camera (Sony, New Jersey, USA). Adherent T cells were quantified from the photographic images using ImageJ software.

Dynamic flow chamber assay. Endothelial cells were seeded in Ibidi µ-slide VI 0.4 ibiTreat chambers the day before treatment was started. After 48 h of cytokine treatment 5×10^5 T cells/ml were flushed over the endothelial cells with a constant shear stress of 0.35 dyn/cm^2. A 3-min video was recorded. To ensure the steady flow of T cells, only the middle minute of the video was analysed. For analysis, potentially interacting T cells as well as rolling, adhering, and tethering T cells were quantified and the sum of all these cells was set to 100%.

Immunohistochemistry. Endothelial cells were seeded at 1×10^5 cells per chamber in a LabTek®II CC chamber slide (Nunc, Rochester, USA), treated with cytokines and fixed with 4% paraformaldehyde. Punch biopsies from lesional and non-lesional skin of 5 patients with severe psoriasis vulgaris or from healthy skin of individuals without inflammatory skin diseases, were taken after written informed consent. This study was approved by the local ethics committee of the University Hospital Frankfurt am Main (144/12); the protocol of the Declaration of Helsinki was followed. The punch biopsies were cut into 4-µm cryosections and fixed with acetone or methanol. All specimens were permeabilized with TBS-T (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween20) and blocked using 5% normal goat serum or 5% bovine serum albumin (BSA). Incubation with primary or isotype antibodies was performed at 4°C overnight. After washing, the samples were incubated with AlexaFluor488 and AlexaFlour594 secondary antibodies. Nuclei were stained with bisbenzimide. Confocal images were generated using a ZeissLSM510 microscope.

Statistical analysis

Statistical calculations were performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA, USA). Results are presented as mean ± standard error of the mean (SEM). Statistical differences between samples were assessed by paired Student's *t*-test. Differences were considered significant at $p < 0.05$.

RESULTS

In order to investigate whether the endothelium in a psoriatic plaque shows signs of insulin resistance, phosphorylation of Akt was examined. Immunohistochemical staining for phosphorylated Akt (serine (S) 473) and CD31, a marker for endothelial cells, revealed phosphorylated Akt in endothelial cells of healthy skin (NN; Fig. 1g, h), while in non-lesional psoriatic skin (PN) only weak phosphorylation of Akt could be detected (PN; Fig. 1d–f) and hardly any endothelial Akt phosphorylation could be found in lesional psoriatic skin (PP, Fig. 1a–c), while the expression of total Akt was comparable in all specimens (16).

To investigate the mechanism that causes the reduction in phosphorylated Akt in dermal endothelial cells in the psoriatic plaque, HDBEC were used, which were previously shown to be an appropriate cell culture

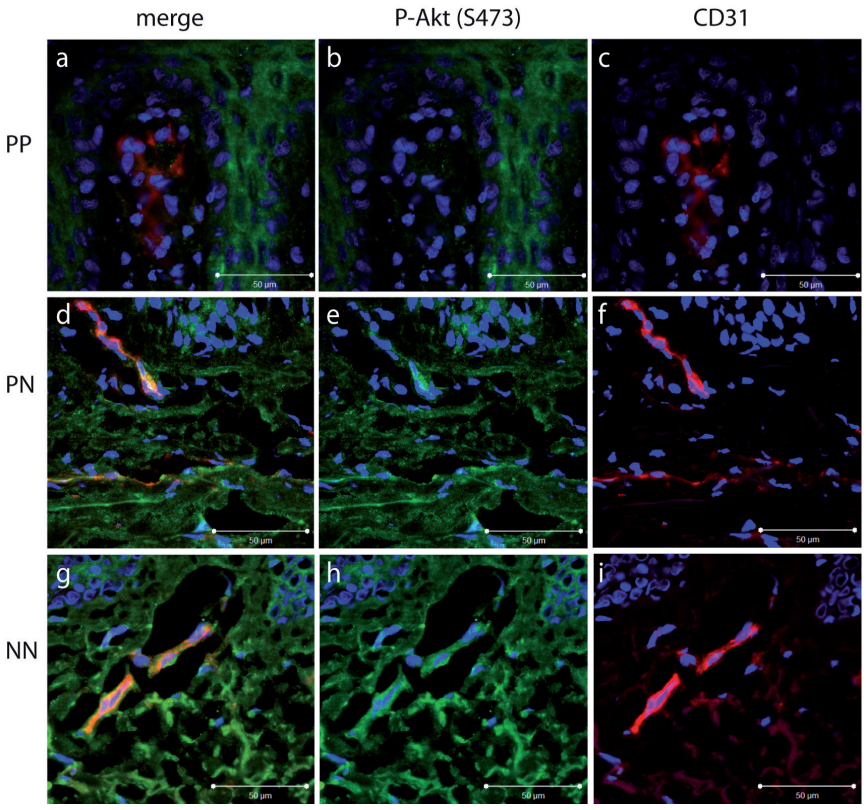

Fig. 1. Phosphorylation of Akt is reduced in micro-vessels of psoriatic skin. Cryosections of punch biopsies from lesional (PP; a–c) or non-lesional (PN; d–f) skin of a psoriasis vulgaris patient or healthy donor (NN; g–i) were stained with P-Akt (S473, *green*) and CD31 (*red*). Nuclei were stained with bisbenzimide (*blue*). Bars represent 50 µm.

model (17). Due to the fact that none of the pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-12, IL-17A, IL-23 and TNF-α, was able to induce stable insulin resistance individually (17), several combinations of these cytokines were tested for their ability to mediate insulin resistance. Only a combination of all pro-inflammatory cytokines (IL-1β, IL-17A, IL-22, IL-23 and TNF-α; cyt mix) induced a strong reduction in insulin-dependent Akt phosphorylation (Fig. 2a), which first appeared after 18 h of cytokine treatment and persisted for up to 6 days (Fig. 2c and d). As HDBEC express the insulin-like growth factor (IGF-1) receptor, which can also activate Akt, we tested whether the pro-inflammatory cytokine mix can also induce resistance to IGF-1. However, the effect seems to be specific to insulin, as HDBEC are still able to respond to IGF-1 after treatment with the pro-inflammatory cytokine mix (Fig. 2b).

An important function of the dermal endothelium is the regulation of lymphocyte extravasation during inflammatory processes, which is mediated via adhesion molecules such as ICAM-1 and E-selectin. Without an inflammatory stimulus hardly any expression of ICAM-1 or E-selectin could be detected (data not shown); thus we co-stimulated HDBEC with TNF-α and analysed the effect of insulin on the expression of adhesion molecules. In this experimental setting, insulin repressed slightly, but

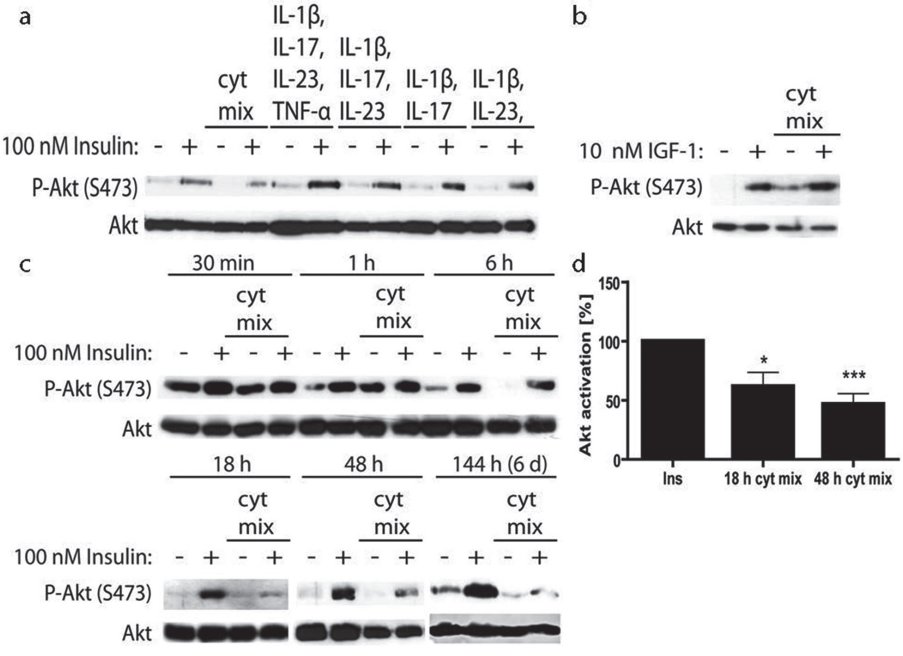

Fig. 2. A pro-inflammatory cytokine mix induces persistent insulin resistance. (a–c) Human dermal blood endothelial cells (HDBEC) were serum-starved and treated with the pro-inflammatory cytokine mix (interleukin (IL)-1β, IL-17A, IL-22, IL-23, and tumour necrosis factor (TNF)-α (20 ng/ml each)) or (a) different combinations of the cytokines as indicated for (a, b) 18 h or (c) the indicated time-points, followed by (a, c) a 10-min insulin (100 nM) or (b) insulin-like growth factor (IGF)-1 (10 nM) stimulation. Cell lysates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Proteins were detected with phospho-Akt S473 and total protein antibodies. (d) Western blot bands of 18 h and 48 h cytokine treatments were analysed densitometrically using ImageJ. Signals were normalized to total Akt bands. The graph represents mean values ± standard errors of the mean (SEM). $n \geq 5$. *$p < 0.05$, ***$p < 0.001$ (paired Student's *t*-test).

significantly, the expression of ICAM-1 and E-selectin (Fig. 3a, b) and thereby had an anti-inflammatory action. To investigate the impact of cytokine-induced insulin resistance on adhesion molecule expression, cells were stimulated with the pro-inflammatory cytokine mix and TNF-α, in combination with insulin (Fig. 3c). The pro-inflammatory cytokine mix, as well as the combination of pro-inflammatory cytokine mix and TNF-α, greatly enhanced the surface expression of ICAM-1 (Fig. 3d). Interestingly, insulin was still able to repress surface expression of ICAM-1, when cells were treated with the cytokine mix (Fig. 3d). In contrast, the surface expression of E-selectin was hardly altered by any of these treatments (Fig. 3e).

Since flow cytometry analysis only measures surface expression of adhesion molecules, total protein expression was examined using Western blotting, which confirmed that ICAM-1 expression was enhanced by TNF-α and even more strongly by the pro-inflammatory cytokine mix (Fig. 3f). Insulin was again able to repress TNF-α-induced ICAM-1 expression. In contrast to flow cytometry analysis, the repressive effect of insulin could not be detected when the cells were pre-stimulated with the pro-inflammatory cytokine mix (Fig. 3f).

As we found that psoriatic cytokines render HDBEC resistant to insulin at the molecular level, while insulin was still able to confer some effects on adhesion molecule presentation, we investigated the biological function of the adhesion molecules using flow chamber approaches.

A de-adhesion assay was performed to analyse the adherence-capacity of T cells to endothelial cells by stimulating an endothelial cell layer with the pro-inflammatory stimulus and insulin. Untreated HDBEC exhibited the lowest binding capacity for T cells, as 70% of T cells de-adhered even at the lowest shear stress value (0.35 dyn/cm^2) (Fig. 4a, black circles). As expected, treatment of HDBEC with either TNF-α, the pro-inflammatory cytokine mix, or the combination, significantly enhanced the adhesion of T cells (Fig. 4a, b). Interestingly insulin increased the adherence of T cells slightly (Fig. 4a, black squares), but did not show any effect on adherence capacity when endothelial cells were pre-treated with the cytokine mix (Fig. 4a, b).

To examine T-cell recruitment to endothelial cells after an inflammatory stimulus in more detail, the capacity of T cells to tether, roll and adhere on the endothelium were analysed. On untreated HDBEC, only approximately 3% of T cells were tethering or adhering and no rolling could be observed. Insulin alone did not affect the behaviour of T cells (Fig. 4c). Upon treatment with TNF-α, the rate of cells interacting with the endothelium increased up to 20% of all T cells and rolling cells could also be

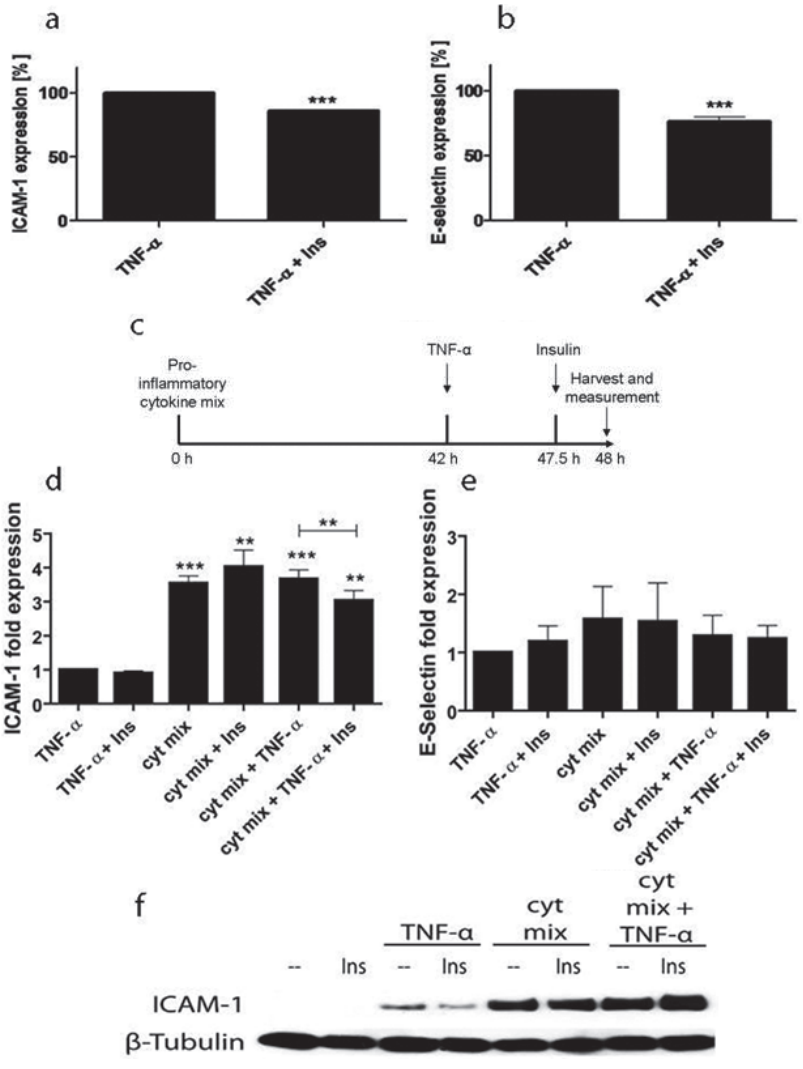

Fig. 3. Insulin mediates anti-inflammatory effects on intercellular adhesion molecule (ICAM)-1, but not on E-selectin. (a + b) Human dermal blood endothelial cells (HDBEC) were treated for 1 h with 1 ng/ml tumour necrosis factor (TNF)-α and 100 nM insulin. (c) HDBEC were stimulated with the pro-inflammatory cytokine mix (interleukin (IL)-1β, IL-17A, IL-22, IL-23, and TNF-α (20 ng/ml each)) for 48 h. After 42 h of incubation, 1 ng/ml TNF-α was added and 30 min before harvest 100 nM insulin was added. (d–f) HDBEC were treated as described in (c). (a–e) Surface expression of ICAM-1 (a, d) and E-selectin (b, e) was measured by flow cytometry. The geometric mean of isotype controls was subtracted from the geometric mean of the samples. Expression levels were normalized to samples stimulated with TNF-α alone. The graphs represent mean values ± standard error of the mean (SEM) of at least 4 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (paired Student's *t*-test). (f) Protein lysates were analysed for the expression of ICAM-1 by Western blotting. β-tubulin was used as a loading control.

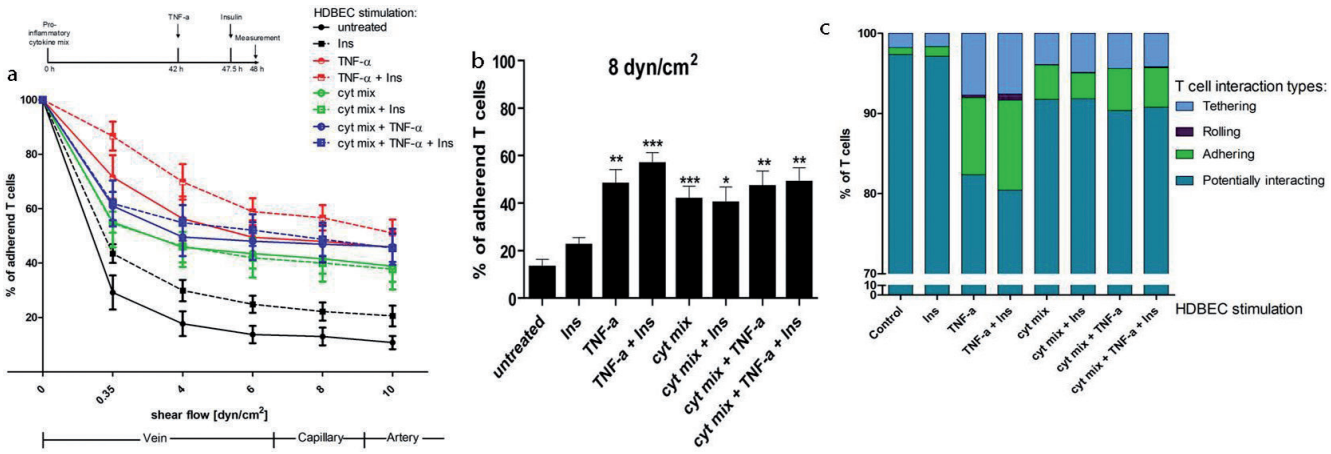

Fig. 4. Insulin exerts pro-inflammatory effects on the functional interaction between T cells and endothelial cells. (a–c) Human dermal blood endothelial cells (HDBEC) were treated as described in Fig. 3c. (a, b) T cells were allowed to adhere to the endothelial cells for 3 min following stepwise increment of shear stress, with each step reflecting the physiological flow in veins, capillaries and arteries. The number of adherent cells was quantified and then normalized to the number of adherent cells before the application of shear stress. $n = 6$. Data show mean ± standard error of the mean (SEM). (b) The graph depicts the values for 8 dyn/cm^2. (c) T cells were perfused over the endothelial cell layer in a constant shear flow of 0.3 dyn/cm^2 and different interaction types were counted. Data show mean values. $n = 13$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test).

detected, while the addition of insulin slightly increased the overall rate of interacting cells. In contrast, the pro-inflammatory cytokine mix led only to a rate of 10% interacting cells, and the addition of TNF-α could not further increase the interactions with the underlying endothelial cells. Under these conditions insulin did not confer any effect on the interactions of T cells with the endothelium (Fig. 4c).

In summary, dermal endothelial cells in the skin of psoriatic patients show a reduced activation of the PI3-K/Akt pathway, which could be a sign of insulin resistance. *In vitro*, we found that a mix of pro-inflammatory cytokines, consisting of IL-1β, IL-17A, IL-22, IL-23, and TNF-α, rendered HDBEC resistant to insulin induction of PI3-K/Akt signalling. Insulin was able to repress TNF-α dependent expression of ICAM-1 and E-selectin, which in the case of ICAM-1 was still present when cells were pre-treated with the pro-inflammatory cytokine mix. Furthermore, interactions of T cells with endothelial cells, such as tethering, adhesion and rolling, were induced with either the pro-inflammatory cytokine mix or TNF-α, while insulin had no significant impact on these interactions.

DISCUSSION

It has been demonstrated previously that psoriatic patients show signs of dermal insulin resistance and that epidermal insulin resistance potentially contributes to the development of the psoriatic plaque (16, 18). Insulin resistance at the endothelial wall of larger vessels is a well-described phenomenon that represents a pathomechanism in the development of atherosclerotic plaques. Since the psoriatic plaque and the arteriosclerotic plaque show great similarities in terms of the involved mediators, cell types and pathophysiological mechanisms (6), we asked whether insulin signalling and, accordingly, resistance to beneficial effects of insulin at the dermal endothelial wall contributes to the pathogenesis of psoriasis.

We found that phosphorylation of Akt is reduced in dermal endothelial cells of psoriatic patients compared with healthy skin, which could be a first sign of endothelial insulin resistance as IRS-1 becomes inhibited under conditions of insulin resistance and is thereby unable to convey signals via the PI3-K/Akt pathway (13). Insulin resistance is induced via the activation of different kinases by pro-inflammatory cytokines. We found that a combination of pro-inflammatory cytokines containing IL-1β, IL-17A, IL-22, IL-23 and TNF-α induces insulin resistance in human dermal blood endothelial cells (HDBEC) *in vitro*. All cytokines in this mix were necessary to induce insulin resistance. This resembles the situation within the psoriatic plaque, where the pro-inflammatory milieu comprises all of these cytokines (19) that are likely to act synergistically (20, 21). However adipokines, such as resistin or leptin (22), or other cytokines, such as IL-33 (23–25), that are known to be elevated in psoriatic plaques may also contribute to this effect via activation of mast cells (26) leading to the secretion of other pro-inflammatory cytokines (22, 23). Although IL-33 seems to confer a protective effect against obesity and insulin resistance in adipose tissue (27), it may be an interesting candidate for further investigations, as IL-33 is secreted by endothelial cells in obese patients (28) and induces expression of adhesion molecules on the endothelium (29).

The biological function of insulin at the dermal endothelial wall seems to be complex and could be time-dependent and context-specific. Insulin reduced TNF-α

induced expression of ICAM-1 and E-selectin, which supports the idea that insulin has anti-inflammatory action. Stimulation of HDBEC with the pro-inflammatory cytokine mix led to enhanced expression of ICAM-1 on the surface, as well as on the total protein level, as also shown by others (30, 31). An increase in E-selectin expression by pro- inflammatory cytokine mix could not be detected, which is in line with a previous study by Erbel et al. (30), who showed no increase in E-selectin expression in human umbilical vein endothelial cells (HUVEC) in response to TNF-α and IL-17A.

Insulin was unable to block the much stronger effect of the pro-inflammatory cytokine mix on ICAM-1 expression, which could be explained by the fact that under these conditions HDBECs are insulin resistant. In contrast, insulin was able to repress ICAM-1 expression in the presence of TNF-α and the pro-inflammatory cytokine mix. These results suggest that this anti-inflammatory effect of insulin on the TNF-α response is not mediated via the PI3-K/Akt pathway, which should be blocked through inhibitory IRS phosphorylation mediated by the pro-inflammatory cytokine mix.

In functional assays, stimulation of HDBEC with pro-inflammatory cytokine mix significantly enhanced the adhesion of T cells to endothelial cells. It is assumed that, in psoriasis, T cells adhere to the functionally altered endothelium, as Lowe et al. (32) could show that normal lymphocytes preferentially adhere to psoriatic endothelium. Interestingly we found that insulin, even though the results were not statistically significant, enhanced interactions between T cells and the endothelium, which would rather argue for a pro-inflammatory role of insulin and is in line with the observations presented by Madonna & De Caterina (33) regarding the interaction of monocytes with HUVEC.

Unexpectedly, it was found that treating cells with TNF-α and the pro-inflammatory cytokine mix leads to fewer interactions between T cells and endothelial cells than TNF-α alone. Based on this data, we suggest that the cytokines used in the pro-inflammatory cytokine mix acted antagonistically or altered the effects of each other and therefore, the expression of distinct adhesion molecules, such as E-selectin, was not enhanced and could not promote the full effect of TNF-α induction. This is supported by studies showing a selective synergism of cytokine actions (34).

The morphology of HDBECs was changed from a slightly rounded, cobblestone-like phenotype (35) to an elongated, spindle-shaped one (data not shown). The altered morphology is thought to be due to differential expression of adhesion molecules influencing the actin cytoskeleton and thereby altering the cellular shape (36), which in turn has an impact on adhesion ligand binding (4).

In summary, this study provides evidence that, in patients with psoriasis, endothelial cells in the dermis show signs of insulin resistance and might therefore also be dysfunctional. This underlines the systemic manifestation of psoriasis, as reduced Akt phosphorylation was also found in blood vessels of non-lesional skin, and supports the model that insulin resistance is an important link between psoriasis and cardiovascular disease. We potentially reveal a new part of the "psoriatic march" (1): local insulin resistance is induced in the skin, affecting the dermal endothelium even before the systemic, and therefore metabolic, dimension of insulin resistance is reached. Interestingly, none of the chosen patients had a pathological level of HbA1c (data not shown). As such, insulin resistance in dermal endothelial cells could be an initial hint towards reduced insulin sensitivity in the skin, which may result in systemic manifestation.

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