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Acute cellular rejection in small-bowel transplantation impairs NCR^{+} innate lymphoid cell subpopulation 3/interleukin 22 axis

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ABSTRACT

Acute cellular rejection (ACR) remains as one of the main causes of graft loss and death in intestinal transplant (ITx) patients. ACR promotes intestinal injury, disruption of the mucosal barrier, bacterial translocation, and organ dysfunction. As epithelial regeneration is critical in reversing these consequences, the functional axis between the innate lymphoid cell subpopulation 3 (ILC3) and interleukin 22 plays an essential role in that process. Natural-cytotoxic-receptor–positive (NCR^{+}) ILC3 cells have been demonstrated to induce intestinal-stem-cell proliferation along with an IL-22–dependent expansion of that population in several intestinal pathologies, though thus far not after ITx. Therefore, we intended to determine the impact of chronic immunosuppression and ACR on ILC3 cells and interleukin-22 (IL-22) production in the lamina propria after that intervention.

Materials and methods: We compared biopsies from healthy volunteers with biopsies from ITx recipients without or with mild-to-moderate ACR, using flow cytometry and the quantitative-PCR.

Results: NCR^{+} ILC3 cells were found to be unaffected by immunosuppression at different time points post-transplant when patients did not experience ACR, but were diminished upon the occurrence of ACR independently of the post-ITx time. Moreover, IL-22–expression levels were notably reduced in ACR.

Conclusion: The NCR^{+}-ILC3/IL-22 axis is impaired during ACR contributing to a delay in or lack of a complete and efficient epithelial regeneration. Thus, our findings reveal that IL-22 analogues could potentially be used as a new complementary therapeutic approach, in conjunction with immunosuppressant drugs, in order to promote mucosal regeneration upon ACR.

1. Introduction

The integrity of the mucosal barrier—whose principal function is absorption and, in conjunction with the immune system, the avoidance of bacterial and fungal translocation—is critical for the maintenance of complete health [1]. After intestinal transplantation (ITx) [2], the development of graft ischemia, chimeric types of changes in the immune-cell composition [3], alterations or variations in the luminal microbiota [1], and the need for using chronic immunosuppression can contribute to an elevated frequency of immunologic insults, such as acute cellular rejection (ACR) [4]; viral, bacterial, or parasite infections [5]; and graft-versus-host disease (GvHD) [6].

All these insults—if the process cannot be controlled [2,7] —can ultimately cause disruptions in the intestinal mucosal barrier; leading to bacterial translocation, the associated sepsis, and eventual patient death.

Innate lymphoid cells (ILCs), a recently characterized population of immune cells, exhibit a lymphoid morphology and are similar to the helper T cells in their induction, activation, and secretory profile; but lack rearrangement receptors [8,9]. ILCs are capable of interacting with

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hematopoietic or nonhematopoietic cells to trigger an inflammatory response or to achieve a homeostatic status in various tissues [10].

The subpopulation-3 ILCs (ILC3s), express the retinoid-related orphan receptor gamma t (RORγt), and the stem-cell receptor c-kit [11,12] and exhibit a crucial role in wound healing and the maintenance of intestinal homeostasis [13]. The natural-cytotoxic-receptor–negative (NCR^-) ILC3s secrete interleukin-17 (IL-17), whereas the NCR^+ ILC3s produce IL-22 [12,14] in response to IL-1b and IL-23. In addition, NCR^+ ILC3s—in expressing the class-II molecules of the major histocompatibility complex (MHCII)—can modulate acquired immunity in a cytokine-independent manner [15,16]. Consequently, recent studies have associated ILC3 dysregulation with certain intestinal pathologies. In Crohn's disease, for example, a reduction in IL-22–producing ILC3s has been observed [17,18]. Furthermore, an increase in IL-17-producing ILC3s that were negative for the neural-cell-adhesion molecule cluster of differentiation 56 (CD56) [19] and in interferon-γ–producing subpopulation-1 ILCs and natural-killer cells (NKs) [17,18] was also documented. Bone-marrow-transplant patients who developed GvHD had lower blood levels of ILC3s than control patients [20]. In patients after ITx, Talayero et al. [21] observed a rise in NCR^+ $CD3^-$ intraepithelial lymphocytes (IELs) and in IL-22 titers, but were not able to make any associations between this observation and the patients' clinical situation.

The main pathway involved in protecting mucosal integrity triggered by ILC3 seems to be the IL-22–IL22-receptor-α (IL-22RA) axis. Because IL-22 promotes a maintenance of the mucosal layer by inducing cell proliferation and inhibiting apoptosis [22], this cytokine participates in epithelial-barrier repair after injuries. IL-22 furthermore regulates nonhematopoietic cells that express IL-22RA, such as the intestinal stem cells (ISCs) present in the base of the Lieberkühn crypt [22]. Studies with mouse and human intestinal organoids indicated that IL-22 administration induced an expansion and proliferation of ISCs along with a formation of intestinal crypts ex vivo [23]. In addition, IL-22 protected ISCs from GvHD and improved their intestinal histological signs in a mouse model [24,25].

Although the ACR targets have been proposed to be epithelial cells [26], not until recently have the cells of the in-transit–amplification zone of the crypt been demonstrated as being the main elements damaged during graft rejection [27]. Furthermore, ISCs are preserved even during severe occurrences of ACR and continue to express IL-22RA. In instances of extensive damage, if the epithelial reparation is incomplete or too slow, the risk for patients becomes increased. T cells are the main target of the immunosuppressive drugs currently used to avoid ACR after ITx, but how these treatments affect the biology of the ILCs is still not clear. Considering that ILCs and T cells share several features; in the work reported here, we aimed at determining whether ILC3 cells and IL-22 production in the lamina propria are modified because of the immunosuppression used, or not, and what the direct impact of ACR is on that lymphoid-cytokine axis.

2. Materials and methods

2.1. Patients

Patients with intestinal transplantation who were in follow-up care from April 2014 to August 2017 were included in this study. The present protocol was approved by the Institutional Review Board of the Favaloro Foundation University Hospital (DDI [1125] 511) and has been performed in accordance with the ethical standards laid down in the declarations of Helsinki and Istanbul. Accordingly, patients or their representatives gave their informed consent to participate in this study. The details of the ITx surgical procedures, the immunosuppression methodology, and the patients' follow-up care have been previously reported [28,29]. Table 1 summarizes the detailed patient data. Six to 8 biopsies were obtained from the distal ileum and divided randomly, with 3–5 being used for clinical histological diagnosis and the other 3 earmarked for the present research protocol. Samples were processed immediately after biopsy and retrospectively classified in groups on the basis of their unequivocal histopathological diagnoses in accordance with the recommendations of the pathology workshop of the VIII Small Bowel Transplantation Symposium [26]. Our study groups were: (1) no rejection (N; ITx biopsies without any signs of histopathology and patients without significant clinical symptoms for 72 h before endoscopy—*e. g.*, abdominal pain, self-limited diarrhea and/or increased ostomy output, respiratory symptoms, or episodes of isolated fever—$n = 15$), (2) mild ACR (MiR; ITx biopsies with histological evidence of incipient ACR; $n = 4$), and (3) the control group (C, ileum biopsies from healthy volunteers who were endoscopically evaluated for colon cancer; $n = 7$). For expression analysis, we incorporated samples with histological evidence of moderate rejection (MoR, $n = 6$). All the ITx patients were treated with either of two standard maintenance immunosuppressive regimens (tacrolimus + corticosteroids + mycophenolate mofetil or tacrolimus + corticosteroids + target-of-rapamycin inhibitors), based on the primery induction used and the clinical needs throughout the follow-up. All the biopsies corresponding to rejection episodes were taken when the rejection was first suspected and then later diagnosed. In all of these cases, therefore, patients were not exposed to immunosuppressive therapeutic regimes against rejection per se.

2.2. Isolation of lymphoid cells from the lamina propria

The intestinal biopsies were collected and transported in ice-cold Hanks's balanced salt solution (HBSS) for immediate processing. The biopsies were first incubated in 5 mM ethylenediaminetetra-acetic acid and 1.5 mM dithiothreitol HBSS with agitation for 25–30 min at room temperature (RT) to eliminate epithelial cells and mucus. The tissues were then digested with collagenase type 1a (62 IU/ml; Sigma-Aldrich, MO, USA) in RPMI 1640 medium (Thermo Fisher, MA, USA) for 60 min at 37 °C. The resulting cell suspensions were filtered through a 70-mm nylon mesh and incubated with ammonium-chloride-potassium (ACK) lysis buffer for 3 min at RT to deplete the erythrocytes present.

2.3. Innate-lymphoid-cell enrichment

Isolated mononuclear cells were enriched in innate lymphoid cells by depletion of the CD4 T lymphocytes through the use of a magnetic-activated cell-sorting system employing separation by adhesion to human-CD4–ligated microbeads (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The $CD4^-$ population isolated was used for flow-cytometry analysis.

2.4. Flow-cytometry analysis

$CD4^-$ mononuclear cells were first treated with 10% (*v*/v)) human serum for Fc-receptor blocking and then incubated for 15 min at RT with the antibodies listed in Table 2 followed by analysis via a FACS-Canto II cytometer (Becton, Dickinson & Co., NJ, USA). The differential expression of the surface proteins listed in Table 3 enabled the identification of all the ILC subsets and the NK population. Expression of the human-leukocyte cell-surface receptor HLA-DR revealed the functional aspects of the cell populations involved. Fig. 1 illustrates the identification scheme used.

2.5. Gene-expression analysis

Entire biopsies were taken by video endoscopy, immediately placed in RNAlater™ stabilization solution (Ambion, TX, USA), and finally stored at −70 °C for total RNA extraction. The RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction (qPCR) were conducted as previously described [30]. Table 2b lists the genes evaluated and the corresponding primer pairs. The gene-expression data

Table 1
Summary of patients included in the study.

a) Samples analyzed by cytometry						
	Group	Age	ITx type	Immunosuppression treatment	Days post-Tx	Rejections
Non-Tx	Control (n = 7)	56.7 ± 7.6		None		
ITx	No rejection (N; n = 15)	9.9 ± 7.6	Isolated	Standard maintenance	1310 ± 908	4.6 ± 2.9
	Mild rejection (MiR; n = 4)	10.0 ± 6.0	Isolated	Standard maintenance	1803 ± 1595	3.5 ± 2.4
b) Samples analyzed for whole-biopsy expression						
	Group	Age	ITx type	Immunosuppression treatment	Days post-Tx	Rejections
ITx	No rejection (N; n = 5)	21.0 ± 17.0	Isolated	Standard maintenance	619 ± 578	1.8 ± 1.8
	Moderate rejection (MoR; n = 6)	14.8 ± 16.7	Isolated	Standard maintenance	684 ± 480	1.3 ± 1.2

ITx, intestinal transplant; N, ITx patients without rejection; MiR, ITx patients with mild rejection; MoR, ITx patients with moderate rejection.

were normalized according to that of the ß-actin gene as a reference. The fold increase was calculated by the Delta-Delta-Ct method with the average of the control (*i. e.*, non-ITx) group as the normalizer.

2.6. Statistical analysis

Comparisons among the groups of data were performed with the Kruskal-Wallis test and Dunn's *post-hoc* test when more than two groups were analyzed, whereas the Mann-Whitney *U* test was chosen to compare data between two groups. All the statistical analyses were performed by means of the GraphPad Prism 5.01 software (San Diego, CA, USA).

3. Results

3.1. Lack of effect of immunosuppressants on innate lymphoid cells

To evaluate the effect of immunosuppressants on ILCs, we compared by flow cytometry the proportion of NKs, ILCs, and ILC3s in the lamina propria of healthy patients and of ITx patients with normal histology and mild rejection at different times post-ITx.

NKs were defined as $CD3^-$ $CD19^-$ $CD127^-$ $CD117^-$ $CD56^+$ $NKp44^{+/-}$ cells within the lymphocyte gate (Fig. 1). The NK frequency did not exhibit differences between the transplanted (and thus immunosuppressed) and nontransplanted (control) groups (Fig. 2 Panel A). As expected, an increase in the activation of NK cells occurred during mild ACR consistent with an inflammatory context (Fig. 2 Panel B; p = .019). Moreover, we defined the ILC population as $CD3^-$ $CD19^-$ $CD127^+$ cells within the lymphocyte gate (Fig. 1). The proportion of total ILCs evidenced a slight diminution in the two transplanted groups with respect to the control group, but this difference was not statistically significant (Fig. 3, Panel A). We next evaluated the relative abundance of the different ILC subsets. ILC1, ILC $CD294^-$ $CD117^-$ (Fig. 1), exhibited the highest percentage in all the groups evaluated, but were not statistically different from one another (Fig. 3, Panel B). Since we did not use the now classic lineage cocktail to select in the lineage-negative gate of the identification scheme, ILC1 could be contaminated with other cells; nevertheless, we did not attempt to characterize this population in greater depth. Moreover, the proportion of ILC2s (ILC $CD294^+$, Fig. 1) and ILC3s (ILC $CD294^-$ $CD117^+$ $NKp44^{+/-}$, Fig. 1) remained comparable among the groups (Fig. 3, Panel B).

Table 3
Cell-surface markers used to identify ILC and NK populations.

Molecule	Description	NK	ILC1	ILC2	ILC3
CD3	LT Co-receptor	−	−	−	−
CD19	LB Co-receptor	−	−	−	−
CD127	IL-7 Receptor	−	+	+	+
CD294	Prostagladin D2 Receptor	−	−	+	−
CD117	c-Kit (stem cell receptor)	−	−	+/−	+
NKp44	Killer activation receptor (KAR)	+/−	−	−	+/−
CD56	Neural cell adhesion molecule (NCAM)	+	−	−	+/−

3.2. Reduced NCR^+ ILC3 levels during ACR

As previously described, the expression of NKp44 receptors on ILC3 cells defines two natural-cytotoxic-receptor (NCR) subpopulations, NCR^+ ($NKp44^+$) and NCR^- ($NKp44^-$) [14]. NCR^+ ILC3s are potent

Table 2
Description of used reagents.

a) Antibodies used for cytometry analysis				
Antibody	Reactivity	Clone	Conjugate	Company
Anti- CD3	Human	SK7	APC-H7	BD
Anti- CD19	Human	HIB19	APC-H7	Biolegend
Anti- CD127	Human	HIL-7R-M21	PE	BD
Anti- CD294	Human	BM16	PERCPCy5.5	Biolegend
Anti- CD117	Human	104D2	PeCy7	BD
Anti- NKp44	Human	P44–8	Alexa 647	BD
Anti- CD56	Human	B159	FITC	BD
Anti- HLA DR	Human	LN3	PE	eBioscience
b) Genes and primers used for analysis by qPCR.				
GeneID	Symbol	Gene description	Cell type	Primer sequences 5′→3’
NM_020525.4	*IL22*	Interleukin 22	ILC3- LTh22	CAACAGGCTAAGCACTGTCA ACTGTGTCCTTCAGCTTTTGC

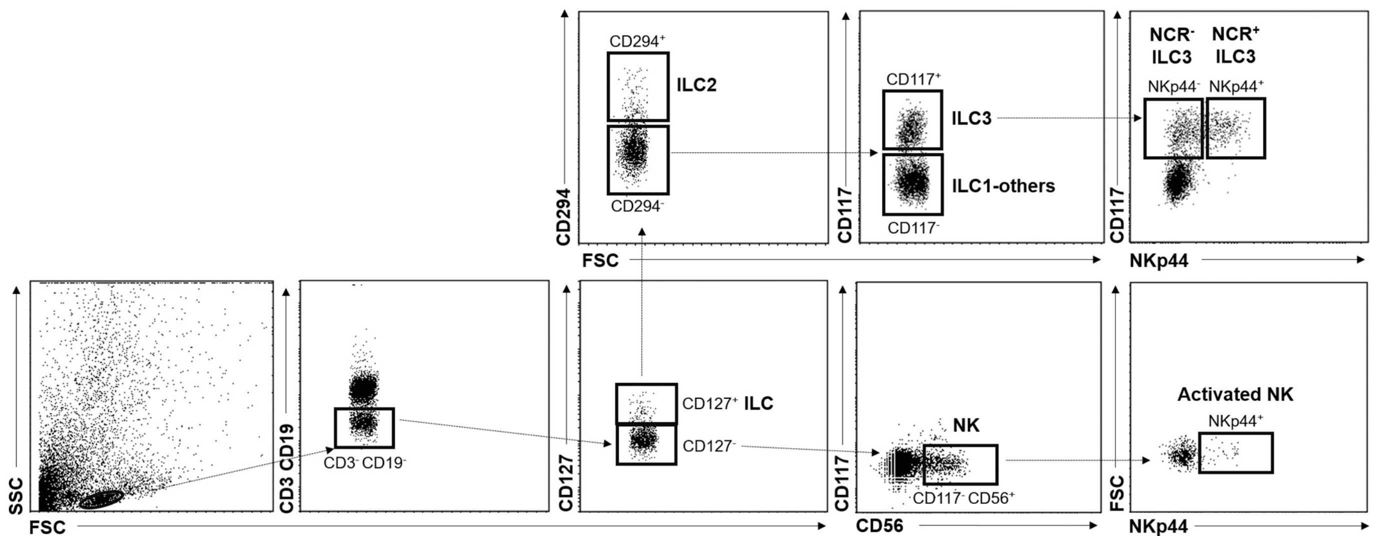

Fig. 1. ILC- and NK-identification scheme. Representative dot plot of flow cytometry analysis of lymphoid cells isolated from lamina propria of fresh biopsies. The arrows between the panels denote the sequential-gating strategy used to identify natural-killer (NK) cells and innate lymphoid (ILC) cells on the basis of the lymphocyte gate. ILC2, ILC subpopulation 2; ILC3, ILC subpopulation 3; NKp44 natural-killer cells with or without cell-surface natural-cytotoxic-receptor expression.

producers of IL-22 and participate in intestinal homeostasis, whereas NCR$^-$ ILC3s secrete mainly the proinflammatory cytokine IL-17. To study the behavior of these two antagonistic subpopulations during ACR, we analyzed the expression level of NKp44 on the ILC3s in all the groups studied (Fig. 1). We determined that the mean percentage of total ILC3s remained constant between the transplanted and the non-transplanted groups (Fig. 3, Panel B and Fig. 4, Panel A). The proportions of NCR$^+$ ILC3s were not different between the control and the ITx normal groups, whereas that subpopulation was significantly decreased in the group experiencing rejection (Fig. 4, Panel B, $p = .005$). Conversely, the percentage of NCR$^-$ ILC3s evidenced a substantial elevation in the rejection patients (Fig. 4, Panel C, $p = .003$).

3.3. Class-II histocompatibility molecules and ILC3s in intestinal transplantation

Since ILC3s have been documented to interact with T lymphocytes through MHCII molecules, we assessed by flow cytometry the expression of MHCII on the ILC3s in the lamina propria isolated from the intestines of ITx patients undergoing immunosuppression (Fig. 5, Panel A). Those ILC3s—and particularly members of the NCR$^+$ subpopulation—expressed MHCII molecules on their surface (Fig. 5, Panel B).

3.4. Reduced IL-22 expression during ACR

To evaluate whether NCR$^+$-ILC3 reduction had any effect on the IL-22 levels, we measured the expression of the *IL22* gene from intact intestinal biopsies. The expression of that locus, on the average, was 12-times lower in the moderate-rejection group than in the transplanted normal group (Fig. 6). Even though this decrease was not significant because of the dispersion of the data ($p = .06$; cf. the error bars in the figure), the *IL22* level nevertheless could be seen to be considerably affected during ACR.

4. Discussion

Many cells and molecules from the innate and acquired immune systems that participate in the inflammatory process have been well characterized. Nevertheless, the recent discovery of the ILC family has revealed an essential role of those cells in initiating, regulating, and resolving inflammation. Even though much information concerning the ILCs came from experimental models, in recent years the presence and functioning in human tissues of those lymphoid elements has been extensively studied [8]. In particular, in the intestine, the participation of the ILC3s in mucosal homeostatic functions has been thoroughly recognized [13,31,32].

Although, after ITx, patients require an aggressive immunosuppression, ACR nonetheless occurs at a high frequency; with the main target of that process being the mucosal epithelial cells. Moreover, as we have previously reported, the most affected epithelial cells are those placed at the crypt transition zone, where cellular proliferation and differentiation occurs [27]. In the present work, we aimed first at evaluating if immunosuppressive drugs had any effect on the ILC constitution and function, and particularly on the ILC3 titers or functioning, in comparison to the classical innate NK cells. To that end, we

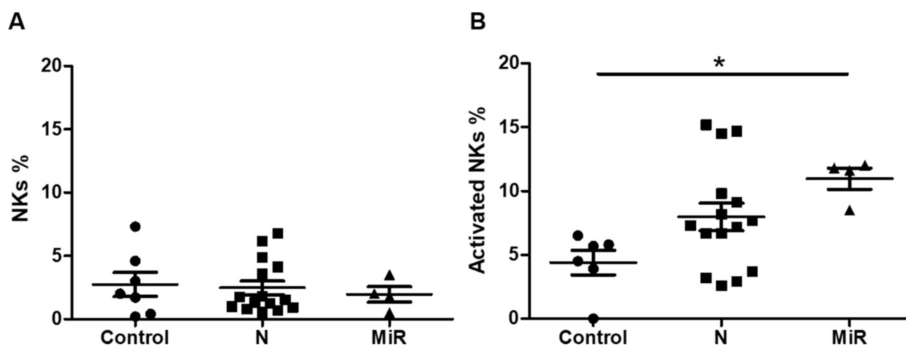

Fig. 2. NK cells in ITx. Flow-cytometry analysis of lymphoid cells isolated from the lamina propria of fresh biopsies in patients non transplanted (Control, $n = 7$), transplanted with normal histology (N, $n = 15$), and transplanted with evidence of mild rejection (MiR, $n = 4$). In the figures the percent total NK cells (Panel A) or activated NK cells (Panel B) is plotted on the *ordinate* for the experimental groups indicated on the *abscissa*. The values are the mean ± SEM, with the groups being compared through the use of the Kruskal-Wallis test along with Dunn's *post-hoc* test. $^*P < .05$.

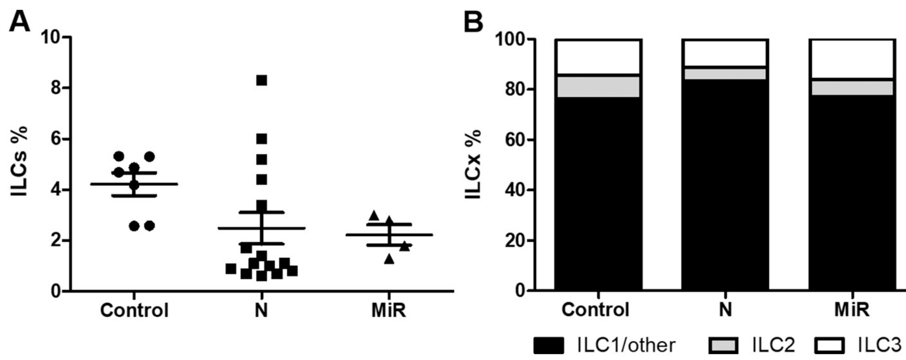

Fig. 3. Effect of immunosuppression on the innate lymphoid cells. Flow-cytometry analysis was performed on lymphoid cells isolated from the lamina propria of fresh biopsies in patients non transplanted (Control, n = 7), transplanted with normal histology (N, n = 15), and transplanted with evidence of mild rejection (MiR, n = 4). Panel A: The percent innate lymphoid cells (ILCs) within the total lymphocytes is plotted on the *ordinate* for the experimental groups indicated on the *abscissa*. Panel B: The percentages of different innate lymphoid cells (ILCx) among the subsets ILC1–ILC3 among the total ILCs is plotted on the *ordinate* for the experimental groups indicated on the *abscissa*. Key to the bar textures: black, ILC1s and/or others; light gray, ILCs of subpopulation 2; white, ILCs of subpopulation 3. The values are the mean ± SEM with the groups being compared through the use of the Kruskal-Wallis test along with Dunn's *post-hoc* test.

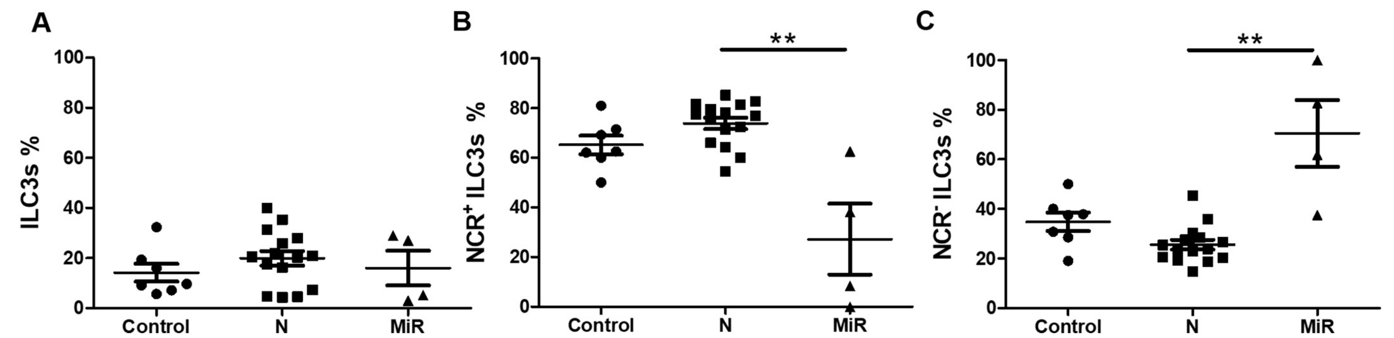

Fig. 4. Reduction in NCR$^+$ ILC3s during ACR. Flow-cytometry analysis of lymphoid cells isolated from the lamina propria of fresh biopsies in patients non transplanted (C, n = 7), transplanted with normal histology (N, n = 16), and transplanted with evidence of mild rejection (MiR, n = 4). In the figure, the percentage of (Panel A) innate-lymphoid-cell subpopulation 3 (ILC3) among the total ILCs, (Panel B) NKp44$^+$ ILC3s (NCR$^+$; N versus MiR, p = .005), (Panel C) NKp44$^-$ ILC3s (NCR$^-$; N versus MiR, p = .003) is plotted on the *ordinate* for the experimental groups indicated on the *abscissa*. The values are the mean ± SEM with the groups being compared through the use of the Kruskal-Wallis test along with Dunn's *post-hoc* test. $^{**}P < .01$.

isolated mononuclear cells from the lamina propria of ileum biopsies from healthy volunteers and from ITx patients with or without signs or symptoms of ACR. After an enrichment in CD4$^-$ cells using magnetic-activated—cell-sorting (MACS) technology, we performed a flow cytometry to select the CD3$^-$-CD19$^-$ population within the lymphocyte gate for downstream ILC and NK gating. The marked progression in the knowledge of and the technology employed in studying ILC biology in recent years has revealed the complexity and heterogeneity of those lymphoid elements [33]. Even today, different research groups work together to establish a consensus on the identification and phenotypic and functional characterization of the ILCs [9,12]. Trabanelli et al. [12], in a comparison of different gating strategies for identifying ILC subsets, recommended the use of 8 markers to exclude different non-ILC lineages in that analysis. Simoni et al. [8], using an exhaustive panel of markers and cytometry by time-of-flight (aka CyTOF), generated a detailed description of human heterogeneity in the ILCs among patients and tissues, in non pathologic states as well as within various pathologic conditions [9]. Our study is somewhat limited in the marker strategy used to define the ILC subsets in that we initiated that work in 2014 using a routinely employed staining before an optimal technique had been universally recognized for identifying those subsets of innate lymphoid cells in humans [34]. The main limitation with the scheme we employed was the possibility of a contamination of the ILC1 subpopulation since those cells are nowadays identified by additional surface markers [12]. Thus, we named this heterogeneous group as *ILC1 and/or others*, being unable to perform a more in-depth analysis at that time.

Our results indicated that no reduction in either the total ILCs or the

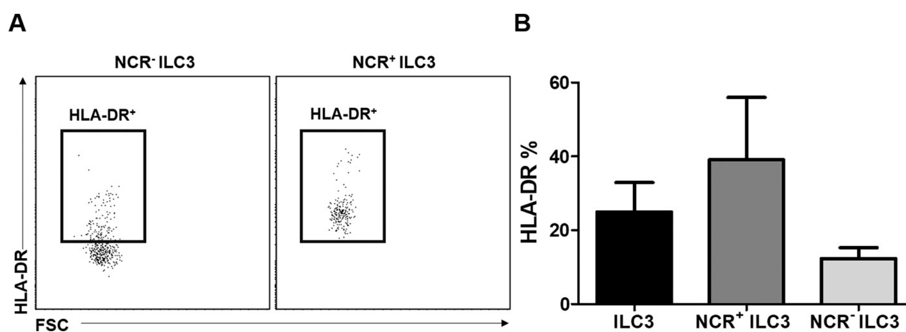

Fig. 5. HLA-II molecules on ILC3 cells after ITx. Flow-cytometry analysis was performed on lymphoid cells isolated from the lamina propria of fresh intestinal biopsies from transplanted patients with normal histology (N, n = 4). Panel A: Representative dot plot of the MHC–class-II HLA-DR expression in NCR$^-$ (left side) and NCR$^+$ (right side) cells. In the panel, the signals from HLA-DR bound to fluorescence-labelled specific antibodies is plotted on the *ordinate* as a function of the forward scatter on the *abscissa*. Panel B: Percentage of cells that express the HLA-DR molecules among the total is plotted on the *ordinate* for the total ILC3s, the NCR$^+$ ILC3s, and the NCR$^-$ ILC3s as indicated on the *abscissa*. The values are the mean ± SEM with the groups being compared through the use of the Kruskal-Wallis test along with Dunn's *post-hoc* test.

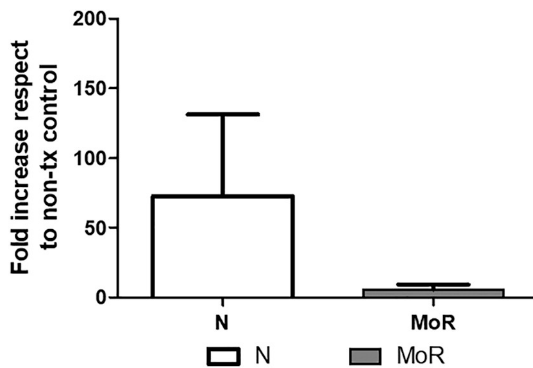

Fig. 6. *IL-22* expression level during ACR The quantitative transcription of the *IL22* gene was evaluated by qPCR in the N ($n = 5$) and the MoR ($n = 6$) groups. In the figure, the fold increase in gene expression relative to the non transplanted-control values—as calculated by the the DDCt method and normalized to the expression of the *ß-actin* gene as a reference—with the average of that group as the normalizer, is plotted on the *ordinate* for the N and MoR samples on the *abscissa*. The values are the mean ± SEM with the groups being compared through the use of the one-tailed Mann-Whitney *U* test.

ILC3 subpopulation occurs in the lamina propria of ITx patients in comparison with that tissue obtained from healthy individuals. Moreover, the expression of MHCII was evident in ILC3 cells from ITx patients under immunosuppression. The same pattern was observed in ITx patients undergoing mild rejection. These findings are consistent with those obtained by Meier et al [30], reporting the effect of immunosuppression on the constitution and function of isolated lymphoid follicles in ITx, and with more recent results indicating the same pattern in the peripheral blood of kidney- and liver-transplant recipients [35]. Although ILC3s have been extensively recognized as immune-regulatory elements through cytokine secretion, recent studies have revealed that those cells can also modulate an acquired response via a cytokine-independent pathway [15,16]. This activity is mediated by MHCII molecules. We therefore evaluated MHCII expression in the ILC3s of ITx patients and observed that the ILC3s express MHCII molecules on their surface, and especially the NCR^+ subgroup. In experimental models, the capability of modulating the response of $CD4^+$ T cells to commensal bacteria has been attributed to ILC3 [15] along with inducing a specific T-cell response after stimulation in vivo and in vitro [16]; with the latter instance involving peripheral NCR^- ILC3s. A report on Crohn's disease, demonstrated that a decrease in MHCII expression contributed to a worsening of the pathology [36]. Furthermore, in ITx patients, intestinal IgA production is not impaired by immunosuppression [30]. The results reported in the present work were in accordance with this finding since $MHCII^+$ ILC3s had been demonstrated to participate in the mucosal IgA production circuit [32]. Our present results indicated that ILC3s expressed MHCII molecules even under maintenance immunosuppression. In addition, Weiner et al. [37] demonstrated that donor ILCs persist for a long-time in the gut after ITx, whereas the donor T-cell population is rapidly replaced [38]. The question then is whether or not MHCII expression on ILC cells contributes to a generation of local chimerism even at a long time after transplant. Further studies are necessary to add more details into this evolving field of intestinal-transplant immunology.

Of interest to us was that Talayero et al. [21] reported an increase in ILC3s in the intraepithelial compartment of transplant patients over the levels recorded in the controls. This finding might result from the analysis of different compartments of the mucosa and a different means of identifying ILC3s. That study indicated an increase in $CD3^-$ IELs that expressed NKp44, CD56, and other markers; but those results were found in separate assays without following the ILC3 identification scheme that we used (cf. Fig. 1). An enhancement in NKp44 expression in $CD3^-$ IELs and in IL-22 levels in ITx patients has been reported, but not during the occurrence of ACR. The functional consequence of such a shift in the abundance of this population still remains unclear [21].

In Crohn's disease, a decrease in IL-22–producing ILC3s along with a consequent increase in IL-23-inducible NK and ILC1—both IFN-γ producers—was described [17,18]. That ILC3s, along with ILC1s, could downregulate the expression of the RORγt and upregulate the immune-cell transcription factor T-bet as well as secrete IFN-γ [39,40]. ILC1s were the most abundant ILC subpopulation in our samples, but no differences were observed between the ITx and the non-ITx groups. In addition, Geremia et al. [19] observed an increase in IL-17–secreting ILC3s in Crohn's disease. Moreover, ILC3-derived IL-2 was recently demonstrated to be essential for maintaining the immunologic homeostasis of the Treg subclass of T lymphocytes along with oral tolerance to dietary antigens in the small intestine. Furthermore, production of IL-2 by ILC3s was significantly reduced in the small intestine of patients with Crohn's disease, with this diminution correlating with lower frequencies of the Treg cells [41].

Intestinal pathologies often exhibit a decrease in homeostatic ILC3s because of a rise in proinflammatory ILCs—such as ILC1, or NCR^- ILC3 cells. Our results revealed a similar pattern in post-ITx ACR patients. A direct link between NKp44 surface expression on the ILC3s and the IL-22 production of those lymphoid cells has recently been demonstrated [14]. In contrast, the $NKp44^-$ subpopulation (NCR^-) produced mainly IL-17. Hence, the NCR^+/NCR^-–ILC3 ratio had an influence on reparation or the inflammatory profile in the immune process. In our patient cohort, we could detect a remarkable NCR^+ ILC3-cell decrease in mild-rejection ITx samples compared with those from transplant patients without rejection. Consistent with this result, the expression of IL-22 in rejection biopsies was notably low. Similarly, in the most recent International Congress of Intestinal Rehabilitation and Transplant Association (CIRTA 2019), a group from Washington reported a decrease in NCR^+ ILC3s (referred to by them as *protective ILC3s*) immediately after ITx followed by a repopulation 1 month postoperatively [42]. That reconstitution of protective ILC3s correlated positively with improved epithelial-barrier function through an increase in IL-22–dependent antimicrobial-peptide expression. In contrast, the abundance of proinflammatory ILC1s and NCR^- ILC3s could be contributing to the initial epithelial-barrier breakdown and early clinical complications. Along those lines, the use of multiparametric flow cytometry and single-cell analysis has recently pointed to an inherent heterogeneity in intestinal ILC1 and ILC3 populations, with possible transitions between them [43]. This inherent plasticity may be operating in the ACR scenario studied in the present work.

Despite a progressive increase in the knowledge within this area, we still need to evolve a more complete understanding of the nature and functioning of the ILCs in each of the processes involved during ITx. Fig. 7 summarizes the changes in the IL-22/IL22RA axis during ACR in view of the present results and the findings published by our group concerning the ISC status and IL-22RA expression in ITx patients [27]. Munneke et al. [20] reported lower levels of ILC3s in bone-marrow–-transplant patients who developed GvHD compared with those who did not. In mouse models of this pathology, intraperitoneal administration of IL-22 induced the recovery of damaged ISCs and improved histologic signs in the intestine [23,24]. This evidence in favor of that form of therapeutic intervention could be extrapolated to the ITx field in the future in order to accelerate epithelial-barrier recovery after exfoliative ACR. An understanding of the impairment in the regulatory axis as the direct consequence of a rejection will provide a novel opportunity for characterizing the use of IL-22 analogues as complementary therapy for early ACR in order to facilitate and/or accelerate intestinal repair.

In summary, our study demonstrates that NCR^+ ILC3 cells, although not affected by immunosuppression at different post-transplant times, are diminished during ACR. Consistent with these findings and the ILC3–IL-22 mucosa-regulatory axis described in this work; IL-22–expression levels are reduced in ACR. In addition, the NCR^+ ILC3s were the principal elements found to express MHCII molecules; whose

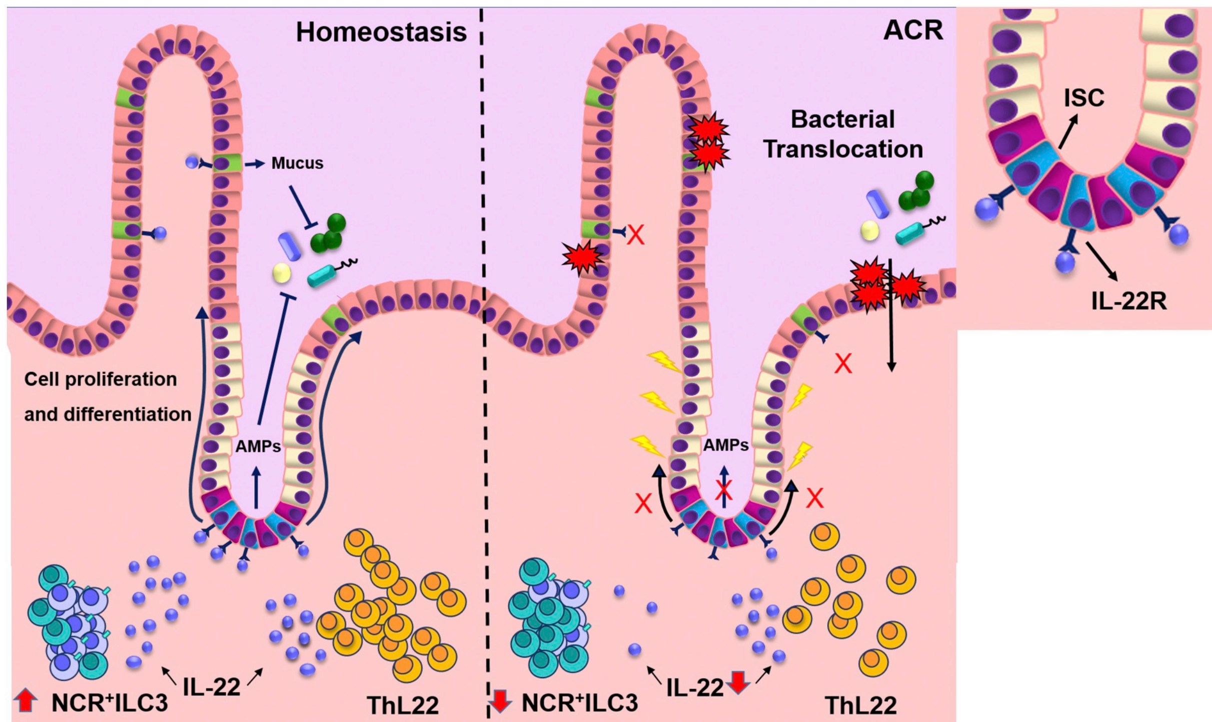

Fig. 7. The IL-22/IL-22RA axis in post-ITx ACR patients: The main IL-22 producers in the intestinal lamina propria in homeostasis (left panel) or during ACR (right panel). The interaction between IL-22 and its receptor on ISCs triggers cell proliferation and differentiation. In ACR, the number of NCR^+ ILC3s as well as the levels of IL-22 decrease leading to an inefficient epithelial repair. Bacterial translocation can occur and, consequently, increases the risk of septicemia. The injury produced by T cells in the transit-amplifying zone of the crypt are indicated by yellow thunderbolts. Key to abbreviations: ACR, acute cellular rejection; ILC3, innate-lymphoid-cell subpopulation 3; IL, interleukin; ThL22 and $CD4^+$, T lymphocytes that secrete IL-22; IL-22RA, IL-22 receptor-α; ISC, intestinal stem cells; NCR, natural cytotoxic-receptor–positive ILC cells; AMP, antimicrobial peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surface presentation, in turn, is able to modulate the acquired immune response. Whether a decrease in NCR^+ ILC3s in ITx patients with ACR implies an altered relationship with the intestinal microbiota, or an impaired capability for epithelium regeneration—or whether a preponderance of NCR^- ILC3s result in an exacerbated inflammatory response—are questions that need to be resolved through future studies.

Declaration of Competing Interest

None.

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