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Potential Antiinflammatory Role of Insulin via the Preferential Polarization of Effector T Cells toward a T Helper 2 Phenotype

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Hyperglycemia in critical illness is a common complication and a strong independent risk factor for morbidity and death. Intensive insulin therapy decreases this risk by up to 50%. It is unclear to what extent this benefit is due to reversal of glucotoxicity or to a direct effect of insulin, because antiinflammatory effects of insulin have already been described, but the underlying mechanisms are still poorly understood. The insulin receptor is expressed on resting neutrophils, monocytes, and B cells, but is not detectable on T cells. However, significant up-regulation of insulin receptor expression is observed on activated T cells, which suggests an important role during T cell activation. Exogenous insulin *in vitro* induced a shift in T cell differentiation toward a T helper type 2 (Th2)-type response, decreasing the T helper type 1 to Th2 ratio by 36%. This result correlated with a corresponding change in cytokine secretion, with the interferon-γ to IL-4 ratio being decreased by 33%. These changes were associated with increased Th2-promoting ERK phosphorylation in the presence of insulin. Thus, we demonstrate for the first time that insulin treatment influences T cell differentiation promoting a shift toward a Th2-type response. This effect of insulin in changing T cell polarization may contribute to its antiinflammatory role not only in sepsis, but also in chronic inflammation associated with obesity and type 2 diabetes. (*Endocrinology* 148: 346–353, 2007)

HYPERGLYCEMIA IN CRITICAL illness is commonly observed and is a strong independent risk factor for morbidity and death (1, 2). Intensive insulin therapy in critical illness aiming to achieve normoglycemia has recently been shown to decrease this risk by up to 50% (3). This might be due in large part to the reversal of glucotoxicity, but a direct beneficial effect of insulin is also possible, *e.g.* via antiinflammatory or antiapoptotic pathways.

Some complex antiinflammatory effects of insulin have been described, mediated via down-regulation of proinflammatory cytokines and reactive oxygen species through inhibition of NF-κB (4), down-regulation of acute phase proteins (5, 6), and conversely, via increasing endothelial nitric oxide synthase (7) and antiinflammatory cytokine secretion (8). In experimental models, intensive insulin treatment during sepsis was able to decrease proinflammatory (IL-1β, IL-6, macrophage inflammatory factor, TNFα) and increase antiinflammatory cytokines (IL-4, IL-10) (9), indicating an immunomodulating effect. Furthermore, antiapoptotic effects of insulin and other growth factors have also been described, and inhibiting sepsis-induced apoptosis of T and B cells is thought to be beneficial. It is still not clear which immune cells are most affected by insulin, nor the mechanisms by which insulin might alter the immune response.

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Abbreviations: IFN-γ, Interferon-γ; IR, insulin receptor; IRS, IR substrate; JNK, c-Jun N-terminal kinase; MMP, metalloproteinase; PBMC, peripheral blood mononuclear cell; Th1, T helper type 1; Th2, T helper type 2.

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T helper type 1 (Th1) or type 2 (Th2) cells are effector T cells important for the modulation of the inflammation process. Th1 cells are important for cell-mediated immune responses, especially for intracellular pathogens, secreting IL-2, interferon-γ (IFN-γ), and TNF, whereas Th2 cells are important for antibody-mediated responses, especially against intestinal pathogens and parasites, secreting IL-4, IL-5, IL-10, and IL-13. A Th1-dominant response characterizes several autoimmune diseases, whereas a predominantly Th2 response is a feature of allergic diseases and asthma (10). IL-4 is an important activator of B cells, and a shift from Th1 to Th2 cytokines could lead to a diminution of cellular immune responses and a stimulation of antibody-mediated responses. Thus, Th2 cells have inhibitory effects on Th1 differentiation and *vice versa* (11), illustrating that an appropriate balance between Th1 and Th2 cells is important to provide optimal host protection by the immune system.

The influence of insulin on T cell development into Th1 or Th2 cells has not been determined so far. It is well accepted that the MAPK pathways play an important role in T cell activation and differentiation. It is known that increased ERK phosphorylation leads to polarization of T cells toward Th2 and, in contrast, phosphorylation of p38 and c-Jun N-terminal kinase (JNK) favors a T cell differentiation toward Th1 (12, 13). The effect of continued activation of the insulin signaling pathway on the MAPK is well established, causing activation of ERK and inhibition of p38 and JNK (14), suggesting it could favor Th2 T cell differentiation. In the present study, we assessed the regulation of insulin receptor (IR) expression on various immune cells and determined whether insulin influences T cell differentiation into Th1/Th2 subtypes by changing the balance of the aforementioned MAPK.

Here, we report that insulin is able to promote Th2 T cell differentiation as shown by a decrease in Th1 to Th2 ratio as well as the IFN-γ to IL-4 ratio *in vitro*. This newly discovered effect of insulin may contribute to a modulation of inflammation and clinical outcome benefit in critical illness, and a reduced response to this effect might contribute to the proinflammatory state found with insulin resistance in obesity and type 2 diabetes.

Materials and Methods

Experimental subjects

All the following experiments were performed with approval of St. Vincent's Hospital Research Ethics Committee (Sydney-Darlinghurst, Australia). To obtain human peripheral lymphocytes we collected EDTA blood samples (40 ml) from 15 healthy male donors (aged 25–30 yr) and four septic patients from the intensive care unit at St. Vincent's Hospital after having obtained written informed consent.

Reagents and flow cytometry

Human peripheral blood mononuclear cells (PBMC) prepared as below were washed with PBS containing 0.25% BSA and stained with FITC-, PE-, PerCP-, or allophycocyanin-conjugated antibodies to various cell surface markers purchased from BD Biosciences (San Diego, CA), with the exception of a biotin-labeled antihuman-IR mouse antibody (NeoMarkers, Fremont, CA). Immunofluorescent staining was performed using standard procedures with appropriate secondary staining reagents (Jackson ImmunoResearch Laboratories, West Grove, PA), and cells were analyzed using BD FACSCalibur (San Diego, CA). Biotin-labeled antihuman IgG2 was used as isotype control for IR assessment. For comparative quantification of IR expression, the mean fluorescence intensity (MFI) of the IR was divided by the MFI of the isotype control, which gives the relative MFI which can be compared among different cell subsets.

For quantification of Th1/Th2 cells we used intracellular cytokine staining for IFN-γ and IL-4 (BD Bioscience PharMingen, San Diego, CA). After surface staining for $CD4^+$ and $CD8^+$, cells were permeabilized using the BD Cytofix/Cytoperm Kit with GolgiPlug (BD Bioscience PharMingen) following the manufacturer's protocol. After the intracellular staining, cells were immediately analyzed by flow cytometry. Apoptosis was measured by FACS counting for Annexin V-positive but propidium iodide-negative (BD Biosciences) stained $CD4^+$ T cells after 72 h activation as stated below.

PBMC isolation and culture

PBMCs were isolated from EDTA blood samples by Ficoll-Paque PLUS (Amersham Pharmacia Biotech) density gradient centrifugation. Human $CD4^+$ T cells were isolated from PBMC preparations by magnetic separation using $CD14^+$ magnetic beads for monocyte depletion, followed by positive selection by $CD4^+$ magnetic beads (MACS; Miltenyi Biotec, Sydney, Australia) reaching a purity of more than 95%.

For stimulation and culture up to 72 h, cells were resuspended in standardized cell culture medium RPMI 1640 containing 11.1 mmol/liter glucose (GIBCO, Invitrogen Corp., Auckland, New Zealand) supplemented with 100 U/ml penicillin/streptomycin (Life Technologies, Rockville, MD) and 10% bovine calf serum (HyClone, Logan, UT), resulting in basal insulin levels of 1.6 μU/ml. Cells in a concentration of 1×10^6 cells/ml were added to tissue culture plates (Nalge Nunc International, Rochester, NY) and kept in a CO_2 incubator at 37 C. Human recombinant neutral insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) or bovine insulin (Calbiochem, San Diego, CA) was added at a concentration of 1000 μU/ml = 7 nM = 40 ng/ml, with no difference in the results between the two species.

T cells were stimulated with plate-bound antihuman CD3 mAb [clone TR66, BD Bioscience PharMingen, coated onto culture plates overnight in PBS (10 μg/ml) at 4 C] in combination with 2 μg/ml soluble antiCD28 (BD Bioscience PharMingen). Cells were harvested at 24, 48, and 72 h and used for FACS or Western blot. Culture medium was used for cytokine protein measurement by ELISA. For proliferation assays, cultures were pulsed with $[^3H]$thymidine (1 μCi/well) 18 h before harvesting and quantified using a β-scintillation counter.

To generate Th1 cells, we added IL-12 (PeproTech Inc., Rocky Hill, NJ) and neutralizing antibodies against IL-4 (BD Bioscience PharMingen) to the culture medium. For Th2 cells, we added IL-4 (PeproTech Inc.) and neutralizing antibodies against IFN-γ (BD Bioscience PharMingen). Cells were harvested after 72 h and reincubated in the same conditions for another 72 h.

B cells were stimulated with 10 μg/ml goat $F(ab')_2$ antihuman μ-chain Ab (Jackson ImmunoResearch Laboratories) and 50 ng/ml IL-4 (PeproTech Inc.) for 72 h. Neutrophils and monocytes were stimulated with 10 ng/ml lipopolysaccharide (Sigma-Aldrich Co., St. Louis, MO) for 24 h.

Measurement of cytokine protein levels in culture medium

The cytokines IFN-γ and IL-4 were measured in the supernatant of stimulated $CD4^+$ T cells using BD OptEIA Sets (BD Bioscience PharMingen). Samples were added in triplicate to 384-well MaxiSorp plates (Nalge Nunc International) and the instructions of the manufacturer's protocol were followed. As substrate for horseradish peroxidase conjugated secondary antibody we used the reagent set A+B from BD Biosciences. The reaction was stopped with 20 μl H_2SO_4 (2 N), and absorbance was measured at 450 nm wavelength using an automated spectrometer by TECAN (Mannedorf/Zurich, Switzerland). A standard curve for each plate allowed us to calculate the corresponding cytokine concentration.

Western blot

Stimulated and cultured $CD4^+$ T cells were harvested at different time points and homogenized in lysing buffer. Proteins were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes using the Novex XCELL II Mini Cell Transblot apparatus (San Diego, CA) at 100 V for 1 h. The membranes were blocked for 1 h in TBS containing 0.1% Tween 20 and 5% skim milk powder at room temperature, briefly rinsed, and incubated overnight at 4 C with rabbit primary antisera raised against pERK, phospho-p38, pJNK, pAKT, total AKT (Cell Signaling Technology, Danvers, MA), pIRS-1 (pY612; Biosource International, Camarillo, CA), and total ERK-1, total p38, total JNK-1, and total IR substrate (IRS) 1 (Santa Cruz Biotechnology, Santa Cruz, CA), all diluted 1:1000.

After washing the membranes with TBS-Tween, an antirabbit or antimouse secondary antibody conjugated to horseradish peroxidase (diluted 1:1000; Amersham Biosciences, Little Chalfont, UK) was applied for 1 h at room temperature. Western Lightning chemiluminescence reagents (PerkinElmer Life Science, Boston, MA) and Hyperfilm ECL High Performance chemiluminescence films (Amersham Biosciences) were used to detect labeled antigen. We used the software IPLab Gel (version 1.5 g; Signal Analytics Corp., Vienna, VA) for quantification and divided the quantity of phosphorylated proteins by the quantity of the corresponding total protein.

Results

IR is absent on resting T cells, but strongly up-regulated after activation

To identify the immune cell candidates for insulin action, we measured the cell surface expression of the IR on all major blood leukocyte subsets using flow cytometry. Blood samples from at least six healthy subjects were processed as described in *Materials and Methods* to isolate PBMC or neutrophils, and stained with antibody against characteristic cell surface immune markers ($CD4^+$, $CD3^+$, $CD8^+$, $CD3^+$, $CD16^+$, $CD56^+$, $CD3^+$, $CD19^+$, $CD14^+$). In the resting state, IR was mainly found on the surface of neutrophils, monocytes, and B cells, but was undetectable on the resting T lymphocytes (Fig. 1, A and D).

For each cell type, we chose an appropriate agent for maximal activation, as described in *Materials and Methods*.

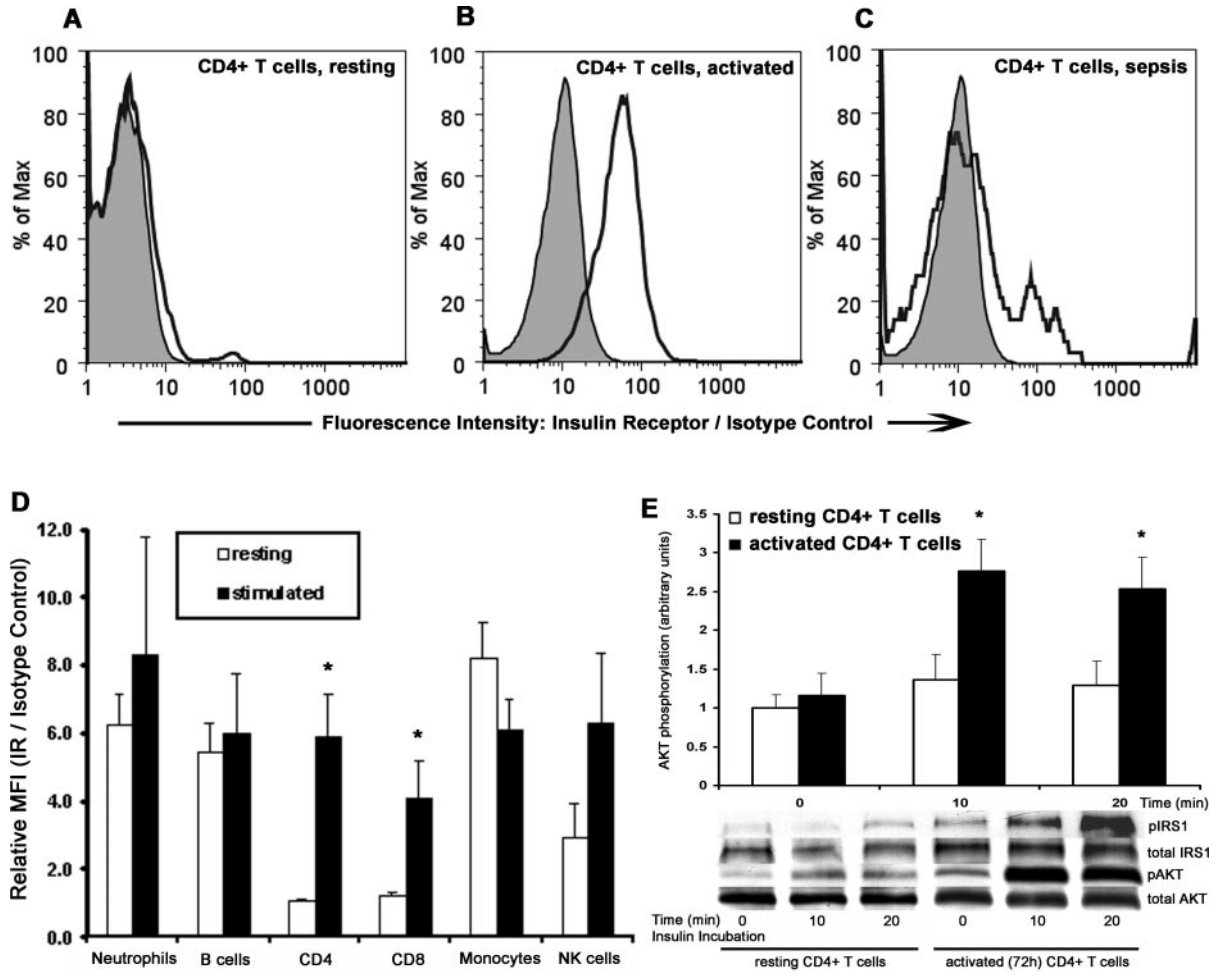

FIG. 1. Resting (A), activated (B), or septic (C) human $CD4^+$ T cells stained for IR (*white*) and corresponding isotype control (*gray*). A shift of the MFI to the right in B and C corresponds to positive staining of IR. The relative MFI, used in D, is the result of the MFI of IR divided by the MFI of isotype control. D, The relative MFI allows comparing the staining intensity for IR between the major leukocyte subsets in the resting or activated state. Results are given as means ± SEM of at least five subjects. *, Significance by $P < 0.005$ with respect to resting cells, as determined by Mann-Whitney *U* test. E, Human $CD4^+$ T cells, resting or activated for 72 h with anti-CD3 and anti-CD28 subjected to incubation with insulin 1000 μU/ml for 10 and 20 min. Results are given as means ± SEM. *, Significance by $P < 0.05$ with respect to time 0, as determined by Mann-Whitney *U* test. n = 3 different subjects and experiments.

The optimal stimulation time was established from time course experiments (data not shown). IR expression on neutrophils, monocytes, and B cells was not significantly changed after stimulation, whereas a strong and statistically significant up-regulation of IR expression was observed on $CD4^+$ T cells (6-fold up-regulation, $P < 0.0002$) and $CD8^+$ T cells (4-fold up-regulation, $P < 0.003$) (Fig. 1, B and D).

This *in vitro* observation was confirmed by a trend *in vivo* because increased IR expression was observed on a subset of peripheral blood T cells from septic patients from our intensive care unit compared with healthy controls (Fig. 1, A–C). Thus, IR expression is regulated on activated T cells and may play a key role during T cell activation and differentiation.

Newly expressed IR on T cells are functional

To confirm the up-regulation of IR on activated T cells, and to confirm that these receptors are functional, we analyzed activation of the insulin signaling pathway by measuring IRS-1 and AKT phosphorylation, in both activated and resting $CD4^+$ T cells. Fig. 1E shows that both IRS-1 and AKT are significantly phosphorylated 10 and 20 min after the addition of insulin to activated T cells, whereas no significant change in IRS-1 and AKT phosphorylation was observed when insulin was added to resting $CD4^+$ T cells. These results not only correlate with the absence of IR on resting cells and its up-regulation on activated $CD4^+$ T cells, but also prove that these receptors are functional and able to stimulate the insulin signaling pathway in activated T lymphocytes.

Insulin does not change activation status or proliferation, but inhibits apoptosis of $CD4^+$ T cells

To investigate the effects that could be mediated by activation of the insulin signaling pathway, we measured as a first step the up-regulation of classical activation markers such as CD25 and CD69 upon cell activation. Fig. 2, A and B, show the FACS analysis of activated $CD4^+$ T cells cultured with or without insulin and stained for these two activation markers, and it clearly shows that there is no difference in

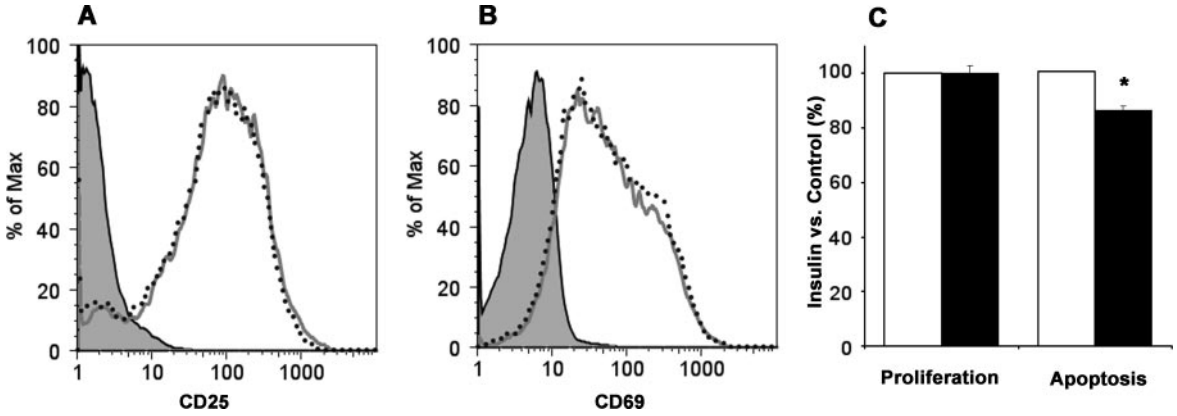

FIG. 2. Sorted CD4$^+$ T cells resting (*gray*) and stimulated with anti-CD3/anti-CD28 for 72 h in the presence (*dotted line*) or absence of insulin (*solid line*), stained for activation marker CD25 (A) and CD69 (B), showing no difference in activation status. C, Sorted and stimulated CD4$^+$ T cells as above in the presence (1000 μU/ml, *black bars*) or absence of insulin (*white bars*). Proliferation was measured by [^{3}H]thymidine incorporation, and apoptosis was measured by staining for Annexin V and propidium iodide. Values are compared with cell cultures without insulin (=100%), error bars represent SEM. *, Significance by $P < 0.05$ with respect to control as determined by Mann-Whitney U test. n = 3 different subjects and experiments.

activation status caused by insulin. Furthermore, we measured proliferation of sorted CD4$^+$ T cells after 72 h activation using [^{3}H]thymidine incorporation assays, but there was no difference found when cells were incubated with insulin (Fig. 2C).

However, when we counted for apoptotic CD4$^+$ T cells after a 72-h activation period using flow cytometry and gating for Annexin V-positive and propidium iodide-negative stained cells, we observed a decrease in apoptotic cells by 14% when compared with baseline ($P < 0.05$) (Fig. 2C).

Insulin promotes T cell differentiation toward a Th2 phenotype

We aimed to investigate whether stimulation of these newly expressed IR during T cell activation can lead to a change in T cell differentiation, especially into Th1 and Th2 T cell effector subsets. Therefore, sorted CD4$^+$ T cells from five healthy subjects were stimulated with anti-CD3 and anti-CD28 antibodies and cultured for 72 h in the presence or absence of insulin. This time frame allowed the cells to maximally express IR on the cell surface and initiate T cell proliferation and differentiation (Fig. 1, A–E).

After 72 h, cells were harvested and stained for intracellular cytokines IFN-γ and IL-4, the key signature cytokines distinguishing Th1 from Th2 cells (11). Cell counts of Th1 and Th2 polarized T cells using flow cytometry demonstrated a decrease of the Th1 to Th2 ratio by 36% ($P < 0.021$) in the presence of 1000 μU/ml insulin, suggesting a skewing of T cell differentiation toward a Th2 cell type (Fig. 3, A–C, and Table 1). This effect on T cell differentiation was clearly dose dependent, with the Th1 to Th2 ratio decreasing from 1.42 without insulin to 1.29 at 50 μU/ml insulin (−9.5%, not significant), to 1.08 at 250 μU/ml insulin (−24%, $P < 0.05$) and to 0.94 at 1000 μU/ml (−36%, $P < 0.05$) (Fig. 3C). In conclusion, insulin plays an unexpected differentiation role during effector T cell polarization.

To clarify whether the observed effect of insulin in T cell polarization is caused by an inhibited Th1 or enhanced Th2 differentiation, we generated Th1 and Th2 cells *in vitro* using Th1- or Th2-promoting culture conditions as previously described (15). These Th1- and Th2-promoting cultures were incubated with or without insulin, and Th1 and Th2 cells were analyzed after 7 d by flow cytometry using intracellular cytokine staining. We demonstrated that addition of insulin did not change the number of Th1 cells obtained from Th1-promoting cultures. In contrast, a significant increase (22%; $P < 0.05$) in the number of Th2 cells in the Th2-promoting cultures was seen in the presence of insulin (Fig. 3D and Table 1). This indicates that insulin is acting mainly by enhancing Th2 differentiation, rather than inhibiting Th1 differentiation. In summary, an increase in Th2 cell number is mainly responsible for the described decrease in Th1 to Th2 ratio caused by the addition of insulin.

Insulin can induce a shift toward a Th2-type cytokine secretion profile

To confirm changes in effector T cell polarization upon insulin stimulation, cytokines secreted by activated T cells were analyzed. Sorted CD4$^+$ T cells were stimulated and cultured as described above, in the presence or absence of insulin. After 24, 48, and 72 h, the culture supernatants were collected and IFN-γ and IL-4 levels were measured by ELISA. After 48 and 72 h of treatment with insulin, IFN-γ levels were decreased by −32 and −21%, respectively ($P < 0.05$), and IL-4 levels increased by 32, 16, and 14% at 24, 48, and 72 h, respectively ($P < 0.05$). This resulted in a marked and significant insulin-driven reduction of the IFN-γ to IL-4 ratio at 48 and 72 h by −39 and −33%, respectively ($P < 0.05$) (Fig. 3E).

Therefore, changes in the phenotype of effector T cells seen in Fig. 3, A–D, were further supported by the preferential secretion of Th2 cytokines.

Altered T cell polarization is associated with increased ERK phosphorylation

Although there are no reports of a role for insulin in the regulation of T cell differentiation, it is well established that

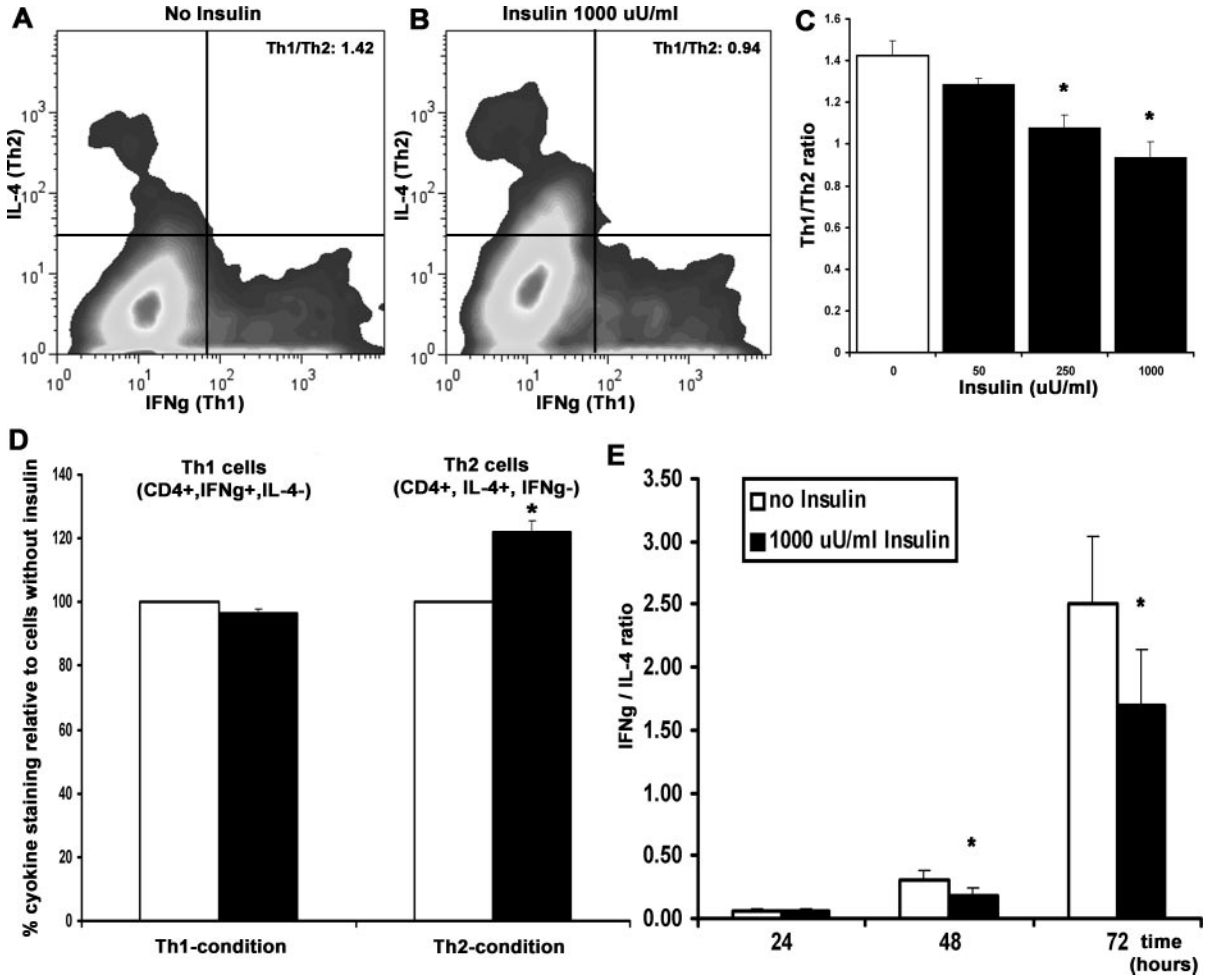

FIG. 3. Sorted $CD4^+$ T cells stimulated for 72 h with immobilized anti-CD3 and anti-CD28 in the presence (B) or absence (A) of 1000 μU/ml insulin. Th1/Th2 populations were defined by intracellular cytokine staining for IFN-γ and IL-4. C, Insulin dose-dependent change in the Th1 to Th2 ratio. D, Sorted $CD4^+$ T cells were stimulated as above, but cultured in Th1-condition (IL-12, anti-IL-4) or Th2-condition (IL-4, anti-IFN-γ) for two periods of 72 h. Th1/Th2 populations were defined as above using intracellular cytokine staining. See Table 1 for cell numbers in C and D. E, IFN-γ and IL-4 were measured using ELISA in the culture media of sorted $CD4^+$ T cells stimulated as above, with or without insulin. *, Significance by $P < 0.05$ with respect to control (cells cultured without insulin), as determined by Mann-Whitney U test. n $\geq$ 3 different subjects and experiments.

insulin can activate some MAPK such as ERK (14). Independently, it is also known that MAPK, especially ERK (in Th2) and JNK/p38 (in Th1) are important regulators of T cell differentiation (12, 13). Therefore, we tested whether the change in T cell polarization caused by insulin treatment is associated with characteristic changes in MAPK phosphorylation, which could lead to activation of transcriptional programs driving T cell polarization (11).

TABLE 1. Th1/Th2 cell numbers after stimulation

	Control	Insulin	Δ Insulin-control (%)
Th1	11.00%	8.20%	−25.5
Th2	2.90%	4.20%	+44.8
Th1 to Th2 ratio	5.1	2.3	−35.8[a]
Th1/2 conditions			
Th1 (Th1 condition)	23.3%	22.5%	−3.4
Th2 (Th2 condition)	2.9%	3.5%	+21.8[a]

Number of Th1 and Th2 cells, expressed as a percentage of total $CD4^+$ T cells, after culture for 72 h (see Fig. 3C) or after culture for 168 h in specific Th1 or Th2 condition (see Fig. 3D), in the presence or absence of insulin.

[a] Indicates significance by $P < 0.05$ with respect to control (cells cultured without insulin), as determined by Mann-Whitney U test. n $\leq$ 3 different subjects and experiments.

Similar $CD4^+$ T cell cultures as described above were used to collect intranuclear and cytoplasmic cell proteins for Western blot analysis at three time points (24, 48, and 72 h), with or without insulin. Fig. 4A shows increasing phosphorylation of ERK, p38, and JNK over time in activated T cells. After 72 h, ERK phosphorylation was clearly enhanced by the presence of insulin, whereas no change in phosphorylation of p38 or JNK was observed at this time point.

Quantification of Western blots from at least eight different normal subjects after 72 h activation *ex vivo* confirmed a significant rise in ERK phosphorylation by 28% ($P < 0.05$) after insulin treatment, whereas no significant changes in p38 and JNK phosphorylation were observed in control cultures at the same time (Fig. 4B). Changes in pERK levels paralleled the observed changes in cytokine production (IL-4) as shown in Fig. 3E, both becoming significant after 48 h. Confirmation

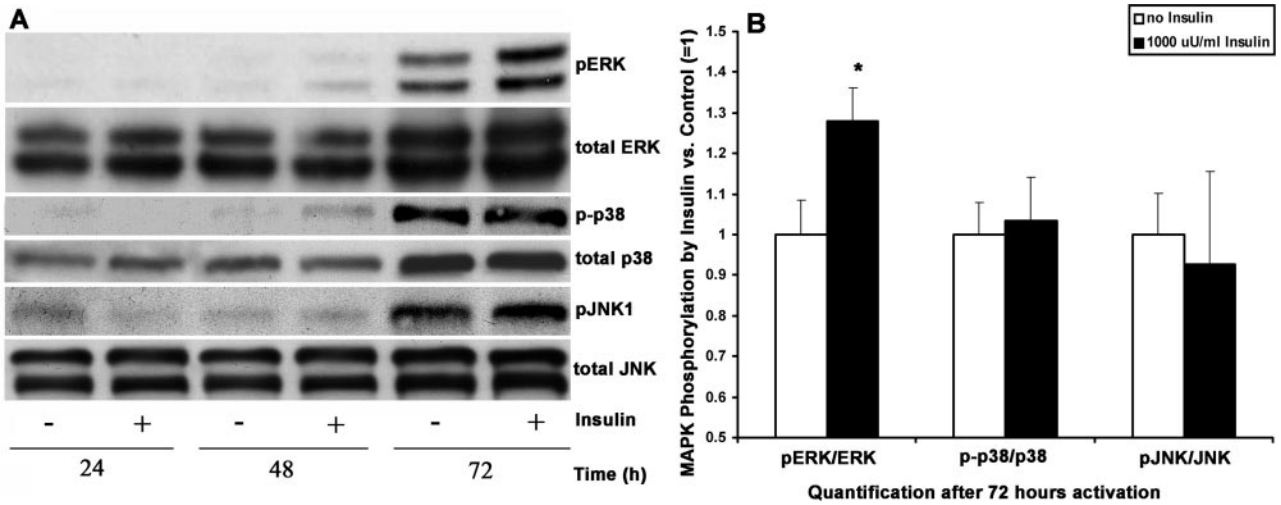

FIG. 4. Sorted $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies for 72 h in the presence or absence of 1000 μU/ml insulin. A, Western blot analysis was performed at 24, 48, and 72 h. B, Quantification of ERK, p38, and JNK phosphorylation at the 72-h time point. Phosphorylated proteins were divided by total proteins. *, Significance by $P < 0.05$ with respect to control (cells cultured without insulin), as determined by Mann-Whitney U test. n = 8 different subjects.

of an increase in ERK phosphorylation in $CD4^+$ T cells after insulin treatment provides a possible mechanism explaining the role of insulin in driving Th2 T cell differentiation.

Discussion

In the present study, we show for the first time that insulin influences T cell differentiation promoting a shift toward a Th2-type response, mainly due to an increase the number of Th2 T cells generated, with corresponding changes in the cytokine secretion profile. The reduction of the Th1 to Th2 cell number ratio and the IFN-γ to IL-4 secretion ratio caused by insulin can be regarded as an important immunomodulatory effect, in accordance with other documented antiinflammatory effects of insulin, including inhibition of NF-κB, reducing proinflammatory cytokines and acute phase proteins as well as increasing production of antiinflammatory cytokines (4–6, 8, 9, 16–18).

Th1 and Th2 are important in the modulation of the immune response. Each subtype is potentially able to counter regulate the other, leading to a dynamic balance that adapts the host immune response according to the specific pathogen. Each subset has specific roles and properties, such as cell-mediated immune responses, especially those targeting intracellular pathogens, which are characteristic of Th1 cell function, and antibody-mediated responses, *e.g.* against intestinal pathogens and parasites, which are more typical of Th2 cell function. Furthermore, a Th1-dominant response is seen in several autoimmune diseases, whereas a predominantly Th2 response is often the feature of allergic diseases and asthma (19–22). Imbalances between Th1 and Th2 cells are often found in these chronic diseases. IL-4 is an important activator of B cells, and a shift from Th1 to Th2 cytokines could lead to a diminution of cellular immune responses and stimulation of antibody-mediated responses. The relative abundance of Th1 and Th2 cells in sepsis depends largely on the nature of microbes driving the infection and its duration (23). Although still controversial, some studies suggest that a Th1/Th2 imbalance favoring a predominance of Th2 cells could be responsible for the immunosuppression observed in sepsis (24). However, these studies used *in vitro* stimulation of T cells, and as such are limited because they do not integrate parameters such as the type of pathogen involved or the time course of sepsis. A shift toward a Th2-response has recently been described as a protective mechanism against an overwhelming proinflammatory immune response, associated with recovery and survival of septic patients (25, 26). In this study, we report that insulin is able to change the balance of Th1 and Th2 in favor of a Th2-type response *in vitro*.

Critical illness and sepsis are often associated with hyperglycemia, a proinflammatory state, and marked insulin resistance. High-dose insulin administration is often necessary to achieve normoglycemia. Furthermore, the excess of proinflammatory cytokines found in severe sepsis is associated with the risk of multiorgan failure and subsequent death. The proinflammatory effect of hyperglycemia has been well demonstrated *in vitro* and *in vivo*, causing an increase in reactive oxygen species generation (27), up-regulation of adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and IL-6 (16), the proinflammatory transcription factors activator protein-1 and early growth response-1, and their regulated gene transcription of metalloproteinase (MMP) 2 and MMP-9 (28).

In contrast, it has been shown that insulin administration and its antiinflammatory properties can counteract the proinflammatory state caused by hyperglycemia. A Belgian study (1) reported a reduction in the level of certain acute phase proteins (C-reactive protein, mannose-binding lectin) and cytokines (IL-6) in patients randomly assigned to intensive insulin treatment. How insulin is involved in directly modulating immune function is not well understood. Previous work has shown that insulin can decrease NF-κB activation and certain proinflammatory cytokines in PBMC (4). In addition, insulin reduces intranuclear early growth response-1, the expression of tissue factor, plasminogen activator inhibitor-1 (17), and the expression of vascular endothelial growth factor and MMP-9 (18).

De novo emergence of IR on T cells upon activation is not a novel finding (29–31), and the presence of IR on other leukocyte subsets has been described already (32). However, this is the first report comparing the expression of IR on all major subsets of human peripheral blood leukocytes in the resting and activated state, and thus demonstrating that *de novo* expression of IR upon activation is a unique property of T cells, and might indicate an important role in modulating T cell response (Fig. 1D). A slight but not significant increase of AKT phosphorylation after short time incubation of resting $CD4^+$ T cells might indicate a weak expression of IR that otherwise is not detectable by flow cytometry or quantitative PCR (Fig. 1E). However, the up-regulation of IR after activation was strong (6-fold) and highly significant. A similar trend of up-regulated IR was seen on $CD4^+$ and $CD8^+$ T cells collected from four septic patients from our intensive care unit. In contrast to our *in vitro* activation, it was only a defined subset of T cells showing IR expression (Fig. 1C), and this subset was also characterized by high expression of CD25 and CD69. Considering that *in vivo* activation of T cell is antigen-specific, and a consequence of direct contact with antigen presenting cells, the observation that not all T cells show a similar degree of activation seems physiological.

Because IR is also expressed on other immune cells such as innate immune cells, we cannot exclude other insulin-mediated protective roles from these cells as an explanation for the reduced inflammation seen in septic patients receiving insulin. However, we postulate that the ability of insulin to modify the polarization of Th cells may be an important contributor to the protective effect of insulin during severe illness. Whether insulin can also prevent observed apoptosis of $CD4^+$ T cells (as shown in our *in vitro* experiments; Fig. 2C) and B cells (33) or modulate T cell differentiation and cytokine secretion in sepsis is currently being investigated by our group in association with an Australian multicenter study conducting a clinical trial on the use of insulin infusion in intensive care.

The insulin concentration used in our experiments (1000 μU/ml = 7 nM) is supraphysiological, but still much lower than in most other *in vitro* experiments, where concentrations up to 100 nM frequently are used. Pharmacological insulin concentrations reached under intensive insulin treatment (*e.g.* 8 U/h) are reported to be up to 125 μU/ml (34), but insulin administration of up to 20 U/h at the start of insulin treatment can increase levels up to 500 μU/ml. Several studies using euglycaemic hyperinsulinemic clamps in septic patients reached levels up to 1250 μU/ml (35). These high levels of insulin have shown to partially overcome the marked insulin resistance encountered in sepsis in regard to glucose metabolism, especially glucose oxidation. In our study, the effect of insulin on T cell differentiation was clearly dose dependant, starting at levels of 50 μU/ml and becoming significant at levels of 250 and 1000 μU/ml (Fig. 3C). In summary, the concentration used in our experiments was chosen to ensure a maximal effect despite possibly developing insulin resistance within T cells.

We also investigated whether insulin affects basic T cell biology such as proliferation, apoptosis, and activation status. As shown in Fig. 2C, there was no change in T cell proliferation in the presence of insulin, but a slight but significant decrease in $CD4^+$ T cell apoptosis. Antiapoptotic effects of insulin have already been described (36), and could be beneficial in the setting of sepsis-induced apoptosis of T and B cells (33). To investigate the possibility that insulin might alter the activation status of stimulated T cells, we stained $CD4^+$ T cells for the classical activation markers CD25 and CD69. As demonstrated in Fig. 2, A and B, there was no change in both activation markers after 72 h of activation in the presence of insulin, which could have explained the observed differences in MAPK phosphorylation. It is known that CD25 and CD69 up-regulation is a slow process, peaking after 24 h of cell activation, and therefore, it is not to be expected to be changed in our short time insulin incubation for 10 and 20 min (Fig. 1E). Nevertheless, we confirmed an identical expression of these activation markers by flow cytometry in this experiment (data not shown).

The important role of MAPK in T cell development and differentiation has been well described, especially the role of ERK activation for Th2 development, and p38 and JNK for Th1 development (12, 13). Insulin stimulates cell proliferation through ERK activation, and it has been shown to negatively act on p38 and JNK after prolonged incubation. It is noteworthy that the incubation time of insulin is important to choose to answer specific research questions, as the observed effects on MAPK phosphorylation can be directly the opposite when comparing short- and long-term incubation (14).

In our experiments, we showed that, in activated $CD4^+$ T cells, insulin increases the phosphorylation of ERK without changing phosphorylation of p38 or JNK after 72 h activation, which is paralleled by a shift toward a Th2-cytokine secretion profile (Figs. 2E and 3, A and B). Selective activation of ERK in T cells in response to insulin is likely to be the reason for increased Th2 T cells generated by insulin treatment as ERK activation is critical for Th2 T cell generation (12, 13).

There is also evidence that IL-4 signaling recruits IRS family proteins downstream of phosphatidylinositol 3-kinase, which are important for growth, survival, and regulation of gene expression in response to IL-4 and IL-13 (37). Furthermore, although Stat6 and JAK-3 are present in equivalent amounts in both Th1 and Th2 cells, IRS-2 protein levels are much lower in Th1 than in Th2 cells (38). Thus, altered sensitivity of Th2 committed cells to insulin, by way of relatively higher levels of IRS proteins may explain the preferential expansion of Th2 cells reported. Thus, we cannot exclude that the observed Th2 profile induction by insulin observed in this study may be due to a preferential expansion of already committed Th2 cells rather than true differentiation of naive T cells into Th2 cells.

In conclusion, we have demonstrated for the first time that insulin influences T cell differentiation, promoting a shift toward a Th2-type response. The association of increased ERK phosphorylation with T cell polarization toward a Th2 phenotype in response to insulin treatment suggests that MAPK phosphorylation is key to the role of insulin as a modulator of effector T cell differentiation. This modulatory effect of insulin is in accordance with other documented antiinflammatory effects of insulin. Whether similar changes in T cell differentiation are observed in response to intensive insulin treatment during sepsis in humans is currently being

investigated. In summary, this study uncovers a novel and so far unappreciated role for insulin and IR in the immune system. A better understanding of the role of insulin on immune cell function is important for improving therapeutic strategies for patients in intensive care and other situations of hyperglycemia and insulin resistance.

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