IL-32 induces indoleamine 2,3-dioxygenase+CD1c+ dendritic cells and indoleamine 2,3-dioxygenase+CD163+ macrophages: Relevance to mycosis fungoides progression

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ABSTRACT
Mycosis fungoides (MF) progresses from patch to tumor stage by expansion of malignant T-cells that fail to be controlled by protective immune mechanisms. In this study, we focused on IL-32, a cytokine, highly expressed in MF lesions. Depending on the other cytokines (IL-4, GM-CSF) present during in vitro culture of healthy volunteers’ monocytes, IL-32 increased the maturation of CD11c+ myeloid dendritic cells (mDC) and/or CD163+ macrophages, but IL-32 alone showed a clear ability to promote dendritic cell (DC) differentiation from monocytes. DCs matured by IL-32 had the phenotype of skin-resident DCs (CD1c+), but more importantly, also had high expression of indoleamine 2,3-dioxygenase. The presence of DCs with these markers was demonstrated in MF skin lesions. At a molecular level, indoleamine 2,3-dioxygenase messenger RNA (mRNA) levels in MF lesions were higher than those in healthy volunteers, and there was a high correlation between indoleamine 2,3-dioxygenase and IL-32 expression. In contrast, Foxp3 mRNA levels decreased from patch to tumor stage. Increasing expression of IL-10 across MF lesions was highly correlated with indoleamine 2,3-dioxygenase and IL-32 expression. Thus, IL-32 could contribute to progressive immune dysregulation in MF by directly fostering development of immunosuppressive mDC or macrophages, possibly in association with IL-10.

KEYWORDS
CTCL, cutaneous T-cell lymphoma; DCs, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; hARP, human acidic ribosomal protein; IDO, indoleamine 2, 3-dioxygenase; MFIs, medium fluorescence intensity; MF, mycosis fungoides; mDC, myeloid dendritic cell; mRNA, messenger RNA; PBMCs, peripheral blood mononuclear cells; Treg, regulatory T cell.

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INTRODUCTION
Cutaneous T-cell lymphoma (CTCL) is characterized by clonal expansion of malignant T-cells, typically exhibiting the phenotype of mature CD4+ memory T-cells. Similar to other malignant diseases, the interaction between malignant T-cells and surrounding non-malignant inflammatory cells is involved in the pathogenesis and progression of CTCL. The most common type of CTCL is mycosis fungoides (MF). MF accounts for approximately 55% of all CTCL cases and has different characteristics than Sézary syndrome, another common type of CTCL. MF initially presents as flat erythematous patches covering limited areas of the body (patch stage). In the patch stage, MF typically exhibits an indolent clinical behavior and the disease can remain stable for many years. Some of the patch lesions progress to indurated plaque lesions (plaque stage), while only limited cases develop large tumors (tumor stage). With disease progression, the malignant T-cells can disseminate to lymph nodes, peripheral blood and internal organs, which carries an unfavorable prognosis. Until now, many T-cell-related factors, including adhesion molecules, chemokines, and cytokines, have been linked to the pathogenesis of MF, particularly as MF is a disease of malignant T-cells. A number of recent reports have focused on immune cytokines as factors linked to increasing growth of T-cells with tumor progression. The role of progression-related cytokines in the tumor microenvironment is unknown, but possible functions include regulation of T-cell growth or survival and/or induction of an immunosuppressive environment that could allow malignant T-cells to escape effective anticancer immune responses. MF lesions contain a complex mixture of leukocytes that includes malignant T-cell clones, other T-cell populations, CD163+ macrophages, and a variety of CD11c+ myeloid dendritic cell (mDC) subsets. Ongoing interactions of T-cells with macrophages as well as dendritic cells (DCs) in lesions could lead to chronic T-cell activation or inflammation associated with these lesions.

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Previously, we and others reported that IL-32, a pro-inflammatory cytokine, is highly and progressively expressed in MF skin. We also showed that MF tumor cells are regarded as the most probable candidates for expressing IL-32. IL-32 promotes proliferation and survival of tumor cells in MF, and among many cytokines that we examined, IL-32 messenger RNA (mRNA) expression level is most strongly related to disease progression. These facts indicate that IL-32 plays important roles in the pathogenesis of MF. Here, we show that CD1c DCs increase with disease progression in MF lesions, and that IL-32 can induce CD14\(^{-}\)HLA-DR\(^{\text{high}}\)CD11c\(^{+}\)CD1c\(^{+}\) cell populations as well as CD163\(^{-}\)CD68\(^{+}\) macrophages from peripheral blood mononuclear cells (PBMCs) of healthy volunteers. IL-32 prompts CD14\(^{-}\)HLA-DR\(^{\text{high}}\)CD11c\(^{+}\)CD1c\(^{+}\) cells as well as CD163\(^{-}\)CD68\(^{+}\) cells to express indoleamine 2,3-dioxygenase (IDO), which is an enzyme that catalyzes the rate-limiting step of tryptophan degradation along the kynurenine pathway, leading to immune suppression and to cancer progression in other systems. Furthermore, IDO is highly expressed in a positive correlation with IL-32 in MF skin and increases with tumor progression. Hence, we propose that IL-32 may contribute to the progression of MF not only by acting on tumor cells, but also increasing CD1c\(^{+}\)DCs and CD163\(^{-}\)CD68\(^{+}\) macrophages in lesions as well as creating the potential for immune escape of tumor cells by IDO expression on DCs and macrophages.

Results

**MF skin contains increasing CD11c\(^{+}\) and CD1c\(^{+}\) cells with disease progression**

We have previously found that IL-32, probably produced by tumor cells, is progressively expressed in MF skin and that IL-32\(^{+}\) cells exist in close proximity to CD11c\(^{+}\) cells. We first did immunohistochemistry to visualize CD11c\(^{+}\) mDCs as well as CD1c\(^{+}\) cells in MF skin lesions. We detected increases in CD11c\(^{+}\) (Fig. 1A) and CD1c\(^{+}\) (Fig. 1B) DCs as MF progresses from patch to tumor stage.

**IL-32 induces CD14\(^{-}\)CD163\(^{-}\)CD68\(^{+}\) cells in combination with GM-CSF**

Because mDCs increase with IL-32 in MF lesions, we investigated whether IL-32 could modulate differentiation of CD11c\(^{+}\) or CD1c\(^{+}\) DCs from monocyte precursors. IL-32 has at least nine isoforms, and occurs in four major splice variant isoforms of the mRNA, namely IL-32\(^{a}\), IL-32\(^{b}\), IL-32\(^{g}\), and IL-32\(^{d}\). Among the four major isoforms, IL-32\(^{b}\) seems to be the most abundant, and IL-32\(^{g}\) the most potent. Hence, we used IL-32\(^{b}\) and IL-32\(^{g}\) for functional experiments. We first examined the effect of IL-32\(^{b}\) and IL-32\(^{g}\) on mDC differentiation. Plastic-adherent human monocytes were cultured in the control medium or medium with varying combinations of IL-4, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-32\(^{a}\), IL-32\(^{b}\), IL-32\(^{g}\), and IL-32\(^{d}\). Among the four major isoforms, IL-32\(^{b}\) seems to be the most abundant, and IL-32\(^{g}\) the most potent. Hence, we used IL-32\(^{b}\) and IL-32\(^{g}\) for functional experiments. We first examined the effect of IL-32\(^{b}\) and IL-32\(^{g}\) on mDC differentiation. Plastic-adherent human monocytes were cultured in the control medium or medium with varying combinations of IL-4, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-32\(^{b}\), and IL-32\(^{g}\). After 5 d, the expression of DC and macrophage markers was assessed. Compared to the control medium alone (gray line), the addition of IL-32\(^{b}\) or IL-32\(^{g}\) (black line) increased the proportion of CD14\(^{-}\) cells as well as the cell population with upregulated CD14 expression (Fig. 2A, top row). Among CD14\(^{+}\) cells, GM-CSF-derived cells have increased CD14 expression upon IL-32\(^{b}\) or IL-32\(^{g}\) addition (Fig. 2A, third row). Among CD14\(^{+}\) cells, the medium fluorescence intensity (MFIs) of CD163 and CD68 were higher on IL-32\(^{b}\) or IL-32\(^{g}\) addition (Fig. 2B and C, third row). On the other hand, with the addition of IL-32\(^{b}\) or IL-32\(^{g}\), CD14\(^{+}\) cells have diminished CD68 expression in other conditions (Fig. 2C). We previously showed...
that IL-4 mRNA expression levels in MF skin were not significantly higher than those in healthy volunteers. Additionally, we confirm that MF skin shows significantly higher GM-CSF mRNA expression compared to healthy volunteers (Fig. S1). These results suggest that in combination with GM-CSF, IL-32 \( \beta \) or IL-32 \( \gamma \) can induce CD14\(^{-}\)CD163\(^{-}\)CD68\(^{-}\) macrophage-like cells.

**IL-32 induces CD14\(^{-}\)HLA-DR\(^{high}\)CD11c\(^{+}\)CD1c\(^{-}\) cells from monocytes**

Regarding CD14\(^{-}\) populations, the addition of IL-32\( \beta \) or IL-32\( \gamma \) alone brought a new population (HLA-DR\(^{high}\)CD11c\(^{high}\)) as well as HLA-DR\(^{high}\)CD11c\(^{int}\) cells, whereas we only detected HLA-DR\(^{high}\)CD11c\(^{int}\) cells in the medium with no other cytokines (Fig. 3A, top row). IL-32-derived HLA-DR\(^{high}\)CD11c\(^{+}\) cells (blue line) showed higher CD86 and CD83 expression compared to HLA-DR\(^{high}\)CD11c\(^{+}\) cells harvested from the medium without a cytokine (red line) (Fig. 3B, top row). The addition of IL-32\( \beta \) or IL-32\( \gamma \) not only increased the population of HLA-DR\(^{high}\)CD11c\(^{+}\) cells, but also strongly induced expression of mature DC markers including HLA-DR, CD86, and CD83 in the medium with GM-CSF (Figs. 3A and B, third row). The treatment with IL-4 alone induced HLA-DR\(^{high}\)CD11c\(^{+}\) cells with comparatively high CD86 expression, whereas the addition of IL-32\( \beta \) or IL-32\( \gamma \) to that medium containing IL-4, slightly antagonized DC induction or CD86 expression (Figs. 3A and B, second row). In the medium with the combination of IL-4+GM-CSF, the addition of IL-32 decreased overall frequencies of CD14\(^{-}\)HLA-DR\(^{high}\)CD11c\(^{+}\) cells, but the differentiation toward maturity of mDCs was accelerated with high expression of CD86 and CD83 (Figs. 3A and B, fourth row). These results indicate that IL-32\( \beta \) or IL-32\( \gamma \) alone can induce CD14\(^{-}\)HLA-DR\(^{high}\)CD11c\(^{+}\) DC-like cells and that DC induction from monocytes is accelerated by the addition of IL-32 to the medium without cytokines or the medium with GM-CSF.

Since we found an increase in CD1c\(^{-}\) cells with disease progression in MF skin, we examined CD1c\(^{-}\) expression among IL-32-induced CD14\(^{-}\)HLA-DR\(^{high}\)CD11c\(^{+}\) cells. Although the expression levels of CD86 and CD83 vary based on the conditions, we detected consistent rising MFIs of CD1c in IL-32-induced CD14\(^{-}\)HLA-DR\(^{high}\)CD11c\(^{+}\) cells compared to the control condition without IL-32 (Fig. 4A). Additionally, IL-32\( \beta \) or IL-32\( \gamma \) induced more CD14\(^{-}\)HLA-DR\(^{high}\)CD11c\(^{+}\)CD1c\(^{-}\) cells compared to the control condition without IL-32 (Fig. 4B). Therefore, we concluded that both IL-32\( \beta \) and IL-32\( \gamma \) can induce more CD14\(^{-}\)HLA-DR\(^{high}\)CD11c\(^{+}\)CD1c\(^{-}\) cells from monocytes.

**MF skin contains IDO\(^{-}\)CD1c\(^{-}\) as well as IDO\(^{-}\)CD163\(^{-}\) cells, both of them are induced by IL-32**

Previously, many DC-related immunoregulatory factors such as IDO have been suggested to be involved in tumor progression.\(^{11,17}\) Hence, we investigated mRNA expression levels of IDO and analyzed the correlation between its mRNA expression level and that of IL-32. IDO mRNA expression levels in MF lesions are higher than those in healthy volunteers (Fig. 5A). Furthermore, we found a significant correlation between IDO.
Figure 3. Changes of CD86 and CD83 expression on CD14<sup>+</sup> HLA-DR<sup>high</sup>CD11c<sup>+</sup> cells by addition of IL-32. Cytokines added to the control medium are written to the left of the panels. (A) Viable CD14<sup>+</sup> cells harvested from the control medium with nothing or from the control media with the addition of IL-32 were selected to detect HLA-DR<sup>+</sup>CD11c<sup>+</sup> cells. The left panels show the cells obtained from the control medium. The middle and right panels represent the cells obtained from the control medium with IL-32 or IL-32γ, respectively. The numbers written within the panels represent the percentages of the circled population (HLA-DR<sup>+</sup>CD11c<sup>+</sup> cells). (B) CD86 and CD83 expression on viable CD14<sup>+</sup> HLA-DR<sup>high</sup>CD11c<sup>+</sup> cells (coming from the circled gate in the corresponding condition of (A)). Red and blue lines represent the cells harvested from the control medium without IL-32 and the control medium with IL-32 or IL-32γ, respectively. One FMO is shown to represent the specificity of CD83 antibody as a dot line.

Figure 4. Increase in CD11c<sup>+</sup> cells from monocytes cultured with the addition of IL-32. Cytokines added to the control medium are shown to the left of the panels. (A) Expression of CD11c on viable CD14<sup>+</sup> HLA-DR<sup>+</sup>CD11c<sup>+</sup> cells. Red and blue lines represent the cells harvested from the control medium without IL-32 and with IL-32β or IL-32γ, respectively. (B) Viable CD14<sup>+</sup> HLA-DR<sup>high</sup> cells were selected to show CD11c (x-axis) and CD11c (y-axis) expression. The left panels show the cells obtained from the control medium. The middle and the right panels represent the cells obtained from the control medium with IL-32β or IL-32γ, respectively. The numbers within the panels show the percentages of the circled population (CD11c<sup>+</sup>CD11c<sup>+</sup> cells).
IDO mRNA expression was significantly correlated with IL-10 mRNA but not with Foxp3 mRNA

Regulatory T cells (Treg) are emerging as a key component of acquired tolerance to tumors, resulting in tumor progression. Increased Treg activity facilitates tumor growth, whereas depletion of Tregs allows for effective antitumor immune responses that would otherwise be undetectable or ineffectual to occur. Since the IDO pathway contributes to regulation of Foxp3+ Treg lineage commitment and function, we next investigated Foxp3 mRNA expression in MF skin (Fig. 7A). Though MF skin showed higher Foxp3 mRNA expression compared to healthy volunteers, its expression levels decreased progressively from patch to tumor stage. Furthermore, we could not find a significant correlation between IDO and Foxp3 mRNA expression. In addition to Treg-related immunosuppressive functions, it has been reported that IDO can induce IL-10, an established immunosuppressive cytokine. Therefore, we also explored IL-10 expression in MF skin. Consistent with a previous report, MF skin exhibited high and progressive IL-10 mRNA expression (Fig. 7B). Additionally, IL-10 mRNA expression was significantly correlated with IDO (Fig. 7B) as well as IL-32 (Fig. 7C). On the other hand, IL-10 mRNA expression was not significantly correlated with Foxp3 (Fig. 7C) even though IL-10 is known to be produced by Tregs. Overall, these data establish distinct pathways for negative immune regulation during MF progression. The highest elevation of Foxp3 is seen in patch stage, but increasing expression of IL-10 occurs from patch to tumor stage. IL-32 is a potential regulator of increasing IDO and IL-10 throughout MF progression.

Discussion

We have previously showed that IL-32 is progressively increased in MF skins. Here, we revealed that IL-32 expression is positively correlated with increasing IDO as well as IL-10 in MF lesions. We found that IL-32 could induce more IDO+CD14+HLA-DRhighCD11c+CD1c+ cells as well as IDO+CD163+CD68+ cells from monocytes, depending on the cytokine environment. IL-32 is associated with numerous pathologies, including infectious diseases, chronic inflammation, and malignant diseases. Regarding the involvement of IL-32 in malignant diseases, IL-32 prompts pancreas cancer cell proliferation, whereas overexpression of IL-32α suppresses cell growth of hepatocellular carcinoma. Whether IL-32 accelerates cell proliferation or not appears to depend on the type of IL-32 isoform, its expression levels, and affected cell types. For example, HEK293T cells expressing high levels of IL-32β or IL-32γ died, but overexpression of IL-32α in human mammalian cell lines did not result in cell death. The immunologic functions of IL-32 have been mainly investigated in...
inflammatory diseases, including rheumatoid arthritis, chronic obstructive pulmonary disease, graft-vs.-host disease, and chronic rhinosinusitis. In rheumatoid arthritis, synovial IL-32 expression correlates with inflammation as well as tumor necrosis factor-α levels, and injection of IL-32 into knees induces joint swelling. Since tumor necrosis factor-α is one of the drivers of synovial inflammation and since IL-32 overexpression increases its levels, it was suggested that IL-32 might be crucial for the autoinflammatory loop found in joints of rheumatoid arthritis patients. However, based on our results, IL-32 might also induce production of macrophages that can mediate joint inflammation and destruction.

Past studies have reported somewhat conflicting data on the ability of IL-32 to modulate DC and macrophage differentiation. Netea et al. showed that IL-32γ antagonized GM-CSF-induced CD1a expression, but increased CD14+ cells. Additionally, the GM-CSF+IL-4-induced CD1a expression was reversed by the addition of IL-32γ to the cell culture. They concluded that IL-32 induced the differentiation of monocytes into macrophage-like cells. On the other hand, Schenk et al. demonstrated that the addition of IL-32 to primary monocytes induced CD1b expression and that the MFI of CD86 were higher on IL-32-derived vs. GM-CSF-derived CD1b+ DCs. They concluded that IL-32 alone can induce functional DCs from monocytes. In our study, IL-32β or IL-32γ in combination with GM-CSF increased CD14 expression levels of the CD14+ population, and IL-32β or IL-32γ with IL-4 decreased CD14+HLA-DRhighCD11c+ populations. Our findings are consistent with Netea et al.’s report. However, IL-32β or IL-32γ alone induced CD14+HLA-DRhighCD11c+ cells with CD86 and CD83 expression, which are regarded as mDCs. Furthermore, the addition of IL-32β or IL-32γ to the medium with GM-CSF induced more CD14+HLA-DRhighCD11c+ cells with high CD86 and CD83. These results are congruent with Schenk et al.’s report. Another finding of note is that the expression levels of CD86 and CD83 on monocytes-derived mDCs, which were raised by the addition of IL-32, were still lower than those of highly matured mDCs (data not shown). We obtained these highly matured mDCs by the established method. Since expression levels of CD86 and CD83 could be referred to as “intermediate,” we think that these IL-32-derived CD14+HLA-DRhighCD11c+ cells are not completely matured, but only show “deregulation toward mature mDCs.” Taken together, differentiation of IL-32-exposed monocytes to DCs or macrophages would depend on other cytokines present in the microenvironment. Thus, upregulation of IL-32 could explain the increase in both DCs and macrophages that typify MF lesions.

Figure 6. Increase in IDO expression on mDCs and macrophages by addition of IL-32. IDO expression on (A) Viable CD14−HLA-DRhighCD11c+ cells and (B) Viable CD14−CD163−CD68+ cells. Red and blue lines represent the cells harvested from the control medium without IL-32 and with IL-32β or IL-32γ, respectively. Cytokines added to the control medium are written to the left of the panels.
Currently, three main subsets of DCs are found in human skin; Langerhans cells, mDCs, and plasmacytoid DCs. Among them, mDCs are most abundant and most common, and mDCs are thought to be best characterized by the presence of the transmembrane integrin molecule CD11c, found at high levels on almost all human mDCs. Thus, another important finding in this study is that the addition of IL-32 results in consistent differentiation of CD14\(^{-}\) HLA-DR\(^{\text{high}}\)CD11c\(^{+}\)CD1c\(^{-}\) DCs in vitro. This CD1c\(^{-}\)mDC subset is best referred to as “resident mDCs” and is regarded as relatively immature mDCs that are capable of inducing only a mild T-cell response. It has been suggested that in the resting state, resident mDCs may be tolerogenic, indicating that the increase in IL-32-derived CD14\(^{-}\) HLA-DR\(^{\text{high}}\)CD11c\(^{+}\)CD1c\(^{-}\) cells itself would lead to tumor-tolerogenic environment.

Another cell type possibly induced by IL-32 is CD163\(^{+}\)CD68\(^{+}\) macrophages. Macrophages are divided into the classically activated (M1) macrophages and the alternatively activated (M2) macrophages by distinct states of polarized activation. M1 macrophages produce type I proinflammatory cytokines, participate in killing intracellular pathogens, and have an antitumorigenic role. Conversely, M2 macrophages, most of them are distinguished from M1 macrophages by the expression of CD163, produce type II cytokines as well as IL-10, and lead to immunosuppression. It has been known that during tumor progression, the macrophage phenotype changes from M1 to M2 and that tumor-associated macrophages, which prompt tumor progression, exhibit an M2-like phenotype. An increase in CD163\(^{+}\) cells in MF skin with disease progression has been already reported, and our findings indicate that abundant IL-32 in combination with GM-CSF may increase CD163\(^{+}\)M2 macrophages in MF lesions, thus contributing to tumor progression.

As described above, the induction of specific cell types, such as CD11c\(^{-}\)-resident mDCs and CD168\(^{+}\) macrophages, could accelerate disease progression. Another possible function of IL-32 in MF progression may be related to IDO induction. The molecular mechanisms underlying tumor-induced tolerance are currently the subject of active research. IDO catalyzes the rate-limiting step of tryptophan degradation along the
As for the involvement of Tregs in MF, their presence in MF Tregs are derived from MF tumor cells or in the kynurenine pathway,10 and both the reduction in local tryptophan metabolites such as kynurenine contribute to the immunosuppressive effects of IDO. It is known that IDO is chronically activated in many patients with cancer47 and that IDO activation correlates with more extensive disease.48 Kynurenine pathway metabolites are natural immunologically active ligands for aryl hydrocarbon receptor, and they suppress anti-tumor immune responses.49,50 IDO can be expressed by multiple cell types, including DCs, macrophages, epithelial cells, eosinophils, endothelial cells, and tumor cells.11 Although the regulation of IDO expression is complex and remains a subject of active investigation, certain types of antigen-presenting cells such as DCs and macrophages seem to be preferentially expressed to functional IDO when challenged with proinflammatory stimuli or exposed to signals from activated T-cells. In cancer patients, IDO-expressing DCs are thought to help suppress the initiation of immune responses to tumor-derived antigens and perhaps help create systemic tolerance to these tumor antigens. In this study, we clearly show that IDO is highly expressed on CD11c+ mDCs and CD1c+ resident mDCs, and, to a lesser extent, on CD163+ M2 macrophages in MF skin. Furthermore, under the condition with IL-32, monocyte-derived CD14+ HLA-DRhighCD11c+ mDCs as well as CD163+CD68+ macrophages are prone to express IDO. Although we did not study the detailed functions of IDO-expressing cells in MF skin, we confirmed that mRNA expression of kynureninase, which is a downstream enzyme of the kynurenine pathway, is upregulated in MF skin and that its expression level correlates to that of IDO (Fig. S2). This suggests that IDO expressed in MF skin would be functional. To our knowledge, this is the first report showing high IDO expression in MF. We also suggest that IL-32 may be one of the candidates that induce IDO expression on mDCs and macrophages in MF skin.

Regarding the immunosuppressive functions of IDO, tryptophan deprivation acts synergistically with kynurenine metabolites to drive de novo differentiation of Foxp3+ Tregs from uncommitted CD4+ T-cells.51-53 In the case of pre-existing, mature Tregs, co-culture with DCs expressing IDO during Treg activation enhances their suppressive functions54 and blocks inflammation-induced destabilization of the Treg phenotype.55-57 As for the involvement of Tregs in MF, their presence in MF skin was confirmed by many authors, but it is not clear whether Tregs are derived from MF tumor cells or infiltrating non-malignant T-cells.58 In our study, Foxp3 mRNA expression in early stage MF (patch and plaque stages) was higher than that in healthy volunteers, but its expression levels decreased gradually from patch to tumor stage. Hence, Tregs might function to restrict T-cell proliferation in early stages of the disease.

IL-10 is an immunosuppressive cytokine and its high expression in MF has been shown by RT-PCR and in situ hybridization.24,25 We showed that IL-10 mRNA expression increases with disease stage and that a significant correlation exists between IL-10 and IDO as well as IL-32 mRNA. This increase in IL-10 expression could potentially disturb antitumor immune responses with MF progression.

In conclusion, we show that IL-32 accelerates the induction of CD1c+ mDCs and CD163+CD68+ macrophages from monocytes. mDCs as well as CD163+CD68+ macrophages express IDO highly with the addition of IL-32. IDO expression increases with disease progression and correlates not with Foxp3 but with IL-32 as well as IL-10 mRNA expression in MF skin. IL-32 upregulation in MF skin might contribute to a tumor tolerogenic environment by induction of specific cell types as well as IDO expression, promoting MF progression.

**Materials and methods**

**Skin and blood samples**

MF skin samples (patch stage; n = 8, plaque stage; n = 8, tumor stage; n = 5) were obtained from the Skin Cancer Center at the Charité – Universitätsmedizin Berlin under its approved protocols. Skin samples from healthy volunteers (n = 8), were collected at The Rockefeller University under its IRB-approved protocols. Informed consent was obtained and the studies were performed in adherence with the principles of the Declaration of Helsinki. Buffy coats used for isolating PBMCs were purchased from the New York Blood Center.

**Immunohistochemistry**

Frozen tissue sections were stained with mouse anti-human CD11c (B-ly6, BD PharMingen) 1:100, CD1c (AD5-8E7, Miltenyi Biotec) 1:10, or IDO (10.1, LSBio) 1:100. Biotin-labeled horse anti-mouse antibody (Vector Laboratories) was used to detect mouse monoclonal antibody. The staining signal was amplified with avidin-biotin complex (Vector Laboratories) and developed with chromogen 3-amino-9-ethylcarbazole (Sigma–Aldrich).

**Immunofluorescence**

Frozen skin sections were fixed with acetone and blocked with 10% normal goat serum (Vector Laboratories) for 30 min. Primary antibodies were incubated overnight at 4°C and bound with the appropriate Alexa Fluor® 488- or 568-conjugated secondary antibody for 30 min at room temperature. Antibodies used were: CD11c (B-ly6, BD PharMingen) 1:100, CD1c (AD5-8E7, Miltenyi Biotec) 1:10, IDO (10.1, LSBio) 1:100, CD163 (5C6-FAT, Acris Antibodies) 1:100.

**Quantitative reverse transcription (RT)-PCR assay**

mRNA was extracted from skin samples using the RNeasy Mini Kit (Qiagen). cDNA was synthesized by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Preamplification RT-PCR was performed using TaqMan® PreAmp Master Mix Kit, primers and probes (Applied Biosystems). The primers and probes used for IL-32 (Hs00992441_m1), IDO (Hs00984148_m1), Foxp3 (Hs01085834_m1), and IL-10 (Hs00961622_m1) were from Applied Biosystems. The sequences of the primers and probe used for human acidic ribosomal protein (hARP) are: hARP-forward: CGCTGCTGAA-CATGCTCAA, hARP-reverse: GTGCGAACACCTGCTG-GATG; hARP-probe: 6-FAM TCCCCCTTCTCCTTTGGGC TGG-TAMRA (GenBank accession no. NM-001002). The
Results were normalized to hARP housekeeping gene and analyzed with Applied Biosystems PRISM 7700.

**Generation of DCs and macrophages**

PBMCs were obtained by density-gradient centrifugation over Ficoll-Paque (GE Healthcare) and resuspended at 4 x 10^6 cells per ml in the complete media. In our study, the complete media was made by RPMI1640 (Gibco) with 5% human AB serum (cellgro), 1% HEPES (Sigma-Aldrich), and 0.5% Gentamicin (Gibco). The cells were plated in culture dishes and allowed to adhere to plastic for 3 h at 37°C, 5% CO₂. Plates were washed with the complete media to remove non-adherent cells. Plastic-adherent cells were cultured in the complete media supplemented with nothing, 25 ng/mL IL-4 (R&D Systems), 100 IU/mL GM-CSF (R&D Systems), 50 ng/mL IL-32β (R&D Systems), 50 ng/mL IL-32γ (R&D Systems), 25 ng/mL IL-4 + 100 IU/mL GM-CSF, 25 ng/mL IL-4 + 50 ng/mL IL-32β, 25 ng/mL IL-4 + 50 ng/mL IL-32γ, 100 IU/mL GM-CSF + 50 ng/mL IL-32β, 100 IU/mL GM-CSF + 50 ng/mL IL-32γ, 25 ng/mL IL-4 + 100 IU/mL GM-CSF + 50ng/mL IL-32β, or 25 ng/mL IL-4 + 100 IU/mL GM-CSF + 50 ng/mL IL-32γ for 5 d. Cultures were supplemented with the same amounts of IL-4 and GM-CSF on days 2 and 4. 25 ng/mL IL-32β or 25 ng/mL IL-32γ was added only on day 2. On day 5, cells were harvested for flow cytometry analysis.

**Flow cytometry**

LIVE/DEAD Fixable Blue dye (Invitrogen) 1:500, QDot® 800-CD14 (TuK4, Invitrogen) 1:500, PerCP/Cy5.5-CD163 (GHI/61, BioLegend) 1:50, Alexa Fluor® 488-CD68 (Y1/82A, BioLegend) 1:20, Alexa Fluor® 700-CD11c (B-ly6, BD Pharmingen™) 1:100, APC-H7-HLA-DR (L243, BD Biosciences) 1:200, BV650-CD86 (2331 FUN-1, BD Horizon™) 1:100, APC-CD83 (HB15e, BD Pharmingen™) 1:50, Brilliant Violet 421™-CD1c (L161 BioLegend) 1:40, and PE-IDO (700838, R&D Systems) 1:20 were used for cell surface and intracellular staining. Fluorescence minus one and isotype-matched control antibodies were used to set up baselines to exclude background from the analysis. After LIVE/DEAD and surface staining, the cells were fixed and permeabilized for IDO and CD68 intracellular staining. Cell acquisition was performed by the LSR II flow cytometer supported by the FACS Diva v6.1.1 software (BD Biosciences). Data were analyzed by using FlowJo v.X (Treestar, Inc.).

**Statistics**

All values obtained from RT-PCR were transformed to log₁₀ before analysis. Analysis between two groups was performed using the Student’s t-test. To analyze more than two groups, one-way ANOVA followed by Tukey’s multiple comparison test was done. Correlation coefficients were determined by using the Spearman’s rank correlation test. p values of < 0.05 were considered statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

H.O., J.G., and J.G.K. designed the experiments; H.O. conducted experiments, analyzed data, prepared figures, and drafted the manuscript; D.H., W.S., and J.G.K. provided skin samples; N.G., and M.M. edited the manuscript.

**References**

1. Wong HK, Mishra A, Hake T, Porcu P. Evaluating insights in the pathogenesis and therapy of cutaneous T-cell lymphoma (mycosis fungoides and Sézary syndrome). Br J Haematol 2011; 155:150-66; PMID:21883142; http://dx.doi.org/10.1111/j.1365-2141.2011.08852.x

2. Korgavkar K, Xiong M, Weinstock M. Changing incidence trends of cutaneous T-cell lymphoma. JAMA Dermatol 2013; 149:1295-9; PMID:24005876; http://dx.doi.org/10.1001/jamadermatol.2013.5526

3. Imam MH, Shenoy PJ, Flowers CR, Phillips A, Lechowicz MJ. Incidence and survival patterns of cutaneous T-cell lymphomas in the United States. Leukemia Lymphoma 2013; 54:752-9; PMID:23004532; http://dx.doi.org/10.3109/10428194.2012.729831

4. Benton EC, Crichton S, Talpur R, Agar NS, Fields PA, Wedgeorth E, Mitchell TJ, Cox M, Ferreira S, Liu P et al. A cutaneous lymphoma international prognostic index (CLIPi) for mycosis fungoides and Sézary syndrome. Eur J Cancer 2013; 49:2859-68; PMID:23735705; http://dx.doi.org/10.1016/j.ejca.2013.04.018

5. Sugaya M, Miyagaki T, Ohmatsu H, Suga H, Kai H, Kamata M, Fujita H, Asano Y, Tada Y, Kadono T et al. Association of the numbers of CD163(+) cells in lesional skin and serum levels of soluble CD163 with disease progression of cutaneous T-cell lymphoma. J Dermatological Sci 2012; 68:45-51; PMID:22884782; http://dx.doi.org/10.1016/j.jdermsci.2012.07.007

6. Schlapbach C, Ochsenbein A, Kaelin U, Hassan AS, Hunger RE, Yawalkar N. High numbers of DC-SIGN+ dendritic cells in lesional skin of cutaneous T-cell lymphoma. J Am Acad Dermatol 2010; 62:995-1004; PMID:20466174; http://dx.doi.org/10.1016/j.jaad.2009.06.082

7. Ohmatsu H, Hummde D, Galati N, Gonzalez J, Mobs M, Suarez-Farinas M, Cardinale I, Mitsui H, Guttmann-Yassky E, Sterry W et al. IL32 is progressively expressed in mycosis fungoides independent of helper T-cell 2 and helper T-cell 9 polarization. Cancer Immunol Res 2014; 2:890-900; PMID:24938282; http://dx.doi.org/10.1158/2326-6066.CIR-13-0199-T

8. van Kester MS, Berg MK, Zoutman WH, Out-Luiting JJ, Jansen PM, Dreef EJ, Vermeer MH, van Doorn R, Willemsz R, Tensen CP. A meta-analysis of gene expression data identifies a molecular signature characteristic for tumor-stage mycosis fungoides. J Invest Dermatol 2012; 132:2050-9; PMID:22513784; http://dx.doi.org/10.1038/jid.2012.117

9. Sugahara K, Sugaya M, Miyagaki T, Kawaguchi M, Fujita H, Asano Y, Tada Y, Kadono T, Sato S. The role of IL-32 in cutaneous T-cell lymphoma. J Invest Dermatol 2014; 134:1428-35; PMID:24226419; http://dx.doi.org/10.1038/jid.2013.488

10. Stone TW, Darlington LG. Endogenous kynurenines as targets for drug discovery and development. Nat Rev Drug Discov 2002; 1:609-20; PMID:12402501; http://dx.doi.org/10.1038/nrd870

11. Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest 2007; 117:1147-54; PMID:17476344; http://dx.doi.org/10.1172/JCI31178
11. Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA. Interleukin-32: a cytokine and inducer of TNFalpha. Immunity 2005; 22:131-42; PMID:15664165; http://dx.doi.org/10.1016/j.immuni.2004.12.003

12. Kang JW, Park YS, Lee DH, Kim MS, Bak Y, Ham SY, Park SH, Kim H, Ahn HJ, Hong JT et al. Interaction network mapping among IL-32 isoforms. Biochimie 2010; 141:248-51; PMID:20472437; http://dx.doi.org/10.1016/j.biochi.2010.01.013

13. Goda C, Kanaji T, Kanaji S, Tanaka G, Arima K, Ohno S, Izuhara K. Involvement of IL-32 in activation-induced cell death in T cells. Int Immunol 2006; 18:233-40; PMID:16410314; http://dx.doi.org/10.1093/intimm/dxh339

14. Heinhuiss B, Koenders MI, van de Loo FA, Netea MG, van den Berg WB, Joosten LA. Inflammation-dependent secretion and splicing of IL-32 [gamma] in rheumatoid arthritis. Proc Natl Acad Sci USA 2011; 108:4962-7; PMID:21383200; http://dx.doi.org/10.1073/pnas.1016005108

15. Heinhuiss B, Netea MG, van den Berg WB, Dinarello CA, Joosten LA. Interleukin-32: a predominantly intracellular proinflammatory mediator that controls cell activation and cell death. Cytokine 2012; 60:321-7; PMID:22878344; http://dx.doi.org/10.1016/j.cytjo.2012.07.010

16. Okazaki T, Chikuma S, Iwai T, Fagarasan S, Honjo T. A rheostat for negative regulation and induction of apoptosis through inactivation of NF-kappaB and Bcl-2. Cancer Letters 2012; 318:226-33; PMID:22198841; http://dx.doi.org/10.1016/j.canlet.2011.12.023

17. Calabrese F, Baraldo S, Bazzan E, Lunardi F, Rea F, Maestrelli P, Turato G, Lokar-Oliani K, Papi A, Zun R et al. IL-32, a novel proinflammatory cytokine in chronic obstructive pulmonary disease. Am J Resp Critical Care Med 2008; 178:894-901; PMID:18703789; http://dx.doi.org/10.1164/rccm.200804-664OC

18. Schenk M, Krutzik SR, Sieling PA, Lee DJ, Ochoa MT, Kabacka J, Sale GE, Hansen JA, Dinarello CA, Deeg HJ. Inhibition of IL-32 activation by alpha-1 antitrypsin suppresses alloreactivity and increases survival in an allogeneic murine marrow transplantation model. Blood 2011; 118:5031-9; PMID:21900190; http://dx.doi.org/10.1182/blood-2011-07-365247

19. Lee AW, Truong T, Bickham K, Fonteneau JF, Larsson M, Da Silva I, Cordoba J, Kruger RJ et al. NOD2 triggers an interleukin-32-dependent human dendritic cell program in lepromatous leprosy. Nat Med 2012; 18:555-63; PMID:22447076; http://dx.doi.org/10.1038/nm.2650

20. Lee AW, Truong T, Bickham K, Fonteneau JF, Larsson M, Da Silva I, Somersens S, Thomas EK, Bhadravaj N. A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. Vaccine 2002; 20 Suppl 4:A8-A22; PMID:12477423; http://dx.doi.org/10.1016/S0264-410X(02)00382-1

21. Zaba LC, Kruger JG, Lowes MA. Resident and “inflammatory” dendritic cells in human skin. J Invest Dermatol 2009; 129:302-8; PMID:18685620; http://dx.doi.org/10.1038/jid.2008.225

22. Zaba LC, Fuentes-Duculan J, Steinman RM, Kruger JG, Lowes MA. Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163+FXIIIa+ macrophages. J Clin Invest 2007; 117:2517-25; PMID:17786242; http://dx.doi.org/10.1172/JCI32282

23. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. J Leukoc Biol 2011; 89:557-63; PMID:21248152; http://dx.doi.org/10.1189/jlb.0710409

24. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004; 25:677-86; PMID:15530839; http://dx.doi.org/10.1016/j.it.2004.09.015

25. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010; 11:889-96; PMID:20856220; http://dx.doi.org/10.1038/nri.1937

26. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A. Macrophage polarization in tumour progression. Seminars Cancer Biol 2008; 18:349-55; PMID:18467122; http://dx.doi.org/10.1016/j.semcancer.2008.03.004
43. Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, Bottazzi B, Doni A, Vincenzo B, Pasqualini F et al. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). Blood 2006; 107:2112-22; PMID:16269622; http://dx.doi.org/10.1182/blood-2005-01-0428

44. Sakai Y, Honda M, Fujinaga H, Tatsumi I, Mizukoshi E, Nakamoto Y, Kaneko S. Common transcriptional signature of tumor-infiltrating mononuclear inflammatory cells and peripheral blood mononuclear cells in hepatocellular carcinoma patients. Cancer Res 2008; 68:10267-79; PMID:19074895; http://dx.doi.org/10.1158/0008-5472.CAN-08-0911

45. Beck AH, Espinosa I, Edris B, Li R, Montgomery K, Zhu S, Varma S, Marinelli RJ, van de Rijn M, West RB. The macrophage colony-stimulating factor 1 response signature in breast carcinoma. Clin Cancer Res 2009; 15:778-87; PMID:19188147; http://dx.doi.org/10.1158/1078-0432.CCR-08-1283

46. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005; 5:263-74; PMID:15776005; http://dx.doi.org/10.1038/nrc1586

47. Schrocksnadel K, Wirleitner B, Winkler C, Fuchs D. Monitoring tryptophan metabolism in chronic immune activation. Clin Chim Acta; Int J Clin Chem 2006; 364:82-90; PMID:16139256; http://dx.doi.org/10.1016/j.cca.2005.06.013

48. Huang A, Fuchs D, Widner B, Glover C, Henderson DC, Allen-Mersh TG. Serum tryptophan decrease correlates with immune activation and impaired quality of life in colorectal cancer. Br J Cancer 2002; 86:1691-6; PMID:12087451; http://dx.doi.org/10.1038/sj.bjc.6600336

49. Opitz CA, Litzenburger UM, Sahm F, Ott M, Tritschler I, Trump S, Schumacher T, Jestaedt L, Schrenk D, Weller M et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature 2011; 478:197-203; PMID:21976023; http://dx.doi.org/10.1038/nature10491

50. Pilotte L, Larrieu P, Stroobant V, Colau D, Dolusic E, Frederick R, De Plaen E, Vytenis C, Wouters J, Masereel B et al. Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. Proc Natl Acad Sci USA 2012; 109:2497-502; PMID:22308364; http://dx.doi.org/10.1073/pnas.1113873109

51. Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vallo A, Orabona C, Bianchi R, Belladonna ML, Volpi C et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. J Immunol 2006; 176:6752-61; PMID:16709834; http://dx.doi.org/10.4049/jimmunol.176.11.6752

52. Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol 2008; 181:5396-404; PMID:18832696; http://dx.doi.org/10.4049/jimmunol.181.8.5396

53. Manches O, Munn D, Fallahi A, Lisson J, Chaperot L, Plumas J, Bhawandwaj N. HIV-activated human plasmacytoid DCs induce Tregs through an indoleamine 2,3-dioxygenase-dependent mechanism. J Clin Invest 2008; 118:3431-9; PMID:18776940; http://dx.doi.org/10.1172/JCI34823

54. Sharma MD, Baban B, Chandler P, Hou DY, Singh N, Yagit A, Amsa M, Blazar BR, Mellor AL, Munn DH. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. Proc Natl Acad Sci USA 2008; 109:2497-502; PMID:18536986; http://dx.doi.org/10.1107/blood-2008-12-195354

55. Sharma MD, Hou DY, Liu Y, Koni PA, Metz R, Chandler P, Mellor AL, He Y, Munn DH. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. Blood 2009; 113:6102-11; PMID:18774576; http://dx.doi.org/10.1172/JCI34823

56. Sharma MD, Hou DY, Baban B, Koni PA, He Y, Chandler PR, Blazar BR, Mellor AL, Munn DH. Reprogrammed foxp3 (+) regulatory T cells provide essential help to support cross-presentation and CD8(+) T cell priming in naive mice. Immunity 2010; 33:942-54; PMID:21145762; http://dx.doi.org/10.1016/j.immuni.2010.11.022

57. Baban B, Chandler PR, Johnson BA, 3rd, Huang L, Li M, Sharpe ML, Francisco LM, Sharpe AH, Blazar BR, Munn DH et al. Physiologic control of IDO competence in splenic dendritic cells. J Immunol 2011; 187:2329-35; PMID:21813777; http://dx.doi.org/10.4049/jimmunol.1100276

58. Krejsgaard T, Oudum N, Geisler C, Waski MA, Woetmann A. Regulatory T cells and immunodeficiency in mycosis fungoides and Sézary syndrome. Leukemia 2012; 26:424-32; PMID:21904385; http://dx.doi.org/10.1038/leu.2011.237

59. Bulh L, Sogard H. Immunohistochemical expression of IL-10 in mycosis fungoides. Exp Dermatol 1997; 6:195-8; PMID:9293392; http://dx.doi.org/10.1111/j.1600-0625.1997.tb00205.x