Insulin Signaling as a Potential Natural Killer Cell Checkpoint in Fatty Liver Disease

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Insulin resistance is a key risk factor in the progression of nonalcoholic fatty liver disease (NAFLD) and may lead to liver fibrosis. Natural killer (NK) cells are thought to exert an antifibrotic effect through their killing of activated hepatic stellate cells (HSCs). Here, we investigated how the interplay between NK cells and HSCs are modified by insulin resistance in NAFLD. Fresh peripheral blood NK cells (clusters of differentiation [CD]56^{dim}, CD16^{+}) were collected from 22 healthy adults and 72 patients with NAFLD not currently taking any medications and without signs of metabolic syndrome. NK cells were assessed for insulin receptor expressions and cytotoxic activity when cultured in medium with HSCs. Fibrosis severities in patients with NAFLD were correlated linearly with elevated serum proinflammatory cytokine expression and insulin resistance severity. At the same time, fibrosis severities inversely correlated with insulin receptor expressions on NK cells as well as with their cytotoxic activities determined by CD107a by flow cytometry. NK cells from donors exhibiting severe fibrosis and insulin resistance exhibited significant mammalian target of rapamycin and extracellular signal-regulated kinase depletion (through NK cell western blot quantitation), increased apoptosis, and failure to attenuate HSC activation *in vitro*. While exposure to insulin stimulated the cytotoxic activity of healthy NK cells, rapamycin prevented this effect and reduced NK insulin receptor expressions. *Conclusion*: Elevated insulin levels in F1 and F2 fibrosis enhances NK cell cytotoxic activity toward HSCs and prevents fibrosis progression by insulin receptors and downstream mammalian target of rapamycin and extracellular signal-regulated kinase pathways. At more advanced stages of insulin resistance (F3 and F4 fibrosis), impaired NK cell activity rooted in low insulin receptor expression and or low serum insulin levels could further deteriorate fibrosis and may likely lead to cirrhosis development. (*Hepatology Communications* 2018;2:285-298)

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most rapidly increasing cause of liver disease in the Western world.[1,2] Steatosis of hepatocytes in early stages of NAFLD[3,4] can induce necro-inflammation and progress to nonalcoholic steatohepatitis (NASH),[5] which increases the risk for cirrhosis and hepatocellular carcinoma development.[6,7] Although risk factors of NAFLD progression to cirrhosis/hepatocellular carcinoma have not been fully clarified, disease progression is closely associated with

Abbreviations: α-SMA, α-smooth muscle actin; ALT, alanine aminotransferase; BMI, body mass index; CD, clusters of differentiation; CD107a, lysosomal associated membrane protein 1; ERK, extracellular signal-regulated kinase; EtOH, ethanol; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HOMA, homeostasis model assessment; HSC, hepatic stellate cell; IgG, immunoglobulin G; IL, interleukin; IRS, insulin-receptor substrate; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NK, natural killer; NKT cell, natural killer T cell; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide.

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insulin resistance and metabolic syndrome.[8,9] Insulin sensitizers, such as thiazolidinediones and vitamin E, improved clinical and histologic features of NASH.[10]

Natural killer (NK) cells (clusters of differentiation [CD]$56^{+}CD3^{-}$) are involved in liver injury, regeneration, and fibrosis responses[11] and mediate an antifibrotic effect through direct recognition and killing of activated HSCs.[12] NK cell impairment in cirrhosis may underlie fibrosis progression, and understanding the mechanisms driving this impairment may uncover targets for NK-focused immune therapy.[13] NK cells from obese donors show significantly lower cytotoxic function compared to those obtained from lean healthy donors.[14] Models in diet-induced overweight animals showed similar body mass index (BMI)-associated NK behavior.[15]

In the current study, we investigated the potential role of insulin resistance through expressions of insulin receptors as a metabolic immune checkpoint leading to NK impairment and eventual NASH progression. Potential cytotoxic alterations in NK cells among patients with different NAFLD phenotypes were assessed. Results indicated that NK cell impairment involved in NAFLD progression to cirrhosis is rooted in low insulin signaling manifested by decreased expression of insulin receptors, extracellular signal-regulated kinase (ERK) pathway components, and mammalian target of rapamycin (mTOR) levels. These findings suggest a new cellular insulin checkpoint through which NK cells contribute to fibrosis in patients with NAFLD.

Patients and Methods

STUDY DESIGN

Histologically documented NAFLD cases of patients aged over 18 years who were evaluated at our hospital between 2013 and 2014 were included in this study. To avoid possible bias of therapeutic interventions, we only included patients who were off therapy at the time of evaluation. On the day of liver biopsy, each patient donated blood for NK cell isolation, which was processed the same day and kept in fixation until biopsy evaluation prior to flow cytometric analysis for insulin receptors. Patients were excluded in case of steroid administration; recent use of warfarin, metformin, thiaglitazones, or insulin for more than 1 month or any use in the previous 2 months; or daily alcohol intake >20 gm/day. Patients with competing etiology for liver disease, including viral hepatitis, autoimmune disease, hemochromatosis, Wilson's disease, alpha-1-antitrypsin disease, alcohol, or other toxicity, were excluded from the study. Approval by the hospital's ethics committee and written informed consent from each patient were obtained.

CLINICAL CHARACTERIZATION

BMI, concomitant diseases, and medications at the time of biopsy were reviewed at the time of the study. Serum levels of liver enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase), insulin resistance (calculated according to the homeostasis model assessment [HOMA]), hemoglobin-A1c levels, serum cholesterol (low-density lipoprotein and high-density lipoprotein), and triglycerides were evaluated at the time of biopsy.

NAFLD ACTIVITY SCORE AND FIBROSIS SCORE

All biopsies were assessed for histologic severity of fibrosis and NAFLD activity score and fibrosis.[16] The NAFLD activity score is determined by the sum of scores for steatosis, lobular inflammation, and ballooning and ranges from 0-8. Fibrosis was staged as

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follows: F0, none; F1, perisinusoidal or periportal; F2, perisinus and periportal; F3, bridging fibrosis; and F4, cirrhosis.

PERIPHERAL BLOOD LYMPHOCYTES AND NK ISOLATION

Heparinized blood samples were obtained from 22 healthy volunteers and 72 patients with NAFLD. Mononuclear cells were isolated by centrifugation over a Ficoll-hypaque density gradient (Pharmacia). After three washes in saline, cells were resuspended in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, as described.[17] Human NK cells were isolated from mononuclear cells by using a magnetic cell sorting kit (Miltenyi Biotec) according to the manufacturer's instructions.

NK–HSC INTERACTIONS

Human LX2 (HSC cell line) cells were cultured to confluence in six-well flasks (Nunc Brand Products, Denmark) with Dulbecco's modified Eagle's medium with 1% fetal calf serum. NK cells (1×10^6) were incubated with insulin (20 IU/mL, mimicking human levels; see Table 1) or rapamycin (2 nM), for 6 hours prior to coculture with LX2 cells. Cells were then washed and cocultured with LX2 cells for 48 hours. Before and following coculturing, harvested cells were washed with phosphate-buffered saline (PBS) and assessed for LX2 and NK markers by fluorescence-activated cell sorting (FACS).

FACS ANALYSIS

Both monocultures and cocultures containing human lymphocytes were tested for the pan-leukocyte marker, using anti-CD45 (peridinin chlorophyll protein complex) and anti-CD3 (fluorescein isothiocyanate [FITC]) antibodies and for NK markers, using anti-CD56 (phycoerythrin [PE]) and anti-CD16 (Pacific blue) antibodies (diluted 1:40; IQ Products, Groningen). For each antibody used, an isotype immunoglobulin G (IgG) labeled with each of the fluorochromes listed served as a control. The obtained cells were stained with anti-CD3 antibody to exclude natural killer T cells (NKT cells). The granzyme-B anti-CD107a (allophycocyanin), also known as lysosomal-associated membrane protein 1 (a degranulation marker for NK activation),[18] and anti-insulin receptors (FITC) were used.

To determine apoptosis and viability of human NK cells, we stained cells, following the manufacturer's instructions, with propidium iodide (PI) and FITC-conjugated annexin V (R&D Systems, Minneapolis, MN), which stain fragmented DNA and phosphatidylserine, respectively. Apoptosis was defined as annexin V + and PI–, while killing was defined as annexin V+ and PI+. Cellular expression of pan-mTOR was determined using anti-mTOR (PE) antibodies (R&D Systems). Briefly, cells were trypsinized and brought to a density of 10^6/mL in staining buffer (saline containing

TABLE 1. CHARACTERISTICS OF NAFLD PATIENTS ACCORDING TO HOMA SCORE

	HOMA Score (homeostasis model assessment–insulin resistance)			
Characteristics	Normal HOMA <3 n = 34	Moderate HOMA 3-5 n = 26	Severe HOMA >5 n = 12	P value*
Age [years]	38.6 ± 10.8	41.1 ± 9.4	36.8 ± 12.6	NS
BMI	29 ± 2.4	28.7 ± 3.3	30.3 ± 2.2	NS
LDL cholesterol [mg/dl]	131.7 ± 26.6	136.1 ± 23.1	113 ± 23.5	NS
HDL cholesterol [mg/dl]	37.8 ± 8.4	36.9 ± 10.4	41.5 ± 5.8	NS
Triglycerides [mg/dl]	147.8 ± 39.5	151.1 ± 33.4	153.5 ± 26.6	NS
ALT [IU/L]	81.3 ± 35.5	78.9 ± 35.0	89 ± 35.7	NS
Fibrosis [Metavir]	1.2 ± 0.8	1.9 ± 0.5	2.8 ± 1.1	0.0002
Inflammatory activity [A]	1.4 ± 0.7	1.6 ± 0.6	2 ± 0.0	0.0006
HbA1c [%]	3.3 ± 0.8	4.9 ± 0.4	3.9 ± 0.3	0.005

*P value between HOMA score severity. Seventy-two histology-proven (within 2 years) cases of NAFLD were included if they lacked other etiology for liver disease, metabolic syndrome, or other metabolic drugs, to avoid their phenotypic and functional effects on lymphocytes. Cases were classified according to three HOMA score groups (<3, 3-5, and >5), and metabolic and histology parameters were studied accordingly. HbA1c levels, liver injury grading, and staging had a significant positive correlation with insulin resistance as determined by HOMA score; however, BMI, ALT, LDL, HDL, and triglyceride serum levels did not correlate with insulin resistance in our cohort.

Abbreviations: HbA1c, hemoglobin-A1C; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

1% bovine serum albumin; Biological Industries, Israel). To determine HSC activation, LX2 cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% saponine in PBS for 20 minutes before being stained with anti-human α-smooth muscle actin (α-SMA)-PE monoclonal antibody (R&D Systems) for 30 minutes at room temperature. All stained cells were analyzed with a flow cytometer (BD LSRFortessa cell analyser, BD Biosciences, Mountain View, CA).

WESTERN BLOT ANALYSIS

Following isolation of NK cells, whole NK cell protein extracts were prepared in NK liver homogenization buffer (50 mmol/L Tris–HCl [pH 7.6], 0.25% Triton-X 100, 0.15 M NaCl, 10 mM $CaCl_2$) and complete mini ethylene diamine tetraacetic acid-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Next, proteins (30 μg per lane) were resolved on a 10% (weight/volume) sodium dodecyl sulfate–polyacrylamide gel (Novex, Groningen, the Netherlands) under reducing conditions. For immunoblotting, proteins were transferred to a Protran membrane. Blots were incubated for 1 hour at room temperature in a blocking buffer containing 5% skim milk and then incubated with mouse anti-human ERK antibodies (diluted 1:1,000; R&D Systems) overnight at 4°C, followed by incubation with peroxidase-conjugated goat anti-mouse and rabbit IgG (diluted 1:5,000; Compiegne, France) for 1.5 hours at room temperature. Immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech Inc., Les Ulis, France).

QUANTIBODY HUMAN CYTOKINE ARRAY

A multiplexed sandwich enzyme-linked immunosorbent assay-based technology was used to simultaneously determine the concentration of multiple cytokines (Cat#QAH-CYT-1; RayBiotech, Inc.). For the culture medium of NK cells, a human interferon-γ Quantikine enzyme-linked immunosorbent assay kit (DIF50; R&D Systems) was used. Data analysis for the multiple cytokines was performed using the Quantibody Q-Analyzer, an Excel-based program.

DEPARAFFINIZATION

Paraffin-embedded sections were placed at 60 °C for 15 minutes, incubated in xylene at room temperature for 15 minutes, and then transferred sequentially into 100% ethanol (EtOH), 95% EtOH, 70% EtOH, and 50% EtOH for 4 minutes each at room temperature. Sections were rinsed in deionized water and stored in PBS.

ANTIGEN RETRIEVAL

To determine the optimal condition for antigen retrieval, we used a buffer (10 mM citrate [pH 6.2], 2 mM ethylene diamine tetraacetic acid, and 0.05% Tween 20) that has been shown to give optimal results with immunofluorescence.[19]

IMMUNOFLUORESCENCE STAINING

Tissues were outlined with 100 μL liquid Blocker KASBLOCK to minimize the volume of antibody solution needed for staining. Samples were incubated with human anti-insulin receptor (diluted 1:30) and human anti-NKp46 (diluted 1:170) antibodies (IQ Products, Groningen, the Netherlands) overnight at 4 °C. Following washes with PBS, secondary antibodies conjugated with cyanine-2 or cyanine-3 were applied for 1 hour at room temperature in the dark to detect anti-insulin receptor and anti-NKp46 antibodies, respectively.

CONFOCAL MICROSCOPY AND IMAGE CAPTURE

Samples were viewed and imaged using a Zeiss LSM 710 confocal laser-scanning system (Zeiss, Germany) attached to a Zeiss Axiovert 135M microscope equipped with a plan-apochromat Zeiss 63 × lens. An argon laser (488 nm excitation line) was used to detect green fluorescence, and an Alexa Fluor laser (552 nm) was used to detect red fluorescence.

STATISTICAL ANALYSIS

All clinical, serum, and histologic parameters were compared between groups using the chi-square test for sex and analysis of variance for all other variables. The paired *t* test was applied to test flow cytometry changes within the NAFLD study groups.

Results

HOMA SCORES ARE ASSOCIATED WITH INCREASED LIVER INJURY

Seventy-two patients with NAFLD fulfilled the inclusion/exclusion criteria, 44 of whom were male

(61%). Mean age at biopsy sampling was 39.4 ± 10.6 years, and mean BMI was 29 ± 2.8. As insulin resistance was proposed as a key risk factor in NAFLD pathogenesis, patients were stratified according to their HOMA score (Table 1). Normal HOMA scores (<3) were observed in 34 (47.2%), moderate scores (3-5) in 26 (36.1%), and severe scores (>5) in 12 (16.7%) of the patients. No significant differences in age, BMI, ALT, triglycerides, high-density lipoprotein, or low-density lipoprotein were noted between HOMA score categories. As expected of the selected patient population, who presented mild metabolic complications and was free of medications, hemoglobin-A1c was within the normal ranges. Increased HOMA scores significantly correlated (Table 1; $P = 0.0002$) with histologically detected necro-inflammatory activity and fibrosis. More specifically, HOMA scores gradually increased with fibrosis score progression and were prominent in patients with advanced fibrosis (Fig. 1A; $P = 0.0002$). Insulin serum levels were higher ($P = 0.02$) in early phases of insulin resistance (pancreatic hypersecretion compensatory phase, HOMA ≤5) accompanied with early fibrosis stages (F0 to F2) but were lower in patients with severe insulin resistance (pancreatic failure, HOMA > 5) and advanced fibrosis (F3 and F4; Fig. 1B). In parallel, inflammatory cytokines (interleukin [IL]1a, IL1b, IL2, IL6, IL12p70, IL13, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α, and matrix metalloproteinase 9) increased ($P < 0.05$) with progression of fibrosis scores (Fig. 1C), supporting the association between increased HOMA scores and advanced liver injury (F3/F4 patients).

ELEVATIONS IN THE FREQUENCY OF CD56^{dim} NK CELLS IN PATIENTS WITH F4 NAFLD

Aside from morbid obesity, total NK cell counts[20,21] have been reported in the peripheral blood of donors with insulin resistance and have declined following weight reduction,[22,23] suggesting an immune metabolic disorder. Because CD56^{bright} NK cells were less numerous (data not shown), our results focused on circulating cytotoxic CD56^{dim} cells. A representative dot plot of the flow cytometry analysis of isolated polymorphonulcear cells following Ficoll separations according to their size and granularity is shown in Fig. 2A, and the whole polymorphonulcear population stained with the pan-leukocyte marker anti-CD45 is shown in Fig. 2B. A gate (Gate 1) was drawn on our potential lymphocyte populations, identified as CD45-positive cells with low side scatter-area expression. To further identify NK cells, cells were stained with both anti-CD56 (pan-NK cell marker) and anti-CD3 (pan-T-cell marker) (Fig. 2C) to include NK cells (CD56$^+$/CD3$^-$) and exclude NKT cells (CD56$^+$/CD3$^+$). The stained population was then gated (Gate 2) in order to identify the CD56^{dim} population (CD56$^+$/CD16$^+$; Fig. 2D). Fibrosis severity groups F3 and F4 showed similar NK cell profile results and were therefore combined. Approximately 60% of the circulating NK cells obtained from low-fibrosis donors (F0 and F2) were cytotoxic CD56^{dim} cells compared to 70% among F1 and healthy donors (Fig. 2E). However, patients with NAFLD with F3-F4 fibrosis scores exhibited elevated levels of circulating cytotoxic CD56^{dim} cells (85%; $P < 0.0001$). The increase in NK cells in patients with NAFLD with fibrosis severity F3-F4 could be a compensatory mechanism due to function loss to kill target (NK impairment). For this reason, NK cell activity experiments were performed on these samples.

INHIBITION OF NK ACTIVITY CORRELATED WITH DECREASED EXPRESSION OF INSULIN RECEPTORS

To study the correlation between NK activation and insulin receptor expressions, NK activation marker CD107a in samples was assessed. CD107a expression was significantly higher in patients in early fibrosis stage (F0, 40.5% ± 19.8% of CD56^{dim}; $P = 0.04$) compared to healthy individuals (15.7% ± 0.6% of CD56^{dim}) (Fig. 3A). However, NK stimulation decreased gradually with increased fibrosis (F1, 39.2% ± 19.4%, $P = 0.03$; F2, 31.1% ± 5.9%, $P = 0.001$), with the percentage of CD107a-positive cells reaching a low of 19.6% ± 12.6% in advanced stages of fibrosis (F3-F4, P not significant compared to healthy, $P = 0.05$ compared to low-fibrosis NAFLD groups). These findings suggest increased NK cell stimulation and activation in the early fibrosis stages (F0-F2) but reduced and impaired activation in late NAFLD stages of liver fibrosis (F3-F4).

We next investigated whether inhibition of NK cell activity in F3 and F4 patients with NAFLD correlated with their severe HOMA scores. Insulin receptor expressions on CD56^{dim} NK cells correlated with patient fibrosis scores (Fig. 3B), with 68.6% ± 11.3% of healthy CD56^{dim} NK cells demonstrating insulin

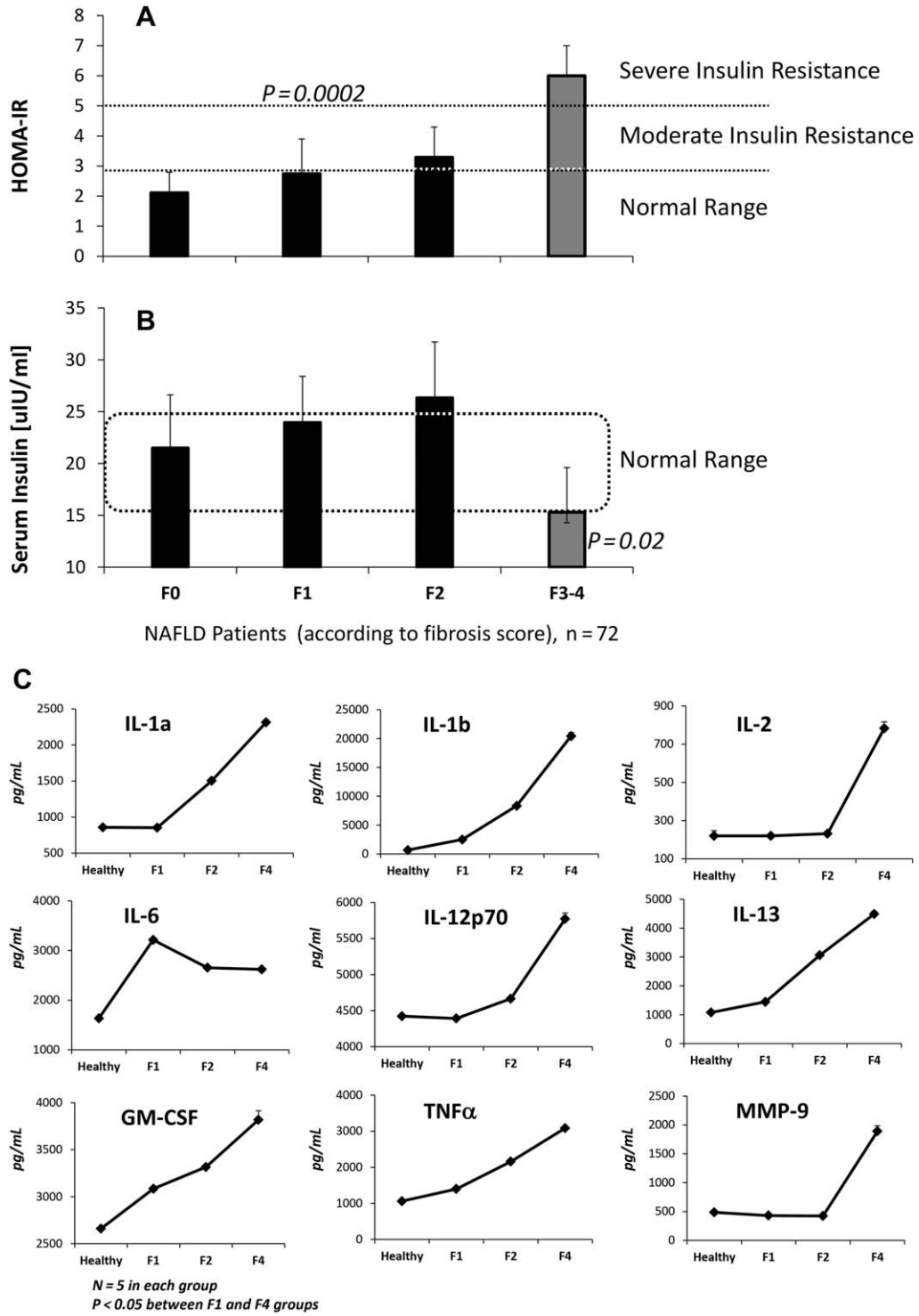

FIG. 1. Insulin resistance correlates with increased liver injury. (A) HOMA scores gradually increased with fibrosis score elevations and were prominent in F3 and F4 fibrosis score. Insulin serum levels increased with increased fibrosis severity to F2; (B) however, decreased levels were seen in patients with advanced fibrosis scores of F3 and F4. (C) Increased proinflammatory cytokine levels in NAFLD F4 patients correlated with increased liver injury. Quantification of the levels measured in three biological and four technical repeats. Data analysis was performed using a Quantibody Q-Analyzer and an Excel-based program; results are presented in pg/mL. Data show mean ± SD. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; HOMA-IR, homeostasis model assessment of insulin resistance; MMP-9, matrix metalloproteinase 9; TNFα, tumor necrosis factor α.

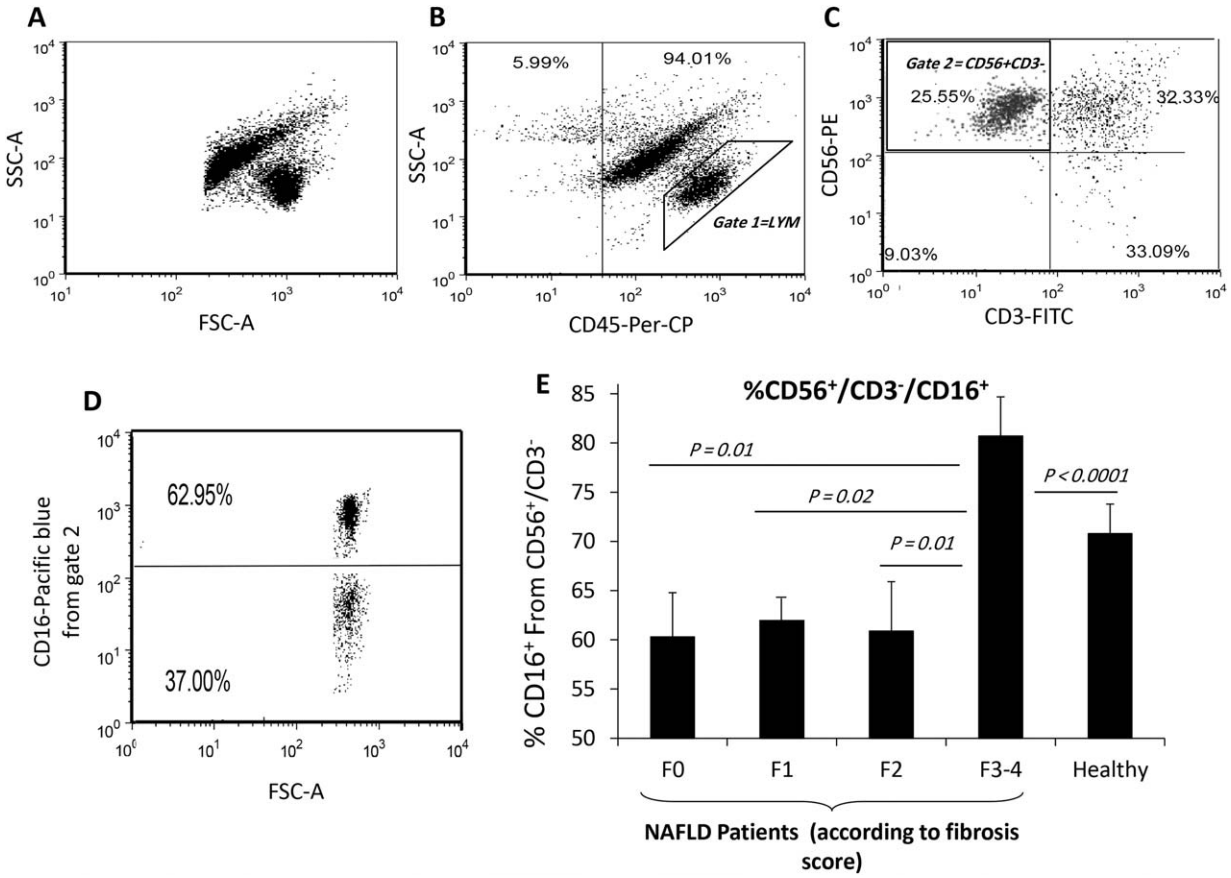

FIG. 2. CD56^{dim} NK cells in patients with NAFLD with different stages of fibrosis. Flow cytometry was used to assess the CD56^{dim} population in patient peripheral blood. Peripheral blood mononuclear cells were purified using the Ficoll-Paque method, as described in Patients and Methods. (A) Dot plot of cells by size (FSC-A) and granularity (SSC-A). (B) Cells were stained for pan-leukocyte markers (CD45-FITC), and the potential lymphocyte population was gated (Gate 1) for CD45+ and low SSC-A. (C) Cells from Gate 1 were further assessed for NK cell content by staining for CD56 (pan-human NK cell marker) and CD3 (T cells). NK cells were gated (Gate 2) according to CD56+CD3–. (D) NK cells were further stained for CD16 to identify the dim populations. (E) Averages of circulating CD56^{dim} NK populations in healthy volunteers and patients with NAFLD. Results are expressed as mean ± SD. Abbreviations: LYM, lymphocyte; Per-CP, peridinin chlorophyll protein complex.

receptor expressions and only 48.8% ± 11.2%, 51.5% ± 10.2%, 50.2% ± 18.6%, and 25% ± 6.3% in the F0, F1, F2, and F3-F4 groups, respectively ($P < 0.05$). No significant differences in NK insulin receptor frequencies were noted between the different early stage NAFLD fibrosis (F1 and F2) groups; however, F3-F4-scored NK cells showed a marked reduction in insulin receptors compared to their F1-F2 counterparts as well as to the healthy group ($P < 0.05$). These observations demonstrated a positive correlation between NK activity, insulin receptor expression, serum insulin levels, and fibrosis scores, namely, patients with severe insulin resistance exhibited NK impairment and diminished insulin activity. This was likely caused by low levels of both serum insulin and NK cell-associated insulin receptor expression.

To further evaluate whether NK cells with decreased insulin receptor expressions differed in their degranulation status from normal levels of insulin receptor expressions, we sorted through flow cytometry gating strategies of NK cells from patients with NAFLD and F4 of high as well as low insulin receptor expressions. Our data showed that NK cells with positive insulin receptors were positive for CD107a expressions, while their insulin receptor negative counterparts showed similar deactivated profiles compared to the isotype IgG controls (Fig. 3C,D). These results indicate a

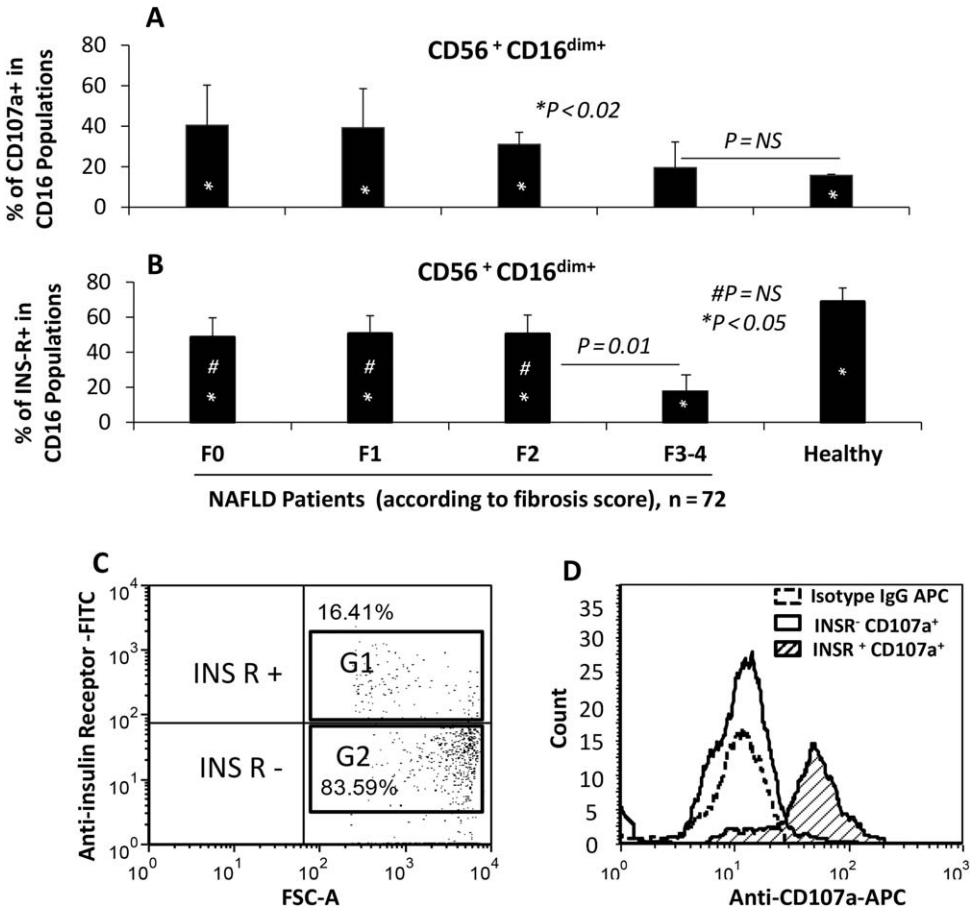

FIG. 3. Changes in insulin receptor expressions correlate with decreased activity of NK cells. (A) CD107a expression by NAFLD CD56^{dim} cells significantly increased in early fibrosis stages (F0-F2), decreased gradually with progressing fibrosis, and remained stable in advanced stages (F3-F4). (B) Insulin receptor expressions on NK cells significantly decreased in all NAFLD groups, with a more prominent decrease in the F4 patients ($P = 0.01$). NK insulin receptor frequencies across early stage fibrosis groups (F0-F2) showed no differences. (C) Dot plot of representative NAFLD F4 fibrosis patient with expressions of insulin receptors. G1 plotted on NK cells with positive insulin receptor (INS R+), and G2 plotted on NK cells with negative insulin receptor (INS R–). (D) Histogram of NK CD107a (APC conjugated) obtained from G1 and G2. Isotype IgG–APC control was used (dotted line). CD107a fluorescence intensities from INSR– (plain histogram) and from INSR+ (lined histogram) are shown. Abbreviations: APC, allophycocyanin; FSC-A, cells by size; G1, Gate 1; G2, Gate 2; INS-R, INSR, insulin receptor; NS, not significant.

correlation between insulin receptor expression and NK cell activity/cytotoxicity.

mTOR/ERK INHIBITION CORRELATES WITH DECREASED CD56^{dim} INSULIN RECEPTOR EXPRESSION AND NK IMPAIRMENT

We assessed the implications of insulin receptor signaling in patient-derived NK cells by quantitating both intracellular pan-mTOR activity and mitogen-activated protein kinase (MAPK) activity (ERK1 pathway).[24,25] mTOR exerts diverse immunoregulatory functions during immune cell activation and differentiation depending on the cell subtype.[25] Insulin signals propagated by recruitment of insulin receptor substrate-1 (IRS-1) to the insulin receptor lead to increased mTOR (mTORC1 and mTORC2) signaling and cell growth.[26,27] The intracellular pan-mTOR activity marker of NK cells was detected by flow cytometry, and ERK phosphorylation was quantitated by western blot. NK cells from healthy donors showed high insulin receptor expression (Fig. 3A) in addition to high expressions of mTOR (Fig. 4A).

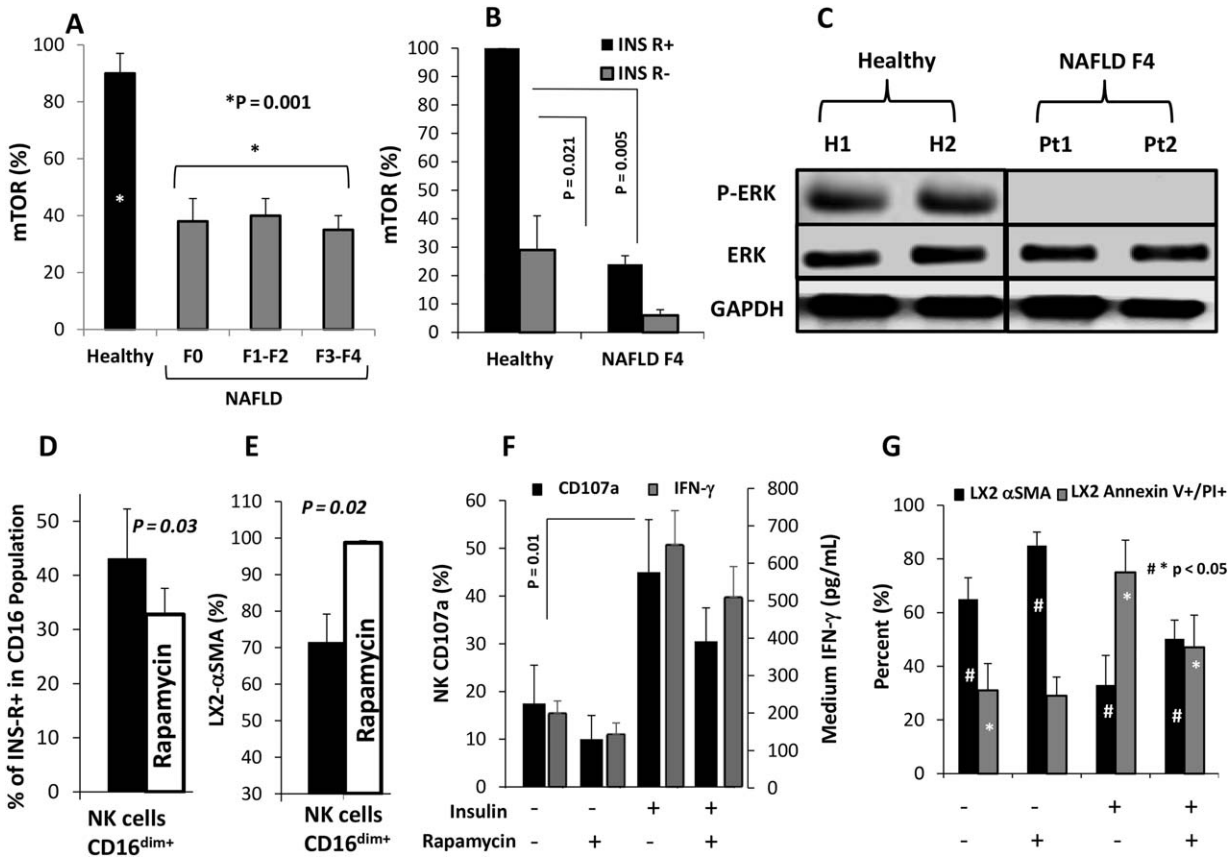

FIG. 4. mTOR and pERK inhibition associated with decreased CD56^{dim} insulin receptor expression and impaired activity. (A) CD56^{dim} NK cells derived from patients with NASH with different fibrosis scores exhibited significant mTOR inhibitions. (B) CD56^{dim} NK cells with high insulin receptors showed high mTOR expressions, while NK cells with low expressions of insulin receptors showed depleted mTOR. Patients with NASH showed reduced mTOR in both NK cell populations with and without the insulin receptors. (C) NASH F4 patients showed dephosphorylated NK ERK. (D) In monocultures, rapamycin induced significant reductions in insulin receptor expressions on the CD56^{dim} NK cells. (E) In cocultures, rapamycin-treated NK cells were unable to reduce LX2 cell activation (α-SMA) ($P = 0.02$). (F) NK incubations with insulin (20 IU/mL) significantly elevated CD16^{dim} NK CD107a expressions (black bars) as well as secreted INF-γ in the medium (gray bars), indicating an increase in their NK cell activity that was reduced by treatments with rapamycin. (G) Pre-incubation of NK cells with insulin significantly improved their cytotoxic activity against LX2 cells, reflected by a reduction of α-SMA (black bars) and stimulated LX2 killings (gray bars). These effects were inhibited by rapamycin. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN-γ, interferon-γ; INS R, INS-R, insulin receptor; p-ERK, phosphorylated ERK.

NAFLD CD56^{dim} NK cells from donors from all fibrosis score groups exhibited significant mTOR depletion ($P = 0.001$; Fig. 4A).

We further sorted NK cells into insulin receptor high and low subsets for a comparative analysis of mTOR percentages through flow cytometry analysis. NK cells with high insulin receptors expressed high mTOR percentages in both healthy and NAFLD donors of F4 fibrosis score; in contrast, NK cells with low insulin receptors showed low mTOR percentages (Fig. 4B). NK cells from patients with NAFLD showed inhibited expressions in mTOR in both insulin receptor-positive and receptor-negative populations ($P < 0.05$) compared to their healthy counterparts. In parallel, western blot analysis showed ERK1/2 to be highly phosphorylated in healthy NK cells while NAFLD NK cells showed no phosphorylation activity (Fig. 4C), indicating impaired insulin signaling.

To mimic the impaired state of NK cells seen in cells derived from NAFLD donors with high HOMA scores, healthy peripheral NK cells were incubated with rapamycin (mTOR inhibitor, 2 nM) for 6 hours. A significant reduction in mTOR activity was achieved

(data not shown) alongside reduced insulin receptor (Fig. 4D). In cocultures, rapamycin-treated NK cells were unable to curb LX2 cell activation (α-SMA), unlike the untreated NK cells ($P = 0.02$; Fig. 4E).

In contrast, incubation of NK CD16^{dim} cells from patients with NAFLD with a normal HOMA score with insulin led to a significant elevation in their CD107a expression as well as secretion of interferon-γ (from 200 ± 32 pg/mL to 650 ± 90 pg/mL), indicating an increase in their activity (Fig. 4F). These effects were inhibited following co-incubations with rapamycin ($P = 0.02$). In addition, insulin significantly deactivated LX2 and improved killing (Fig. 4G). These effects were modulated by the use of rapamycin through reductions of mTOR and inhibited NK cell activity, which as a consequence, activated HSCs in the coculture model. These results indicate that normal serum insulin levels as well as adequate expression of insulin receptors are required to enhance NK activity and reduce liver fibrosis. Conversely, their deficit causes impaired NK function.

INSULIN RECEPTORS ON LIVER NK CELLS

In light of the findings on peripheral NK cells, we sought to evaluate insulin receptor expression on liver NK cells from the same patients. As described in Patients and Methods, liver NK cells were stained for NKP46 and insulin receptors. Confocal microscopy images of NK cells from patients with NAFLD and with fibrosis scores of F1 and F4 with insulin receptor expression present are shown in Fig. 5 (merged images show cells co-expressing NKp46 and the insulin receptor [pink]).

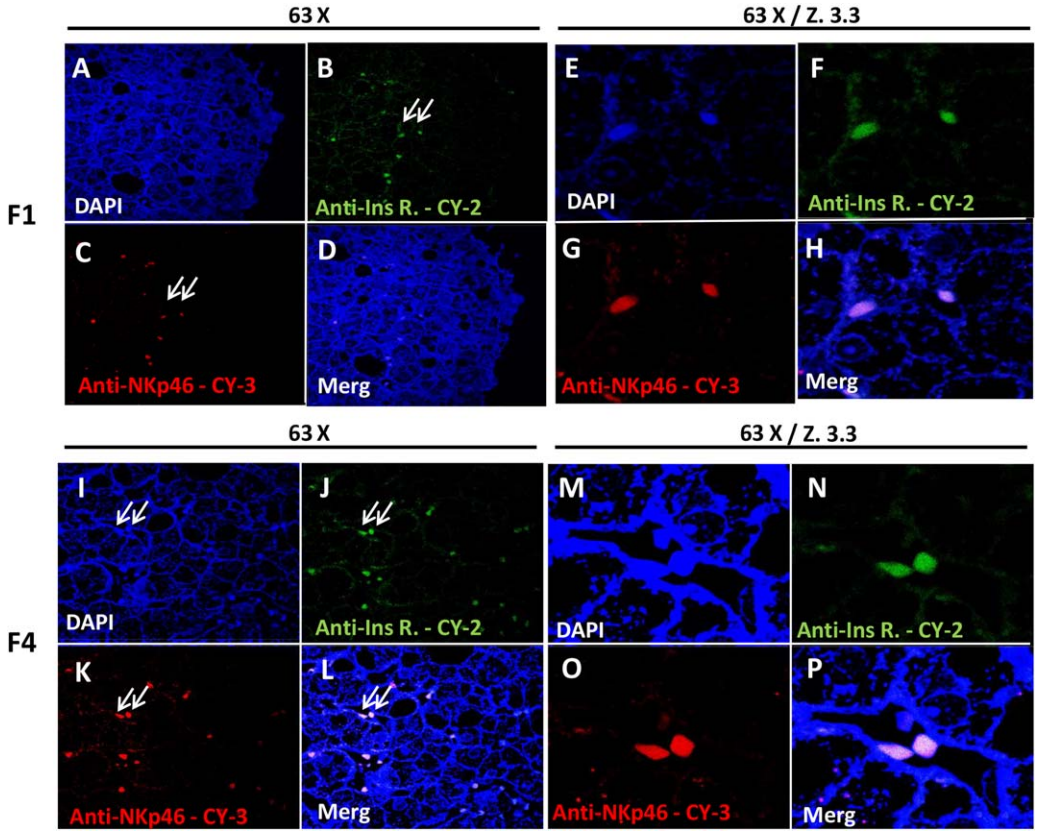

FIG. 5. Liver NK cells express insulin receptors. Liver biopsies from NAFLD patients with fibrosis score F1 (A-H) and F4 (I-P) with both 63 × and 63 × / Z. 3.3 lens magnifications, stained with DAPI nuclear content (blue), Cy-2 conjugated insulin receptor (green), Cy-3 conjugated NKp46 (red), and merged images showing cells co-expressing NKp46 and the insulin receptor (pink). The arrows show selected NK cells in the different staining conditions. Abbreviations: CY, Cy, cyanine; DAPI, 4′,6-diamidino-2-phenylindole; Ins R, insulin receptor; Merg, merged images.

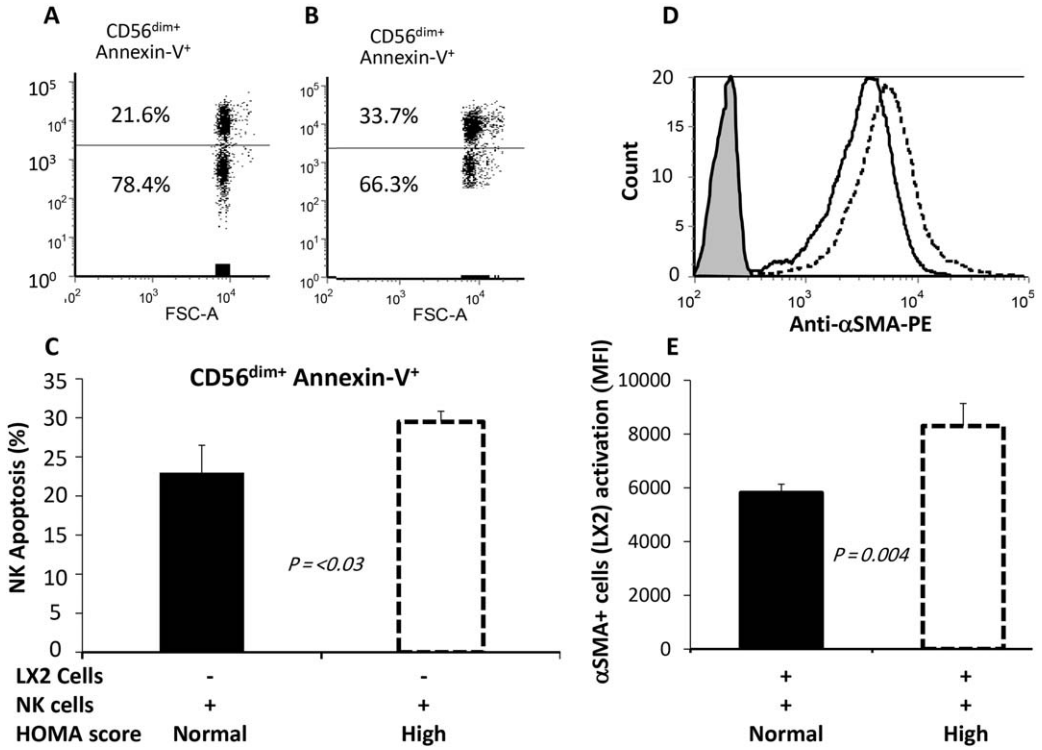

FIG. 6. NK cells from elevated HOMA score donors fail to induce HSC killing. (A) Representative dot plot of apoptotic $CD56^{dim}$ NK cells from normal HOMA score and (B) from high HOMA score (F4) patients. (C) Mean count of apoptotic (Annexin V^+) $CD56^{dim}$ NK cells from patients with NAFLD with high HOMA scores increased compared to those with normal HOMA scores ($P < 0.03$). (D) Representative histogram of LX2 cell activation following cocultures with NK cells from patients with a normal versus patients with a high HOMA score. Gray histogram shows IgG isotype control. (E) Mean α-SMA fluorescence intensities show high LX2 activation following coculture with NK cells collected from patients with a high HOMA score ($P < 0.004$). Abbreviations: FSC-A, cells by size; MFI, mean fluorescence intensity.

NK CELLS ISOLATED FROM ELEVATED HOMA SCORE DONORS FAIL TO INDUCE HSC KILLINGS

Because severe HOMA scores correlated with NK impairment in advanced NAFLD fibrosis, we evaluated whether this impairment involves apoptosis, which in turn contributes to fibrosis severity. To this end, $CD56^{dim}$ cells isolated from patients with NAFLD with varying severities of insulin resistance were cocultured with LX2 cells for 24 hours. $CD56^{dim}$ NK cells from high HOMA score patients (F4) showed high rates of apoptosis compared to those collected from NAFLD donors with normal HOMA scores ($P < 0.03$; Fig. 6A-C). LX2 cells cocultured with NK cells from high HOMA score patients showed higher levels of α-SMA, indicating LX2 activation compared to LX2 cocultures with NK cells of patients with a normal HOMA score ($P < 0.04$; Fig. 6D,E). These data were correlated between severe insulin resistance and advanced fibrosis and loss of the antifibrotic properties of NK cells.

Discussion

Insulin resistance is a key risk factor in the pathogenesis of type 2 diabetes mellitus and NAFLD.[28,29] The current study assessed 72 samples collected from patients with NAFLD, with half of these patients histologically proven as NASH. Our patient population was free of medical therapies, which avoided possible pharmacologic effects. As reported by others,[30] a significant positive correlation was observed between patient HOMA scores and liver injury, including inflammation (histology and cytokines) and fibrosis staging (Table 1; Fig. 1). High HOMA scores (>3)

correlated with histologically defined liver damage severity (Table 1), classified by fibrosis score ($P = 0.0002$) and necro-inflammatory activity ($P = 0.0006$), and was associated with increased serum levels of inflammatory cytokines (Fig. 1C). Differences in HOMA score and liver injury were not affected by age, BMI, ALT, or lipid metabolism (Table 1).

Altered insulin signaling was investigated in the current study as a potential immune checkpoint for NK cell activity (activation or impairment) in NASH patients with varying severities of insulin resistance. Our findings indicate that serum insulin, acting through insulin receptors, directly enhances cytotoxic NK activity toward HSCs, alleviating fibrosis in early hyperinsulinemic stages of insulin resistance. Reduced serum insulin levels and/or reduced expression of insulin receptors on NK cells prevents NK cell activation and induces their impairment by apoptosis. Thus, the attenuated insulin pathway in patients with a high HOMA score impairs NK function and could promote disease progression to cirrhosis.

The NK cells are a key component of the innate immune system.[31] Human NK cells can be classified as $CD56^{dim}$ and $CD56^{bright}$ cells, with $CD56^{dim}$ NK cells accounting for up to 90% of all NK cells in peripheral blood and spleen. Although, equal numbers of $CD56^{dim}$ and $CD56^{bright}$ NK cells are found in the liver,[31] a direct correlation has been found between liver and peripheral NK function.[32] Liver $CD56^{dim}$ NK cells resemble circulating conventional peripheral blood NK cells.[31] According to our data (Fig. 2), the proportion of circulating $CD56^{dim}$ NK cells in patients with early stage fibrosis was significantly lower than in healthy volunteers, but for patients with F3/F4 scores, the proportion was significantly higher than in healthy volunteers.

$CD56^{dim}$ NK cells demonstrated activity in the early NAFLD stages of fibrosis but were in an inactive state in cases of advanced fibrosis (Fig. 3A). These observations corroborate the reported decrease in NK cell function with cirrhosis progression.[32] As HOMA score correlates with liver injury severity, we hypothesized that it may play a role in NK function. Thus, insulin receptor expression by $CD56^{dim}$ NK cells and their response to serum insulin exposure were investigated. Although the role of NKT and NK cells has been studied in the pathogenesis of NAFLD,[31,32] to the best of our knowledge, this is the first observation describing the presence of insulin receptors on peripheral blood-derived NK cells and the first to show differences between healthy volunteers and patients with NAFLD in receptor expression profiles (Fig. 3B), reflecting a kind of "immune NK checkpoint."

The phosphorylation of IRS-1 and IRS-2 and activation of phosphatidylinositol 3-kinase and mTOR signaling have been implicated in the mediation of the metabolic effects of insulin,[33] in addition to the phosphorylation of Shc and activation of Ras, Raf, MAPK kinase, and MAPKs Erk 1 and 2.[34] To investigate the role of the insulin receptor and its downstream signaling pathways in NK cell activity, we adapted a previously established functional test technique[35] of *in vitro* NK cell monocultures and coculture with the LX2 human HSC line. Neither mTOR expression nor phosphorylated ERK was detected in NK cells isolated from patients with NAFLD with advanced fibrosis and severe insulin resistance (Fig. 4). In addition, $CD56^{dim}$ NK cells from such patients exhibited a greater degree of apoptosis when compared to active $CD56^{dim}$ NK cells isolated from patients with NAFLD and a normal HOMA score but suffering from a lower level of fibrosis (Fig. 6A-C). The $CD56^{dim}$ NK cell population demonstrating a high incidence of apoptosis was unable to kill HSCs (LX2) in coculture (Fig. 6E). Therefore, impaired $CD56^{dim}$ NK cells in patients with a high HOMA score are unable to reduce fibrosis, which may explain the high fibrosis scoring.

Rapamycin, an mTOR inhibitor, alters glucose homeostasis, induces insulin resistance,[27] and inhibits NK cell cytotoxicity that contributes to immunosuppression.[36] Accordingly, rapamycin treatment evoked insulin receptor down-expression in $CD56^{dim}$ NK cells derived from patients with NAFLD with normal HOMA scores (Fig. 4D), impaired NK killing potential (Fig. 4F), and attenuated fibrosis (Fig. 4E); this mimicked NAFLD with high HOMA scores (suggesting an *in vitro* model for NK impairment by rapamycin induction of HOMA score). Conversely, $CD56^{dim}$ NK cells derived from patients with high HOMA scores were activated after insulin stimulation, likely the results of normal insulin receptor expression (Fig. 3B). Therefore, insulin-induced stimulation of $CD56^{dim}$ NK cells from patients with a normal HOMA score enhances the killing activity of these cells (Fig. 4F) and reduces HSC activation (Fig. 4G). Pioglitazone hydrochloride insulin sensitizer increased NK CD107a and mTOR levels ($P < 0.04$) and prevented down-expression of insulin receptors (data not shown).

In conclusion, we propose that HOMA scores dictate $CD56^{dim}$ NK cell activity and possibly that of

other immune cells and contribute to functional alterations of NK cells. Thus, insulin can be viewed as an NK activator and provides an antifibrotic effect in early hyperinsulinemic stages. However, states of high HOMA scores with low serum insulin levels and/or low receptor expression contribute to NK cell apoptosis and functional impairment by depletion of mTOR and phosphorylated ERK. These findings can explain fibrosis progression in NAFLD/NASH. $CD56^{dim}$ NK cell dysfunction in insulin-resistant patients may also explain the higher incidence of liver and non-liver malignancies in this patient population.

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