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The anti-angiogenic insulin receptor substrate-1 (IRS-1) antisense oligonucleotide, aganirsen, impairs AU-rich mRNA stability by reducing 14-3-3β-tristetraprolin protein complex reducing inflammation and psoriatic lesion size in patients

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Running title: Aganirsen improves clinical signs of psoriasis

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Abbreviations: CK-16, cytokeratin 16; *h*EC, human endothelial cell; IRS-1, insulin receptor substrate-1; ITT, intent-to-treat; PGA, physician's global assessment; PASI, psoriasis area severity index; SPN, soluble perinuclear-nuclear fraction; TTP, tristetraprolin protein; TSS, total severity sign score.

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Abstract

Increased inflammation and aberrant angiogenesis underlie psoriasis. Here, we report that the inhibition of IRS-1 expression with aganirsen resulted in a dose-dependent reduction ($P<0.0001$) in IRS-1 protein at the cytoplasm, while IRS-1 protein remained quantitatively unchanged in the perinuclear environment. Aganirsen induced a dose-dependent increase in serine-phosphorylated IRS-1 ($pIRS\text{-}1_{Ser}$) in the soluble perinuclear-nuclear fraction (SPN), inducing IRS-1-14-3-3β protein association ($P<0.001$), thereby impairing 14-3-3β-tristetraprolin protein (TTP) complex and AU-rich mRNA's stability ($P<0.001$). Accordingly, aganirsen inhibited ($P<0.001$) *in vitro* the expression of interleukin-8 (IL-8), IL-12, IL-22 and tumour necrosis factor alpha (TNFα), four inflammatory mediators containing mRNA with AU-rich regions. To demonstrate the clinical relevance of this pathway, we tested the efficacy of aganirsen by topical application in a pilot, "double-blind", randomized, dose-ranging study in 12 psoriatic human patients. After 6-weeks of treatment, Least Square means differences with placebo were -38.9% (95%CI [-75.8%; -2.0%]) and -37.4% [-74.3%; -0.5%] at the doses of 0.86 and 1.72 mg/g, respectively. Lesion size reduction was associated with reduced expression of IRS-1 ($P<0.01$), TNFα ($P<0.0001$) and VEGF ($P<0.01$), reduced keratinocytes proliferation ($P<0.01$), and the restoration ($P<0.02$) of normal levels of infiltrating $CD4^+$ and $CD3^+$ lymphocytes in psoriatic skin lesions. These results suggest that aganirsen is a first in class of a new generation of anti-angiogenic medicines combining anti-inflammatory activities. Aganirsen-induced down-regulation of inflammatory mediators characterized by AU-rich mRNA likely underlies its beneficial clinical outcome in psoriasis. These results justify further large-scale clinical studies to establish the dose of aganirsen and its long-term efficacy in psoriasis.

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Introduction

Psoriasis is a common chronic disease occurring in approximately 2% of the population in Western countries (Nestle et al., 2009; Sabat et al., 2007). Extensive work has established that increased inflammation, aberrant angiogenesis and vascular remodelling as well as keratinocyte hyper-proliferation are involved in the pathogenesis of psoriasis (Heidenreich et al., 2009).

T-cell–mediated immune response to an as-yet unknown auto-antigen seems to play a key role in the pathogenesis of psoriasis. This deregulated immune response is primarily driven by $CD4^+$ T cells with a T(h)1 and/or T(h)17 phenotype (Nestle et al., 2009; Coimbra et al., 2012; Nograles et al., 2008), and by the production of TNFα (Caldarola et al., 2009; Orlinick and Chao 1998). A significant increase in the development of the microvasculature (angiogenesis) has been observed in psoriatic lesions compared with healthy skin (Heidenreich et al., 2009), especially in the early phase of the development of the lesions (Henno et al., 2010). Vascular endothelial growth factor (VEGF), which has a central role in angiogenesis, is up-regulated in psoriasis (Detmar et al., 1994), and its levels of expression represent a good indicator of active psoriatic arthritis (Heidenreich et al., 2009).

Conventional systemic therapies for psoriasis, such as methotrexate, cyclosporin A, retinoids or psoralen and ultraviolet A (PUVA) therapies, can result in long-term toxicity and may not be effective. The development of novel therapies targeting inflammatory factors including TNFα, IL-12 and IL-22 now provide new and efficient treatment options (Weger 2010). Nonetheless, because both angiogenesis and inflammation are involved in the development of psoriasis, we speculated that aganirsen (GS-101) could be efficient in psoriatic patients. Aganirsen is an antisense oligonucleotide that inhibits the expression of IRS-1 and has anti-angiogenic activities including inhibition of both VEGF and IL-1βexpressions (Al-Mahmood et al., 2009; Cloutier et al., 2012; Andrieu-Soler et al., 2005). In this study, we show that by

inhibiting IRS-1 expression in the cytoplasmic compartment, aganirsen impaired 14-3-3β-TTP complex formation leading to the inhibition of the expression of several cytokines with AU-rich mRNA, including IL-8, IL-12, IL-22 and TNFα. We tested the efficacy of aganirsen by a 6-week topical application in patients with chronic mild-to-moderate plaque psoriasis and showed that aganirsen reduced plaque area. This was associated with a reduction in the expression of VEGF and TNFα protein as well as a normalization of the levels of $CD4^+$ and $CD3^+$ lymphocytes, and reduced keratinocyte proliferation in psoriatic skin lesion biopsies. These data strongly support that the combined anti-angiogenic and anti-inflammatory properties of aganirsen synergized to improve the clinical status of the patients.

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Materials and Methods

Real-Time Reverse Transcription-Polymerase Chain Reaction Assay. The human microvascular endothelial cell line (*h*EC) was provided by Dr E.W. Ades (Center for Disease Control and Prevention, Atlanta, GA, USA) who established this line by transfecting human dermal endothelial cells with SV40A gene product and large T antigen (Ades et al., 1992). Cells were cultured in EGM2-MV medium (Lonza, Levallois, France). After exposure to various concentrations of aganirsen (0-10 μM) or vehicle for 24 h, hEC (5×10^5 cells/ml) total mRNAs were isolated using the NucleoSpin RNA II kit. RNA yields and purity were assessed by spectrophotometric analysis. The real-time RT-PCR was performed as described previously (Al-Mahmood et al., 2009). In brief, 0.5 μg of total RNA was reverse-transcribed with random hexamer primers and Moloney murine leukemia virus (200 U; Invitrogen), and the synthesized cDNA was used immediately for real-time PCR amplification using the DNA-binding dye SYBR Green I for the detection of PCR products and the following primers: TNFα (sense, 5′-GCTGCAGCACATTATAATACAGAGA-3′; antisense, 5′-GGTGTTTGTCGCGACTCC-3′); IL-8 (sense, 5′-AGTGGACCACACTGCGCCAAC-3′; antisense, 5′-CCACAACCCTCTGCACCCAGT-3′); IL-12 (sense, 5’-GAATGCAAAGCTTCTGATGGA-3’; antisense 5’-GTGGCACAGTCTCACTGTTGA-3’); IL-22 (sense, 5’-CCCTCAATCTGATAGGTTCCAG-3’; antisense 5’-GCAGGTCATCACCTTCAATATG-3’) and GAPDH (sense, 5′-TGAAGGTCGGAGTCAACGGA-3′; antisense 5′-CATTGATGACAAGCTTCCCG-3′). The real-time PCR reactions were carried out with the DNA Light Cycler 480 (Roche Diagnostic, Meylan, France). The results were quantified using the equation: $Copy_{TF}/Copy_{GAPDH} = 2C(\mathbf{t})_{GAPDH} - C(\mathbf{t})_{TF}$. All PCR products were analyzed by electrophoresis on a 1.5% agarose gel, visualized with ethidium bromide, and analyzed using the Genesnap 6.00.26 software

(Syngene, Frederick, MD). Densitometric analysis was performed using GeneTools Analysis Software version 3.02.00 (Syngene).

Subcellular protein fractioning and protein quantification. Subcellular protein fractioning was realized using the Thermo Scientific subcellular protein fractionation kit for cultured cells (Product No. 78840; Pierce Biotechnology, Rockford, USA) according to the manufacturer's instructions. The fractionation kit permitted the separation and preparation of five fractions: membrane, cytoplasmic, soluble peri-nuclear and nuclear (SPN), chromatin-bound and cytoskeletal protein fractions from hECs. IRS-1 protein was only detected in the cytoplasmic and the SPN fractions. The protein content of the different fractions was measured by Bradford's method and adjusted. For purity control within the subfractions, equivalent amounts of proteins were resolved by SDS-PAGE, followed by the transfer of proteins to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with 5% defatted milk solution in PBS-0.5% Tween for 1 h, followed by two washes, and immunoblotting with an anti-epidermal growth factor receptor (EGFR) mAb (clone D38B1, rabbit mAb, Cell Signaling Technology Inc., Danvers, MA) as plasma membrane marker; an anti-heat shock protein p90 (Hsp90) (cytoplasmic fraction marker) (clone C45G5, rabbit mAb, Cell Signaling Technology Inc., Danvers, MA); an anti-histone deacetylase 2 (HDAC 2) mAb (rabbit mAb, Cell Signaling Technology Inc., Danvers, MA) and an anti-transcription factor Sp1 mAb as markers of the soluble nuclear and peri-nuclear fraction; an anti-vimentin mAb (clone D21H3 rabbit mAb, Cell Signaling Technology Inc., Danvers, MA) as a marker of the cytoskeletal fraction; an anti-histone 3 mAb as a marker of the chromatin-bound insoluble nuclear fraction. The results of the purity controls of the obtained fractions were shown in supplemental Figure 1.

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Serum-deprived hECs were incubated with different concentrations of aganirsen or scramble oligonucleotide at 37°C under 5% CO_2 for 6 h. After three washes with ice-cold PBS, cells were suspended with the protein extraction buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 25 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 1 µM phenylmethylsulfonyl fluoride). The protein content was measured by Bradford. IRS-1 concentration in the extracts was determined by the Path-Scan Total IRS-1 Sandwich ELISA kit (Cell Signaling Technology Inc., Danvers, MA) according to the manufacturer's instructions. The data were collected from four separate experiments performed in duplicate and expressed relative to control cells (vehicle).

To confirm the results obtained with the Path-Scan Total IRS-1 Sandwich ELISA kit, equal volumes of the adjusted cell extracts were also resolved by SDS-PAGE, followed by the transfer of proteins to a PVDF membrane. The membrane was incubated with 5% defatted milk solution in PBS-0.5% Tween for 1 h, followed by two washes, and immunoblotting with either an anti-IRS-1-HRP conjugate (1:300 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GAPDH-HRP conjugate (Santa Cruz Biotechnology, Inc.), goat anti-TNFα (1:200 dilution, reference sc52746; Santa Cruz Biotechnology, Inc.), and anti-goat-HRP conjugate (1:20 000 dilution; reference sc2302; Santa Cruz Biotechnology, Inc.). Proteins were then monitored by ECLplus (GE Healthcare, Chalfont St. Giles, UK).

For the quantification of IL-8, IL-12, IL-22 and TNFα in the culture medium, hECs in culture medium without serum were incubated with different concentrations of aganirsen or scramble oligonucleotide at 37°C under 5% CO_2 for 24 h. Culture medium was recovered and used to quantify TNFα, IL-8, IL-12 and IL-22 using human TNF sandwich ELISA kit (reference 900-k25, Peprotech, France) and human IL8, IL-12 and IL-22 sandwich ELISA kits (reference

900-k18, 900-K96 and 900-K246, Peprotech, France), respectively, according to the manufacturer's instructions.

Immunoprecipitation. Human ECs in culture medium without serum were incubated with different concentrations of aganirsen or scramble oligonucleotide at 37°C under 5% CO_2 for 6 h. The cells were washed twice with cold PBS, and lysed with 1% Triton X-100 buffer for 1 h at 4°C. The protein 14-3-3 was immuno-precipitated by adding 4 µg of anti-14-3-3β mAb (reference sc628; Santa Cruz Biotechnology, Inc.) and the immune complexes were harvested with protein G-Sepharose beads (Santa Cruz), washed with lysis buffer, resolved in SDS–PAGE, and proteins were transferred to PVDF membrane and revealed with the indicated antibody.

Pilot clinical study. The clinical study was approved by the institutional ethic committee and was conducted in accordance with Good Clinical Practices (GCPs) including International Conference of Harmonization (ICH) Guidelines and all applicable local laws and regulatory requirements. It was designed as a "double-blind", randomized, control *versus* placebo, dose-ranging, Latin-square design single-centre study from the department of dermatology of the Military Hospital of Tunis (see Study protocol and results as supplementary file). It was set up to evaluate the safety and the efficacy of two doses of aganirsen *versus* placebo in 12 patients with chronic mild-to-moderate plaque psoriasis (Feldman and Krueger 2005). Each patient had 3 psoriasis plaques (including 2 refractory lesions; refractory area are elbow and knee). Each lesion was treated either by a low-dose (0.86 mg/g) or a high-dose (1.72 mg/g) of aganirsen or the ointment alone (placebo; 60 % paraffin oil and 40 % white Vaseline, v/v). At the inclusion and randomization visit (Visit 1), the investigator selected for treatment at least 2 discoids or plaques from 2 refractory sites (elbow and knee) representative of the disease

and a third plaque in a refractory or not refractory site. At Visit 1 (inclusion in the study) and at Visit 2 (3-week post-inclusion), 75 tubes were delivered, *i.e.* 25 tubes of each type (A, B and C). The investigator completed a form that was given to the patient together with the ointment tubes explaining what type (A, B and C) of ointment tube must be used for each plaque. The order of plaques corresponded to the order recorded in the case report form. "A" and "B" types were assigned to refractory sites only. "A" ointment tubes were used for the first plaque, "B" ointment tubes were used for the second plaque and "C" ointment tubes were used for the third plaque. Always the same one type of ointment tube was used to treat a given psoriasis plaque throughout the study for a given patient.

Each index psoriasis plaque was photographed at inclusion before starting the treatment and every three weeks. A study nurse was trained to take these photos according to standards specifically developed for this study.

Population of the pilot clinical study. Before starting the pilot clinical study, the percutaneous absorption of aganirsen was studied quantitatively *ex vivo* on human dermatome skin biopsies mounted in Franz™ diffusion cells. Results showed that single topical application of aganirsen ointment resulted in a therapeutic concentration in both the epidermis and the dermis (supplemental Fig. 2A-2B). No change in protocol of the pilot clinical study occurred. The main characteristics of the included patients were summarized in Table 1.

Immunohistochemistry analysis. We collected 3 types of biopsies from 3 patients randomly selected at the end of the trial: healthy skin; placebo-treated psoriatic skin; and aganirsen (0.86 mg/g)-treated psoriatic skin. Tissue sections were stored at -80°C. For immuno-labelling, they were thawed at room temperature, followed by incubation with 3% of a bovine serum albumin solution in phosphate buffer solution (PBS) for one hour at room temperature.

Then, they were labelled with the indicated mAb diluted to 1/100 in PBS/bovine serum albumin 3% for 1 to 2 hours; anti-TNFα mAb (mouse anti-human TNFα; clone 52B83; reference sc-52746; Cliniscience; Montrouge, France), and anti-VEGF mAb (clone C-1; Santa Cruz), overnight at 4°C for anti-human CD3 polyclonal antibody (Affymetrix eBioscience reference 14-0038-82) and anti-human CD4 polyclonal antibody (Affymetrix eBioscience reference 14-0048-82). Tissue sections were then washed with PBS at room temperature, and incubated with the horse anti-mouse IgG (H+L)-Fluorescein conjugate (reference FI-2000; Cliniscience) diluted to 1/100 in PBS for 1 hour. Following washes with PBS, tissue sections were mounted in aqueous medium (Vectashield; Vector. Ref: H-1500). The labelled tissue sections were examined with a Leica DMR Fluorescence microscope equipped with a DC300F camera for image acquisition. The quantification of the labelling was performed using ImageJ software from NIH Image and staining data were normalized and presented as % labelling area per lesion area. For each labelling, the "n" corresponds to the number of tissue sections analysed. Immunohistochemistry procedure and analysis were repeated 3 times in independent series of experiments using the same tissue sections. This measure was made blinded to treatment.

Study endpoints. The primary efficacy endpoint was the percentage evolution of area of plaque size between baseline and week 6 as measured on photographs. This measure was made blinded to treatment. This efficacy assessment relied on a millimetric surface scale measurement of target lesions (pictures). All lesions within the scale square were measured. The sum of all areas of all lesions within the square was used. Therefore, the primary efficacy parameter was defined as:

$$\frac{\text{area at Visit 3} - \text{area at Visit 1}}{\text{area at Visit 1}} \times 100$$

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Major secondary efficacy endpoints were the percentage change in area after 3 weeks from baseline and change after 3 weeks and after 6 weeks from baseline in Total Severity Sign score (TSS) on physical examination and in Physician's Global Assessment (PGA). Safety assessments were made at all visits by investigating adverse events, serious adverse events, and routine hematologic and laboratory values.

Statistical analysis. Primary and secondary efficacy analyses were based on the intent-to-treat (ITT) population. The ITT population included all randomized patients. Safety analyses were based on the dataset comprising all randomized patients who received at least one dose of study treatment. All statistical tests were two-sided at the 5% significance level. The power level of the study was not considered given the small sample size. Normality of areas of target lesions, percentage changes in area of target lesions and lesion diameter variables were checked visually and with the Kolmogorov-Smirnov test and Anderson-Darling test (Anderson and Darling 1954). All parameters related to psoriatic lesion data were summarised by treatment group (placebo, 0.86 mg/g and 1.72 mg/g) and target lesions. Continuous parameters, such as primary efficacy, were compared using ANOVA. All data were analysed using the SAS software version 9.2.

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Results

Aganirsen reduced IRS-1 protein expression in the cytoplasmic fraction, but not in the soluble peri-nuclear/nuclear (SPN) fraction. Aganirsen dose-dependently inhibited IRS-1 expression in hEC (Fig. 1, A and B) and human keratinocytes (Fig. 1, C and D). Subcellular fractionation of hEC permitted the separation of five fractions: membrane, cytoplasmic, SPN, chromatin-bound and cytoskeletal protein. IRS-1 protein was only detected in the cytoplasmic and the SPN fractions. Closer examination of IRS-1 protein expression revealed that aganirsen inhibited IRS-1 protein expression in the cytoplasmic fraction only (Fig. 1E) and not in the SPN fraction (Fig. 1F). Analysis of the serine phosphorylation state of IRS-1 protein revealed that pIRS-1_{Ser} was undetectable at the cytoplasmic compartment (Fig. 1G); in contrast, there was a dose-dependent increase in pIRS-1_{Ser} in the SPN fraction (Fig. 1H).

Aganirsen impairs 14-3-3β-TTP complex leading to the inhibition of TNFα expression. In the SPN fraction, the increased serine phosphorylation of IRS-1 (Fig. 1H) paralleled the increased association of IRS-1 with the protein 14-3-3β. Increasing amounts of IRS-1 co-immunoprecipitated with the protein 14-3-3β (Fig. 2A); this was accompanied with decreasing quantities of the zinc finger protein TTP co-immunoprecipitated with 14-3-3β (Fig. 2B). These results suggest that the association 14-3-3β/IRS-1 impaired the formation of the complex 14-3-3β/TTP. TTP is a hyper-phosphorylated protein that destabilizes mRNA by binding to their AU-rich elements (Cao et al., 2003; Blackshear et al., 2003). The mRNA of many cytokines contains AU-rich elements including those of TNFα, IL-8, IL-12 and IL-22 (Cao et al., 2003). The scramble oligonucleotide had no effect on the membrane-bound TNFα in hEC (Fig. 2D̃) In contrasṭtthere was a dose-dependent decrease in membrane-bound TNFα in hEC incubated with aganirsen (Fig. 2D). This dose-dependent inhibition of TNFα expression by aganirsen was confirmed by the measurement of the secreted TNFα protein in

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the culture medium (Fig. 2F) and by the measurement of TNFα transcripts by qRT-PCR (Fig. 2G). In addition, aganirsen led to a dose-dependent inhibition of IL-8 expression both at the translational (Fig. 2H) and the transcriptional levels (Fig. 2I), of IL-12 expression both at the transcriptional (Fig. 3A) and the translational levels (Fig. 3B), and of IL-22 expression both at the transcriptional (Fig. 3C) and the translational levels (Fig. 3D).

Efficacy of aganirsen in the treatment of psoriatic lesions. Treatment of 12 patients with topical application of 0.86 mg/g or 1.72 mg/g of aganirsen for 6 weeks led to a significant reduction ($p<0.05$) in the area of the treated lesions compared to placebo (Fig. 4A and 4B, Table 2). By contrast, a slight increase in the lesion area was observed (median 22%) in the placebo-treated group. Least square means (LSMeans) differences with placebo were -38.9% (95%CI [-75.8%; -2.0%]) and -37.4% [-74.3%; -0.5%] for the 0.86 mg/g and 1.72 mg/g groups, respectively. A significant decrease in aganirsen-treated lesion size was observed as early as 3 weeks (Fig. 4A and 4B, Table 2) of treatment compared to placebo ($p<0.01$). LSMeans differences with placebo were -37.3% [-62.7%;-11.9%] and -38.3% [-63.8%; -12.9%] for the 0.86 mg/g and 1.72 mg/g groups, respectively.

We found no difference in the evolution of the TSS score ($p=0.63$ and $p=0.82$ at week 3 and week 6, respectively, Table 2) between the 3 groups. At baseline, TSS had a median of 6, at week 3 a median of 4 and at week 6 a median of 3 in the placebo group. In the 0.86 mg/g-treated group, the median TSS score was of 5 at both baseline and week 3, and of 4 at week 6, whereas in the 1.72 mg/g-treated group, the median TSS score was 6 at baseline, 4 at week 3 and 3 at week 6. Likewise, the PGA score remained stable over the 6-week period and was similar in the three groups (Table 2).

Safety of Aganirsen. Safety analysis was carried out on the 12 patients. Aganirsen safety was

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good without any reported adverse effects. Patients reported no irritation, burning or exudation. In each treatment group, mild itching was reported for 1 lesion at baseline, no itching was reported at 3 weeks and a moderate itching for 1 lesion at 6 weeks.

Aganirsen inhibits IRS-1 and VEGF expression in psoriatic lesions of patients. Treatment with aganirsen (0.86 mg/g) reduced IRS-1 expression by 81±12% (p=0.0132; n=22) in human psoriatic skin lesions relative to placebo-treated psoriatic skin lesions (Fig. 5A, and supplemental Fig. 3). No difference in IRS-1 expression was detected between biopsies of healthy skin and placebo-treated lesions (Fig. 5A). In psoriatic lesion biopsies, we found that VEGF labelling was lowered by 42±8% (p=0.0074) in (0.86 mg/g)-treated psoriatic lesion biopsies compared to placebo-treated pathologic skin lesions (Fig. 5B).

Effects of aganirsen on TNFα-expression, inflammatory cell recruitment and keratinocyte proliferation in psoriatic lesions of patients. Treatment with aganirsen (0.86 mg/g) was also associated with a lower TNFα labelling by 77±11% (p=0.0047; n=14) **(**Fig. 6A and supplemental Fig. 4) in skin biopsies of psoriatic patients compared to placebo-treated psoriatic skin samples. Measurement of $CD4^+$ labelling indicated that psoriatic skin contained 65±21% more cells (p=0.013, n=12) than healthy skin. In contrast, $CD4^+$ labelling in aganirsen (0.86 mg/g)-treated pathologic tissue samples was lower by 43±6% (n=12; p=0.035) compared with placebo-treated pathologic tissues (Fig. 6B and supplemental Fig. 5). Similar results were obtained with $CD3^+$ labelling. The psoriatic skin contained 115±40% more (p=0.0089, n=14) $CD3^+$ labelling than healthy skin, confirming our precedent results with $CD4^+$ cells and supporting further the role of inflammation in psoriasis. $CD3^+$ labelling in aganirsen-treated pathologic skin biopsies was reduced (47±6%; p=0.0355) compared to placebo-treated pathologic tissues (Fig. 6C and supplemental Fig. 6); we found $CD3^+$-

labelling to be similar between healthy skin tissues and aganirsen-treated skin tissues. Finally, after 6 weeks of treatment with aganirsen, expression of Ki-67 and CK-16 were lower by 59±3% ($p=0.0132$, $n=10$) and by 46±8% ($p=0.0265$, $n=5$), respectively, compared to placebo-treated lesions (Fig. 7A-7B and Fig. 8A-8B). Taken together, these findings demonstrate that aganirsen has potent anti-inflammatory effects and inhibits both the infiltration of lymphocytes and the proliferation of keratinocytes in the psoriatic skin.

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Discussion

This study demonstrates that aganirsen is the first in its class of a new generation of anti-angiogenic medicines that combines both anti-angiogenic and anti-inflammatory activities and could be an effective treatment for patients with psoriasis. This statement is supported by several direct evidences including: *i*) *in vitro*, aganirsen induced IRS-1/14-3-3β association which impaired 14-3-3β/TTP complex, leading to the inhibition of the expression of cytokines with AU-rich mRNA including IL-8, IL-12, IL-22 and TNFα; *ii)* in psoriatic patients, aganirsen inhibited the expression of both VEGF and TNFα, reducing thereby the infiltration of inflammatory $CD4^+$ and $CD3^+$ lymphocytes; and accordingly *iii)* aganirsen inhibited keratinocyte proliferation and consequently reduced psoriatic lesion area in patients. Psoriasis is a chronic inflammatory disease where many inflammatory cytokines, including IL-12, Il-17, IL-22 and TNFα, play important pathological roles. Thus, the most desired therapy is one that targets altogether these inflammatory factors. The mRNA of IL-12, IL-17, IL-22 and TNFα shares one characteristic, containing AU-rich elements at their 3'-untranslated regions (Bak and Mikkelsen 2010; Khabar 2010). TTP is a mRNA-binding protein with high-binding specificity for the so-called class II AU-rich elements within the 3′-untranslated mRNAs (Cao et al., 2003; Blackshear et al., 2003); specific binding of TTP to the AU-rich elements results in the destabilization and the decay of the mRNAs (Carballo et al.,1998; Lai et al.,1999). TTP occurs in a complex with 14-3-3 preventing TTP from binding to and/or directing mRNA to the degradation machinery (Johnson et al., 2002; Zhao et al., 2011). Our results show that aganirsen-induced association of IRS-1 with 14-3-3β protein impairs TTP-14-3-3β complex, revealing a novel regulatory role for IRS-1. Indeed, IRS-1 associates with many of the seven isoforms of 14-3-3 proteins (Ogihara et al., 1997; Kosaki et al., 1998; Xiang et al., 2002; Oriente et al., 2005); yet, the functional consequence for these associations was unknown. Our results are therefore the first to demonstrate that by promoting

the association of IRS-1 to 14-3-3β protein and impairing TTP/14-3-3β complex, aganirsen inhibits the expression of IL-8, IL12, IL22 and TNFα at both the transcriptional and the translational levels. This is in line with the importance of the liberation of TTP from the TTP/14-3-3 complex, and with the role of TTP in the regulation of expression of AU-rich elements in the destabilization and the decay of mRNAs (Carballo et al.,1998; Lai et al.,1999). These results, combined with those showing that aganirsen inhibited VEGF and IL-1β expression (Al-Mahmood et al., 2009; Andrieu-Soler et al., 2005), identify aganirsen as a dual-target therapeutic agent that is highly desirable for the treatment of psoriasis.

Topical applications of aganirsen lead to therapeutic concentrations in both epidermis and dermis of human skin (supplemental Fig. 2A and 2B). It has been suggested that the impaired barrier function of psoriatic lesions (Schittek 2011) and the amphipathic nature of aganirsen (Cloutier et al., 2012) could facilitate uptake of antisense drugs in general and aganirsen in particular. In agreement, topical applications of aganirsen decreased psoriatic plaque area in both groups of patients (0.86 and 1.72 mg/g) compared to placebo. Furthermore, aganirsen had a rapid onset of action as illustrated by the significant decrease in lesion area after 3 weeks of treatment of -14% and -13% in the 0.86 and 1.72 mg/g treated groups, respectively. By contrast, the plaque area increased by ~15% in the placebo-treated group over this period. We did not find any significant differences in the evolution of TSS and PGA scores either after 3 or 6 weeks of treatment. This is most likely related to the short term of the trial; at least 12-16 weeks of treatment are required to confirm drug efficacy against psoriatic lesions (Papp et al*.,* 2012a,b). In addition, our study did not reveal a dose-dependent effect of aganirsen; both 0.86 and 1.72 mg/g led to a similar decrease in psoriatic lesion area. These results suggest that the two doses tested in this pilot study induced a maximal effect, at least within the timeframe of the current protocol. Lower daily doses of aganirsen combined with a longer period of treatment will therefore have to be tested in future

clinical studies. Importantly, aganirsen was well tolerated locally and no serious adverse effects were observed in any group. Nonetheless, our study was neither large enough nor of sufficiently long duration to ascertain uncommon adverse effects.

Psoriasis is characterized by chronic inflammation and enhanced lymphocytes infiltration (Nestle et al., 2009; Coimbra et al., 2012). Consistently, psoriatic lesions contained more $CD4^+$ and $CD3^+$ labelling compared to biopsies of healthy skin. Lymphocyte recruitment requires blood vessels, and aberrant angiogenesis has been linked to psoriasis (Sabat et al., 2007; Heidenreich et al., 2009; Detmar et al., 1998). In agreement with the anti-angiogenic property of aganirsen, our results showed that VEGF expression was significantly lower in psoriatic lesions treated with aganirsen when compared to placebo-treated psoriatic lesions, although VEGF expression was not elevated in the latter compared to that in the healthy skin. In contrast, $CD31^+$ and $CD34^+$ labelling was increased in psoriatic lesions compared to the healthy skin, and this increase was normalized following the treatment with aganirsen. On aggregate, these results suggest that the anti-inflammatory activity of aganirsen rather than its anti-angiogenic activity underlies the observed beneficial clinical outcome. VEGF is, nonetheless, chemo-attractant (Sawano et al., 2001; Ferrara et al., 2003) and promotes lymphocyte rolling and adhesion in skin microvessels (Detmar et al., 1998). Consequently, we would like to suggest that the inhibition of VEGF expression by aganirsen contributes to limit the recruitment of immune cells within the psoriatic lesions. This would explain the important decrease in TNFα protein in aganirsen-treated psoriatic lesions, which is mostly produced by activated leukocytes (Orlinick and Chao 1998).

Psoriasis is characterized by epidermal thickening and keratinocyte hyper-proliferation, which is directly linked to the inflammation within the psoriatic lesion (Nestle et al., 2009; Sabat et al., 2007). The expression of VEGF and TNFα was reduced by aganirsen in psoriatic lesions compared to placebo-treated lesions, and was associated with more than 50%

inhibition of keratinocyte proliferation as measured by Ki-67 and CK-16 labelling. The loss of CK-16-associated fluorescence following a 6-week therapy is a crucial finding since CK-16 is an important marker predicting therapeutic outcome (Krueger et al., 1995). The potent inhibitory action of aganirsen on keratinocyte proliferation is most probably linked to its anti-inflammatory action through inhibition of the expression of VEGF, TNFα, IL-12 and IL-22.

Finally, the levels of VEGF and TNFα were elevated in the biopsies of healthy skin, confirm the global pro-inflammatory status of these patients (Nestle et al., 2009; Sabat et al., 2007; Weger 2010). Many anti-inflammatory drugs targeting TNFα, IL-12, IL-17 and 1L-23 have been approved or are in advanced development stage for the treatment of psoriasis (Coimbra et al., 2012; Papp et al., 2012a). Unlike aganirsen that is active topically, these orally administrated new drugs have been, however, associated with numerous side effects. Nonetheless, these new biologics have been used in patients with moderate-to-severe forms of psoriasis, while aganirsen was used in mild-to-moderate forms of the disease (PASI score ranged 4-7) and therefore cannot be compared.

In conclusion, our results demonstrate that aganirsen inhibits the expression of cytokines, which mRNA contains AU-rich elements at their 3'-untranslated regions, including TNFα,IL-1β, IL-8, IL-12 and IL-22, known targets of both inflammation and angiogenesis. On the other hand, we confirme that aganirsen is a potent inhibitor of VEGF expression, the only known growth factor that induces inflammation and promotes angiogenesis and lymphangiogenesis (Al-Mahmood et al., 2009; Ji, 2012; Koch et al., 2011). Despite the limitations of our trial (the small sample size and the short-term duration of the treatment), these results suggest that aganirsen is a dual anti-angiogenic and anti-inflammatory agent that could represent an innovative safe and effective alternative for the treatment of psoriasis, a disease in need for new and safe therapeutic options.

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Authorship Contributions

Participated in research design: A.F., N.D., S.A-M. Conducted experiments: S.C., B.D., M.F., C.L. and A.K. Contributed new reagents or analytic tools: J-P.C., S.A-M.. Performed data analysis: S.A-M. Wrote or contributed to the writing of the manuscript: S.A-M.

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Footnotes

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Conflict of interests

Bernadette Darné, Amin Kadi, Maryline Favier, Corinne Lesaffre, and Neijib Doss declare no conflict of interest. Jean-Pascal Conduzorgues is a consultant in product formulation for Gene Signal. Sylvie Colin and Salman Al-Mahmood are cofounders of Gene Signal, have stock ownership and are employees of Gene Signal. Antoine Ferry detains stock ownership of Gene Signal.

Figure legends

Figure 1: Aganirsen reduced IRS-1 protein expression in the cytoplasmic compartment, but not in the SPN. A) Equivalent amounts of proteins extracts from hEC incubated with increasing concentrations of aganirsen were used to quantify IRS-1 protein by Western blot (upper panel). Membranes were stripped and re-probed with anti-GAPDH mAb to control for equal loading (lower panel). B) The same hEC proteins extracts as in A were used to quantify IRS-1 protein by ELISA sandwich assay. C) Equivalent amounts of proteins extracts from human keratinocytes incubated with increasing concentrations of aganirsen were used to quantify IRS-1 protein by Western blot (upper panel). Membranes were stripped and re-probed with anti-GAPDH mAb to control for equal loading (lower panel). D) The same human keratinocyte protein extracts as in C were used to quantify IRS-1 protein by ELISA sandwich assay. E) Equivalent amounts of the cytoplasmic fractions from hEC incubated with increasing concentrations of aganirsen were resolved by SDS-PAGE, transferred to PVDF membranes and probed with anti-IRS-1 mAb (Upper panel). The membranes were stripped and re-probed with anti-GAPDH mAb to control for equal loading (lower panel). F) Equivalent amounts of SPN fractions from hEC incubated with increasing concentrations of aganirsen were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-serine-phosphorylated IRS-1 (pIRS-1) mAb (Upper panel). The same membranes were stripped and re-probed with anti-actin mAb to control for equal loading (lower panel). G) Equivalent amounts of the cytoplasmic fraction from hEC incubated with increasing concentrations of aganirsen were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-serine-phosphorylated IRS-1 (pIRS-1) mAb. H) Equivalent amounts of SPN fractions from hEC incubated with increasing concentrations of aganirsen were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-serine-phosphorylated IRS-1 (pIRS-1) mAb. Where indicated: *, $p < 0.05$; **, $p<0.001$; ***, $p<0.0001$ *versus*

vehicle-treated group (0.9% NaCl). Scr corresponded to the scramble oligonucleotide (10 μM).

Figure 2: Aganirsen impairs 14-3-3β/TTP complex leading to the inhibition of both IL-8 and TNFα expression. A) Human EC incubated with increasing concentrations of aganirsen were lysed and proteins were immunoprecipitated with anti-14-3-3β mAb. Equivalent amounts of the immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membranes and probed with anti-IRS-1 mAb. B) The same equivalent amounts of immunoprecipitates as in A were resolved by SDS-PAGE, transferred to PVDF membranes and probed with anti-TTP mAb. C) The membranes used in A were stripped and re-probed with anti-14-3-3β mAb to control for equal loading in A and B. D) Human EC were incubated with increasing concentrations of aganirsen were lysed and equivalent amounts of proteins were resolved by SDS-PAGE, transferred to PVDF membranes and probed with anti-TNFα mAb to reveal the membrane bound TNFα. E) The PVDF membranes used in D were stripped and re-probed with anti-actin mAb to control for equal loading in D. F) Human EC were incubated with increasing concentrations of aganirsen and TNFα mRNA was measured by qRT-PCR and expressed as mean ± S.E.M. (n = 4). G) Human EC in serum-deprived culture medium were incubated overnight with increasing concentrations of aganirsen and the culture supernatants were collected to quantify secreted TNFα proteins by ELISA sandwich assay. Results are expressed as pg of TNFα per ml of culture supernatant and presented as mean ± S.E.M. (n = 4). H) Human EC were incubated with increasing concentrations of aganirsen and IL-8 mRNA was measured by qRT-PCR and expressed as mean ± S.E.M. (n = 4). I) Human EC in serum-deprived culture medium were incubated over-night with increasing concentrations of aganirsen and the culture supernatants were collected to quantify secreted IL-8 protein by ELISA sandwich assay. Results were expressed as pg of IL-8 per ml

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of culture supernatant and presented as mean ± S.E.M. (n = 4). Where indicated; *, $p < 0.05$; **, $p<0.001$; ***, $p<0.0001$ *versus* vehicle-treated group (0.9% NaCl). Scr correspond to the scramble oligonucleotide (10 μM).

Figure 3: Aganirsen inhibits both IL-12 and IL-22 expression. A) Human EC were incubated with increasing concentrations of aganirsen and IL-12 mRNA was measured by qRT-PCR and expressed as mean ± S.E.M. (n = 4). B) Human EC in serum-deprived culture medium were incubated overnight with increasing concentrations of aganirsen and the culture supernatant was collected to quantify secreted IL-12 protein by ELISA sandwich assay. Results were expressed as pg of IL-12 per ml of culture supernatant and presented as mean ± S.E.M. (n = 4). C) Human EC were incubated with increasing concentrations of aganirsen and IL-22 mRNA was measured by qRT-PCR and expressed as mean ± S.E.M. (n = 4). D) Human EC in serum-deprived culture medium were incubated overnight with increasing concentrations of aganirsen and the culture supernatant was collected to quantify secreted IL-22 protein by ELISA sandwich assay. Results were expressed as pg of IL-22 per ml of culture supernatant and presented as mean ± S.E.M. (n = 4). Where indicated: *, $p < 0.05$; **, $p<0.001$; ***, $p<0.0001$ *versus* vehicle-treated group (0.9% NaCl). Scr correspond to the scramble oligonucleotide (10 μM).

Figure 4: Topical application of aganirsen reduced psoriatic lesion area compared to placebo. (A) Treatment of 12 patients with topical application of 0.86 mg/g or 1.72 mg/g of aganirsen led to a significant reduction at 3 (†: p<0.01) and 6 weeks (‡: p<0.05) in the treated lesions compared to placebo. Data are means±SD. (B) Representative images of the evolution of psoriatic lesions of patients treated with aganirsen at 1.72 mg/g (upper panel), patients treated with aganirsen at 0.86 mg/g (middle panel) and patients treated with placebo (lower

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panel). Visit 1, visit 2 and visit 3 correspond to baseline, after 3 week and after 6 week of treatment, respectively.

Figure 5: Topical application of aganirsen for 6 weeks decreased both IRS-1 and VEGF expression in psoriatic lesion biopsies. (A) Inhibition of IRS-1 expression (measured by immunofluorescence using an IRS-1 antibody) after daily topical treatment for 6 weeks with aganirsen (0.86 mg/g of ointment) in psoriatic skin lesion biopsies from patients. Data are means±SEM. *, p=0.0008 *vs.* placebo-treated psoriatic lesions. (B) Inhibition of VEGF expression after daily topical treatment for 6 weeks with aganirsen (0.86 mg/g of ointment). Data are means±SEM. *, p=0.0074 *vs.* placebo-treated psoriatic lesions. (C) Representative images of vascular endothelial growth factor (VEGF) labeling in human skin biopsies using anti-VEGF monoclonal antibody in healthy untreated (left panel), psoriatic lesions placebo-treated (middle panel) and aganirsen-treated (right panel). Negative controls (secondary antibody only) are presented in the top row of images, cell nuclei are stained with DAPI in blue (second row of images), immunostaining of VEGF in green (third row of images) and the merge images incorporate both nuclei and VEGF immunostaining-derived fluorescence (fourth row of images).

Figure 6: Topical application of aganirsen inhibits TNFα, $CD4^+$ and $CD3^+$ lymphocyte infiltration in psoriatic lesions. Twice daily topical treatment for 6 weeks with aganirsen at 0.86 mg/g of ointment inhibited TNFα expression (A) in psoriatic skin lesions and reduced the levels of infiltrated $CD4^+$ (B) and $CD3^+$ (C) lymphocytes compared to placebo-treated psoriatic lesions. Data are means±SEM. A: *, p=0.047 *vs.* pathologic placebo-treated and ‡, p=0.007 *vs.* healthy skin; B: *, p=0.002 *vs.* pathologic placebo-treated, †: p<0.0001 *vs.* healthy skin; C: *, p=0.0355 *vs.* pathologic placebo-treated, †: p=0.0089 *vs.* healthy skin.

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Figure 7. Topical application of aganirsen inhibits keratinocyte proliferation in psoriatic lesions. (A) Twice daily topical treatment for 6 weeks with aganirsen at 0.86 mg/g of ointment inhibited keratinocyte proliferation as evidenced by the reduced expression of the keratinocyte proliferation marker Ki-67 in psoriatic lesions. Data are means±SEM. *, p=0.0022 *vs.* pathologic placebo-treated and †, p=0.021 *vs.* healthy skin. (B) Representative images of Ki-67 labelling in human skin samples using anti-Ki-67 monoclonal antibody in healthy non-treated (left panel) and psoriatic lesions either placebo-treated (middle panel) or aganirsen-treated (right panel). Negative controls (secondary antibody only) are presented in the top row of images. Cell nuclei are stained with DAPI in blue (second row of images), immunostaining of Ki-67 in green (third row of images) and the merge images incorporate both nuclei and Ki-67 immunostaining-derived fluorescence (fourth row of images).

Figure 8. Topical application of aganirsen inhibits cytokeratin 16 expression in psoriatic lesions. (A) Twice daily topical treatment for 6 weeks with aganirsen at 0.86 mg/g of ointment inhibited keratinocyte proliferation as evidenced by the reduced expression of CK-16 in psoriatic lesions. Data are means±SEM. *, p=0.0265 *vs.* pathologic placebo-treated. (B) Representative images of CK-16 labelling in human skin samples using anti-CK-16 monoclonal antibody in healthy non-treated (left panel), and psoriatic lesions either placebo-treated (middle panel) or aganirsen-treated (right panel). Negative controls (secondary antibody only) are presented in the top row of images. Cell nuclei are stained with DAPI in blue (second row of images), immunostaining of KC-16 in green (third row of images) and the merge images incorporate both nuclei and KC-16 immunostaining-derived fluorescence (fourth row of images).

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Table 1. Main characteristics of the patients included in the pilot clinical study.

Number of patients	12 (2 women and 10 men)
Average age of patients	48 ± 14 years
Mean body weight of patients (at baseline)	75.8 ± 11.9 kg
Number of non somkers patients	6 (50%)
Number of somkers patients	4 (33.3%)
Number of former smokers patients	2 (16.7%)
Mean heart rate	77.0 ± 5.4 bpm
Mean blood pressure	Systolic (SBP): 133 ± 11 mmHg
	Diastolic (DBP): 71 ± 8 mmHg
Psoriasis diagnosis	>2 years
Psoriasis area severity index (PASI) score	4 to 7; median = 6
Percentage of body surface area (BSA)	3 to 6 %; median = 5% (#1 scale)
Number of refractory lesions	11/12 (91.7%) in placebo group
	10/12 (83.3%) in 0.86 mg/g group
	11/12 (91.7%) in 1.72 mg/g group
	7 (58.3%) in placebo group
Lesions at the upper limb	6 (50%) in 0.86 mg/g group
	8 (66.7%) in 1.72 mg/g group

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Table 2. Study clinical endpoints.

Variables		Placebo	Aganirsen 0.86mg/g	Aganirsen 1.72mg/g
% evolution in lesion area (visit 3)*		24.5 ± 41.7	-14.4 ± 38.6‡	-12.9 ± 52.4‡
% evolution in lesion area (visit 2)		18.5 ± 30.9	-18.8 ± 28.4†	-19.8 ± 31.4†
TSS score**	Baseline	5.5 ± 1.0	5.7 ± 1.5	5.5 ± 1.2
6 Weeks		3.4 ± 1.2	3.6 ± 1.9	3.2 ± 1.3
Physician global assessment (PGA)	Baseline	2.5 ± 0.7	2.5 ± 0.8	2.5 ± 0.7
6 Weeks		3.7 ± 1.1	3.7 ± 1.2	3.9 ± 1.0
Tolerance***				
Prurit	Baseline	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6
6 Weeks		0.2 ± 0.6	0.2 ± 0.6	0.2 ± 0.6
Change in irritation		0 ± 0	0 ± 0	0 ± 0
Change in burning		0 ± 0	0 ± 0	0 ± 0
Change in exsudation		0 ± 0	0 ± 0	0 ± 0
Change in itching after 6 Weeks	-1	1 (8.3%)	1 (8.3%)	1 (8.3%)
0		10 (83.3%)	10 (83.3%)	10 (83.3%)
2		1 (8.3%)	1 (8.3%)	1 (8.3%)

*Percentage evolution (mean±SD) between week 6 (Visit 3) and week 3 (Visit 2) and baseline (Visit 1) in area of treated psoriasis plaque as measured by photography; two types of diameters were measured, both at baseline, 3 weeks and 6 weeks after treatment: sum of diameters (mm) from the CRF and diameters (cm) as measured on pictures. ‡: $p<0.05$ *vs.* placebo; †: $p<0.01$ *vs.* placebo

** Total severity sign score (TSS): sum of signs (redness/erythema, scale/crusting,

thickening/elevation) and symptoms (pruritus) using 4-point scales (e.g. 0=None/clear, Mild =1, Moderate =2, Extensive=3). Score varies from 0 to 12 for each lesion

***Local tolerance of treatment: patient's assessment on a 4-point scale of: irritation, itching, pruritus, sensation of burning and exudation.

For Physician Global Assessment (PGA), three items were noted: erythema, induration and scale, according to Feldman and Krueger 2005.

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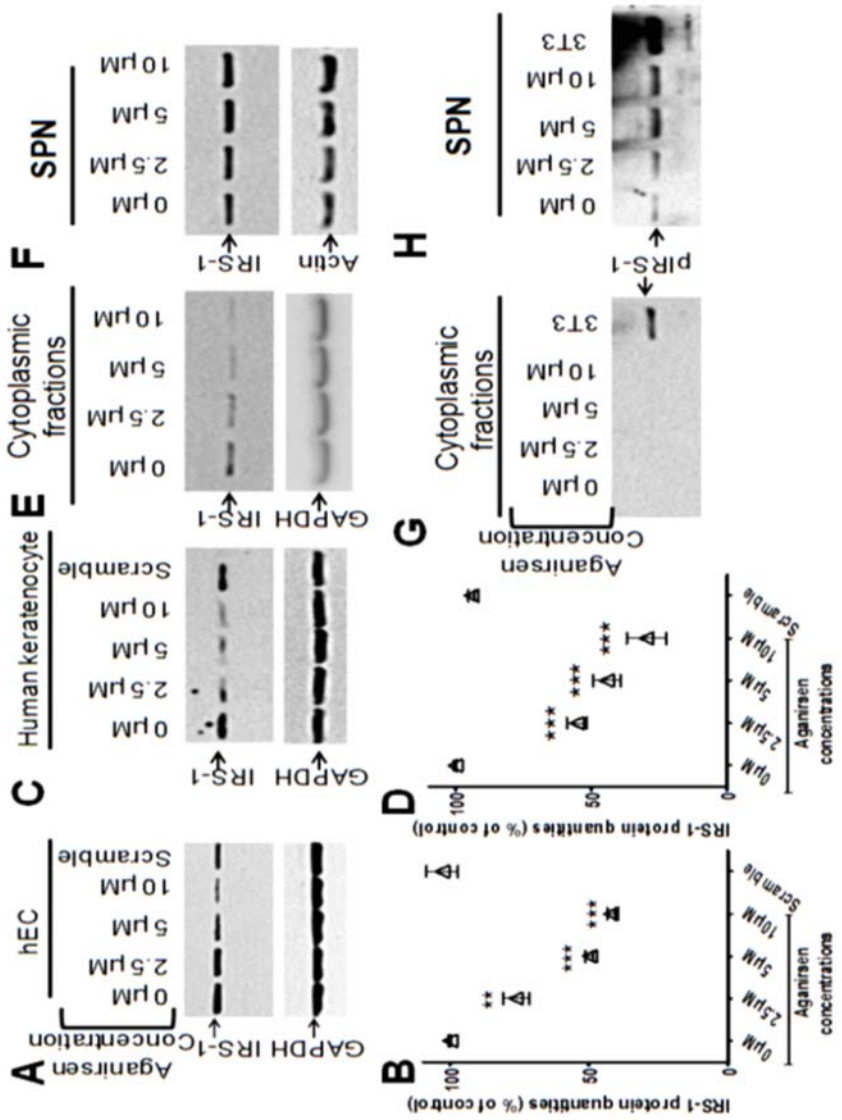
Figure 1



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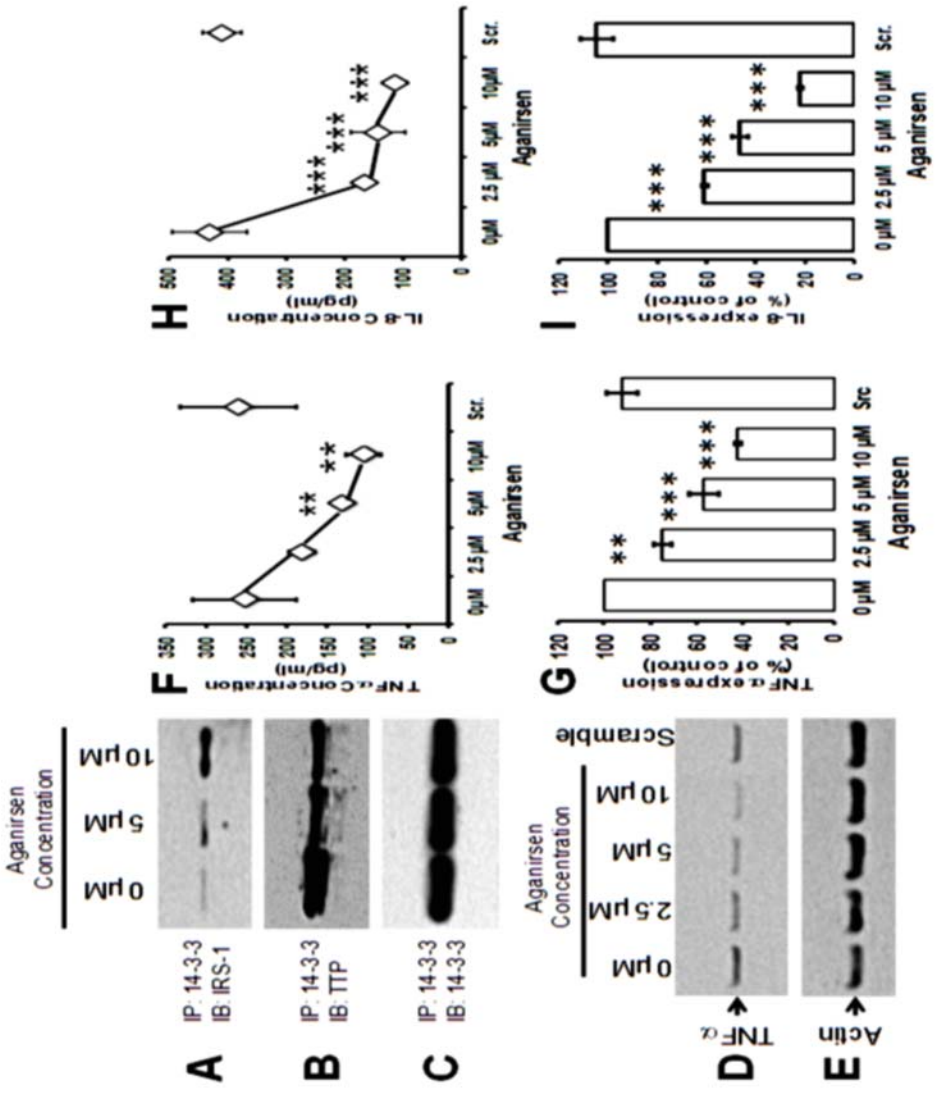

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Figure 3

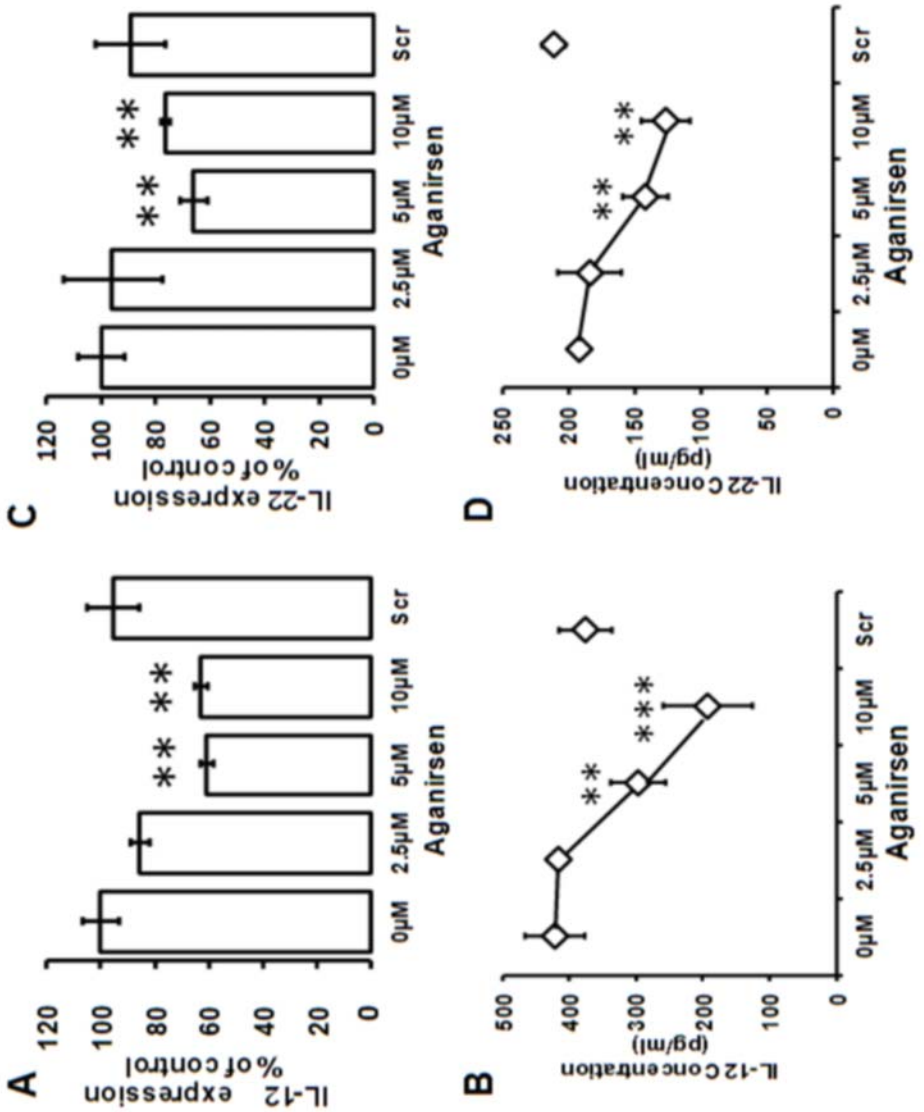

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Figure 4

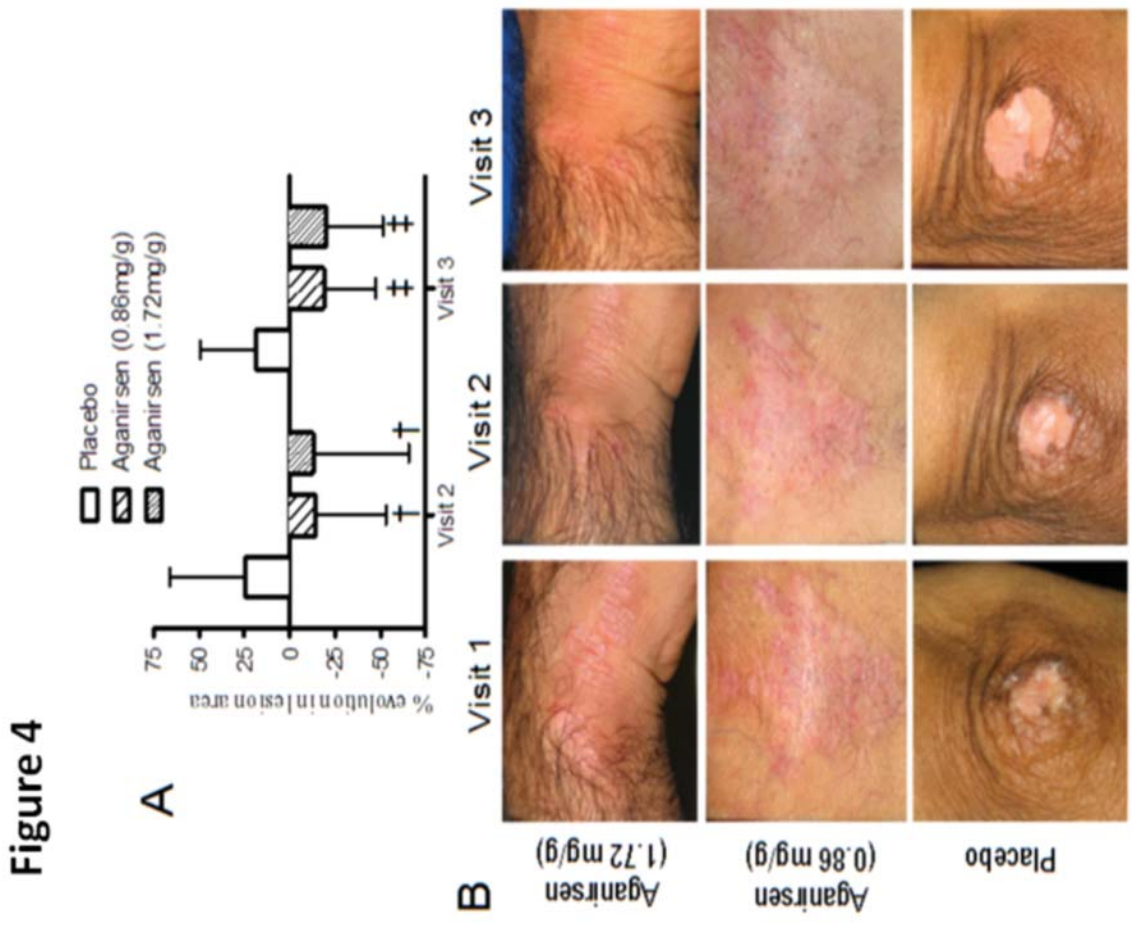

Figure 5

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Figure 6

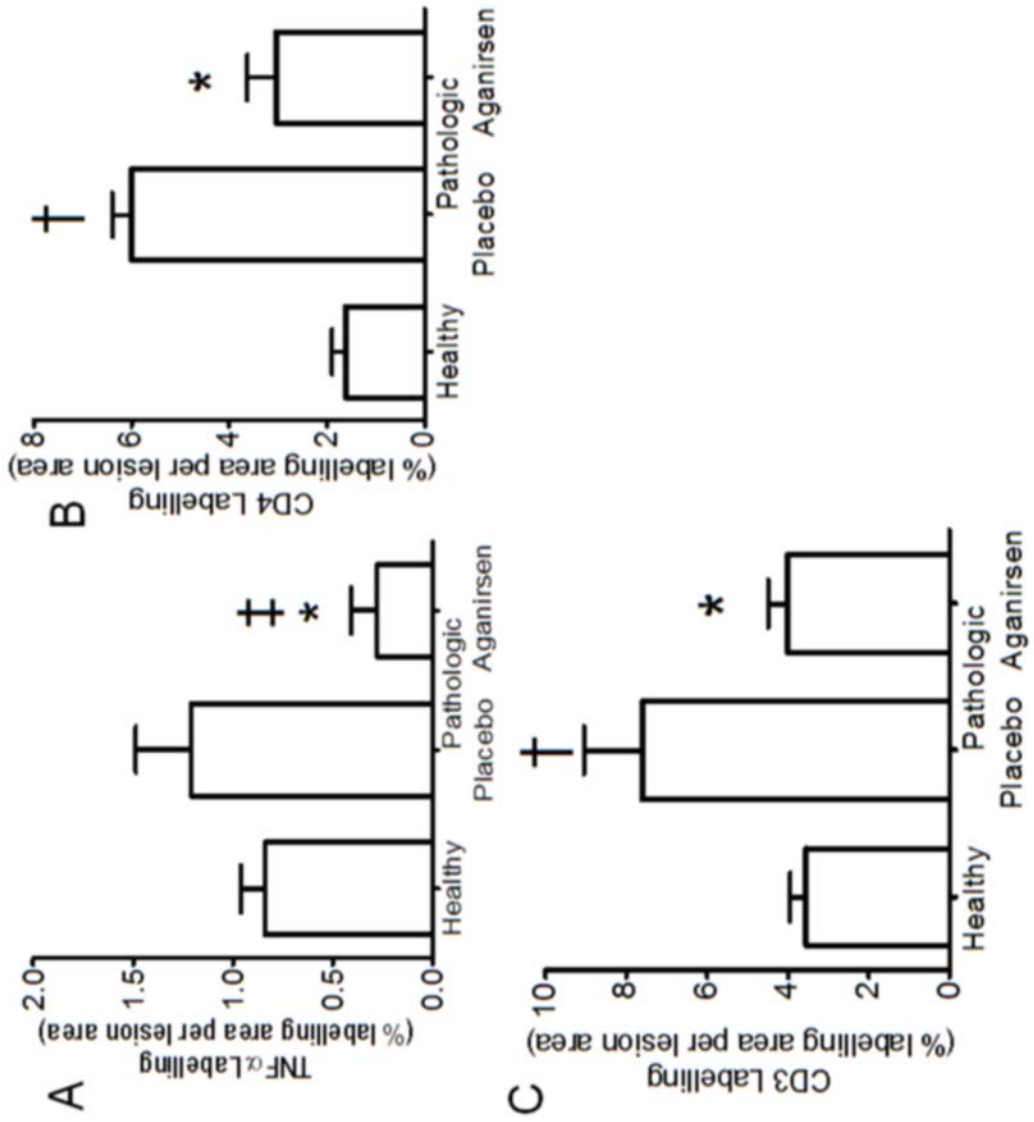

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Figure 7

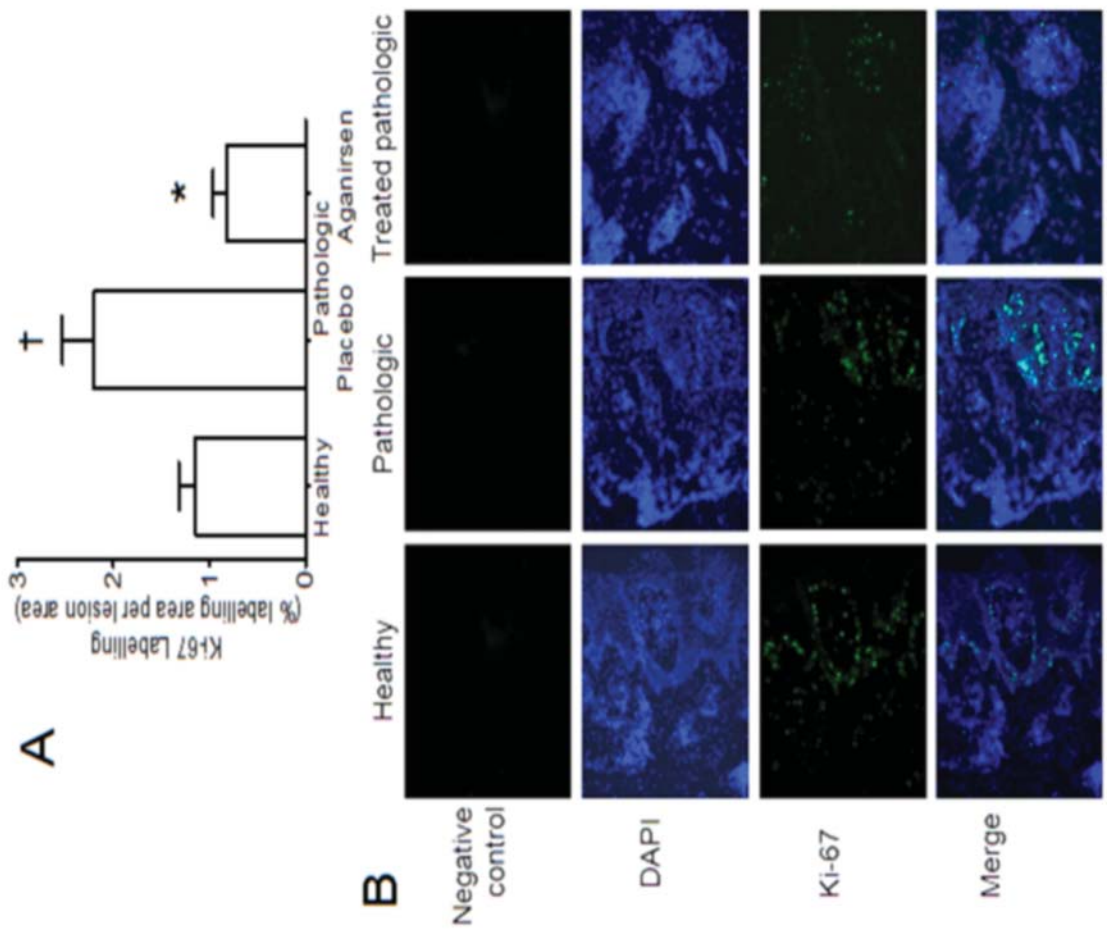

Figure 8