Expansion of Circulating CD49b⁺LAG3⁺ Type 1 Regulatory T Cells in Human Chronic Graft-Versus-Host Disease


TO THE EDITOR

Chronic graft-versus-host disease (cGVHD) remains the main cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (AHSC). cGVHD is a multisystem, immune-mediated disease characterized by tissue fibrosis and immune dysregulation of T and B cells (MacDonald et al., 2017). The pathophysiology of cGVHD involves homeostatic abnormalities of thymus-derived regulatory T cells (Tregs, described as CD4⁺ FOXP3⁺IL-2Ra/CD25hiIL-7Ra/CD127low), including low Treg numbers, decreased thymic export, and increased susceptibility to Fas-mediated apoptosis (Matsuoka et al., 2010). In addition, cGVHD is associated with elevated levels of IL-7 and functional IL-2 deficiency (Matsuoka et al., 2013). Treatment of cGVHD with low-dose subcutaneous IL-2 has proven to be safe and increase Treg numbers (Koreth et al., 2011). The role of other cell subpopulations with regulatory functions and their possible usage for the treatment of cGVHD is under investigation (Schneidwind et al., 2013).

Type 1 adaptive regulatory T cells (Tr1s) are FOXP3-negative CD4 T cells that secrete high amounts of IL-10, have immunosuppressive properties, and are found more frequently in AHSC patients with regulatory functions and their possible usage for the treatment of cGVHD is under investigation (Schneidwind et al., 2013).

Herein, we prospectively analyzed Tr1 and Treg frequency, phenotype, and function in the peripheral blood of 14 AHSC patients with active cGVHD, 11 with cGVHD in remission, 12 without cGVHD, and 12 healthy donors. All samples were collected following written informed consent according to the Declaration of Helsinki. This study received the agreement of the local ethics committee. Tr1s were defined as CD3⁺CD4⁺CD45RA⁻CD49b⁺LAG3⁺ and Tregs as CD3⁺CD4⁺CD25hiCD127⁻. Patients’ characteristics are summarized in Supplementary Table S1.

Tr1 frequencies were higher in AHSC patients with active cGVHD than in patients with cGVHD in remission (10% and 2.3%, respectively;

Abbreviations: AHSC, allogeneic hematopoietic stem cell transplantation; cGVHD, chronic graft-versus-host disease; Treg, thymus-derived regulatory T cell; Tr1, type 1 adaptive regulatory T cell

Accepted manuscript published online 16 May 2020; corrected proof published online 13 August 2020 © 2020 The Authors. Published by Elsevier, Inc. on behalf of the Society for Investigative Dermatology.

N Talvard-Balland et al.
Increased Tr1 Cells in Human cGVHD

TO THE EDITOR

Chronic graft-versus-host disease (cGVHD) remains the main cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (AHSC). cGVHD is a multisystem, immune-mediated disease characterized by tissue fibrosis and immune dysregulation of T and B cells (MacDonald et al., 2017). The pathophysiology of cGVHD involves homeostatic abnormalities of thymus-derived regulatory T cells (Tregs, described as CD4⁺ FOXP3⁺IL-2Ra/CD25hiIL-7Ra/CD127low), including low Treg numbers, decreased thymic export, and increased susceptibility to Fas-mediated apoptosis (Matsuoka et al., 2010). In addition, cGVHD is associated with elevated levels of IL-7 and functional IL-2 deficiency (Matsuoka et al., 2013). Treatment of cGVHD with low-dose subcutaneous IL-2 has proven to be safe and increase Treg numbers (Koreth et al., 2011). The role of other cell subpopulations with regulatory functions and their possible usage for the treatment of cGVHD is under investigation (Schneidwind et al., 2013).

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Conversely, tTreg frequencies were lower in patients with active cGVHD than patients with cGVHD in remission (5.5% and 9.5%, respectively; \( P = 0.03 \)), patients without cGVHD (11.2%, \( P = 0.02 \)), and healthy donors (10.9%, \( P = 0.01 \)) (Figure 1b). Therefore, the Tr1:tTreg ratio was reversed in patients with active cGVHD compared with patients with cGVHD in remission (2.0 and 0.4, respectively; \( P < 0.001 \)), patients without cGVHD (0.4, \( P < 0.001 \)), and healthy donors (0.5, \( P < 0.001 \)) (Figure 1b).

Three patients experienced partial remission of cGVHD after low-dose IL-2 treatment and showed an early drop in Tr1 frequencies (from 24% to 13% on average) and increase in tTreg frequencies (from 6% to 13.2% on average) one week after treatment. Two of these patients were further analyzed 12 and 24 months later while they had ongoing remission of cGVHD. Notably, the frequencies of Tr1s and tTregs and the Tr1:tTreg ratio remained at values comparable to those in healthy donors (Figure 1c).

In all study subjects, Tr1s expressed lower levels of IL-2R\(\alpha\) than tTregs (\( P < 0.001 \)) but higher levels than conventional T cells (\( P < 0.001 \)). Conversely, levels of IL-7R\(\alpha\) in Tr1s were higher than in tTregs (\( P < 0.001 \)) but similar to those in conventional T cells (Figure 1d). These results are consistent with the previously observed increased levels of IL-7 in patients with cGVHD (Matsuoka et al., 2013), which could
stimulate Tr1 expansion. They are also in line with the absence of expansion of Tr1s after IL-2 treatment. The mechanisms that lead to Tr1 drop after IL-2 treatment remain to be understood but could involve IL-7 deprivation, as IL-7 levels were shown to decrease in the serum of patients treated with IL-2 (Matsuoka et al., 2013).

Next, we measured IL-10 secretion of CD4 T cells following anti-CD3/CD28 stimulation, as described previously (Gagliani et al., 2013). As shown in Figure 2a and b, patients with active cGVHD had increased frequencies of IL-10–secreting CD4 T cells compared with patients with cGVHD in remission (2.6% vs. 0.5%, respectively; \( P = 0.02 \)), patients without cGVHD (1.0%, \( P = 0.03 \)), and healthy donors (1.4%, \( P = 0.19 \)). The proportions of IL-10–secreting cells correlated with those of Tr1s across all patient groups (\( r = 0.7, P = 0.003 \), Figure 2c), and IL-10–secreting cells were significantly enriched within the CD45RA+CD49b+LAG3+ subset of CD4 T cells (Figure 2d). Finally, we assessed the suppressive capacity of bead-purified IL-10–secreting CD4 T cells on autologous CD4 T-cell proliferation in response to anti-CD3/CD28 stimulation. As shown in Figure 2e, IL-10+ CD4 T cells significantly suppressed autologous CD4 T-cell proliferation as compared with IL-10− CD4 T cells. Altogether, these data indicate that CD3+CD4+CD45RA−CD49b+LAG3+ cells have the hallmark functional properties of Tr1s, and that Tr1s are expanded in active cGVHD.

Several papers reported an impaired recovery of circulating functional CD4+CD25+ Tregs in patients with active cGVHD compared with patients without cGVHD after AHSTC or healthy donors (Alho et al., 2016; Matsuoka et al., 2013, 2010; Zorn et al., 2005). The role of Tr1s in the maintenance of immune tolerance to
non—self-antigens after AHST may be complex (Gagliani et al., 2013; Roncarolo et al., 2011), especially as the functional plasticity of these cell subsets has been demonstrated. In inflammatory conditions, IL-10—producing Tr1s can derive from the differentiation of tTregs (Le Buane et al., 2011). Immunoregulatory mechanisms are generally induced in the context of immune activation and inflammation. We may then speculate that increased Tr1 levels could represent a compensatory mechanism to (i) the inflammatory environment and (ii) the impaired Treg recovery in these patients. Notably, the increase in Tr1 levels may not be sufficient to circumvent ongoing cGVHD. Thus, our data do not preclude that Tr1s may play a beneficial role to prevent cGVHD and be used in an adoptive transfer therapy. Additional in situ studies of Tr1 localization and proportion in skin tissue would improve our understanding of the regulatory properties of Tr1s in the setting of cGVHD. Future in vivo studies should allow us to optimize adoptive transfer strategies to treat this disease.

Data availability statement

Datasets related to this article can be found at https://flowrepository.org/experiments/2596, hosted at Flow Repository.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

The authors thank Christelle Doliger and Nicolas Setterblad (Plateforme Technologique de l’Institut de Recherche Saint-Louis). This work was supported by Société Française de Dermatologie and Association d’Entraide des Greffés de Moëlle Osseuse. Nana Talvard-Balland was supported by the Ligue Nationale contre le Cancer. Adèle de Masson was supported by the Institut National du Cancer/Institut Thématique Multi-Organismes Cancer.

AUTHOR CONTRIBUTIONS

Conceptualization: ADM, JDB, GS, SCZ, HLB; Data Curation: ADM, HLB, SD, VS; Formal Analysis: ADM, NTB, JDB; Investigation: ADM, NTB, JDB; Writing - Original Draft Preparation: ADM, NTB, JDB, GS, SCZ, Writing - Review and Editing: NTB, ASGD, DM, HLB, FC, MR, RPDL, AX, FSDF, NP, SD, VS, AB, MB, AB, SCZ, GS, JDB, ADM

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These authors contributed equally to this work.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.04.018.

REFERENCES


TO THE EDITOR

ACE2 Expression on the Keratinocytes and SARS-CoV-2 Percutaneous Transmission: Are they Related?

Introduction

Human angiotensin-converting enzyme 2 (ACE2) is an important cell receptor for coronavirus entry into cell. ACE2 is ubiquitously expressed among human tissues. Previous studies have shown that ACE2 was highly expressed in the small intestine, testis, heart, and kidney; moderately in the lungs, colon, liver, and skin; and least expressed in the blood, spleen, and bone marrow (Li et al., 2020). The skin, the respiratory tract, and the digestive tract are the borders between the human body and the external environment, and they are vulnerable to the virus infection because of the expression of ACE2.

In the Journal of Investigative Dermatology, Xue et al. (2020) utilized single-cell RNA sequencing to examine the expression of ACE2 in skin tissues and confirmed that the ACE2 was mainly expressed in keratinocytes, which might provide possible routes for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to invade human cells. Their findings provide a novel insight into the potential transmission route through the skin.

A previous study showed that the virus can survive on the surface for several days (van Doremalen et al., 2020), and the viral RNA has been detected in stool and urine samples from patients who were diagnosed with severe acute respiratory syndrome and coronavirus disease 2019 (COVID-19) (Cheung et al., 2020). Besides, rising summer temperatures mean less clothing and an increased risk of skin exposure to the virus in the environment. These may increase the risk of skin contact with the virus and the virus invading the body through skin damage. However, whether there is a percutaneous transmission is still unknown.

COVID-19 skin manifestations were first reported in the study of Guan et al. (2020), in which 2 of 1099 patients diagnosed with COVID-19 showed a skin rash. Another Italian study reported that 20.4% of 88 diagnosed patients developed multiple types of cutaneous manifestations, including erythematous rashes, widespread urticaria, and chickenpox-like vesicles (Recalcati, 2020). However, SARS-CoV-2 PCR results were negative on skin lesion biopsies from diagnosed patients with skin manifestation (Ahouach et al., 2020). Likewise, ACE2 is also abundantly expressed in the testis (Li et al., 2020). What is more? Despite the presence of significant testicular parenchymal damage in male patients with COVID-19, no virus was detected in the testis and semen samples (Holtmann et al., 2020; Pan et al., 2020; Yang et al., 2020). These pieces of evidence suggest that direct infection of the virus is unlikely to be the cause of cutaneous and testicular manifestations in COVID-19; the pathologic changes in skin and testis are probably nonspecific reaction of COVID-19.

Skin barrier and immunity prevent virus infection

The skin barrier defends diverse external disturbances, and the human innate immune system protects the body from the invasion of pathologic microorganism (Handfield et al., 2018). Cutaneous innate immunity is the first line of immune defense, which restricts the virus from dispersing from the skin and activates the adaptive immune response. Meanwhile, intrinsic immunity provides an immediate and direct antiviral defense mediated by host intrinsic restriction factors. In contrast, host intrinsic immunity is immediately triggered and mediates antiviral defense action. To detect the invading viruses, skin cells express a variety of pattern-recognition receptors, such as toll-like receptors and C-type lectin receptors (Kawamura et al., 2014).

In the study of Xiao et al. (2017), they have found that the fomite transmission route of severe acute respiratory syndrome played a negligible role when working alone but had a much more effect when working in combination with the airborne transmission. Furthermore, there is no clinical evidence on skin-to-skin infection so far. These pieces of evidence imply that the coronavirus cannot infect the skin solely through skin contact and that skin immunity may play an important role in protecting our body from the coronavirus infection.

The invasion of coronavirus is not just about ACE2

The S protein on the SARS-CoV-2 surface is bound to the peptidase domain of ACE2, another protein TMPRSS2 primes the S protein–related viral entry, but the amino acid transporter B₀AT1

Abbreviations: ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

Accepted manuscript published online 15 October 2020; corrected proof published online 23 November 2020

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SUPPLEMENTARY MATERIALS AND METHODS
Subjects
This prospective study was conducted at Saint-Louis Hospital from January 2014 to June 2015. The diagnosis and staging of chronic graft-versus-host disease were made at study inclusion using the National Institutes of Health criteria (Lee et al., 2015). All samples were collected following written informed consent. This study received the agreement of the local ethics committee. Patients’ characteristics are shown in Supplementary Table S1.

Flow cytometry
Peripheral blood mononuclear cells were isolated by density gradient centrifugation (LSM, PAA Laboratories) and stained using combinations of the following antibodies: anti-CD3-Krome Orange (clone UCHT1), anti-CD4 TITC (SFCI12T4D11), and anti-CD25 PE-Cy7 (B1.49.9) (all from Beckman Coulter, Brea, CA); anti-CD45RA PerCP-Cy5.5 (HI100, Biolegend, San Diego, CA); anti-CD127 APC-eFluor 780 (eBio-RDR5), anti-CD49b APC (P1H5), and anti-Foxp3 PE (PCH101) (all from eBioscience, San Diego, CA); anti-LAG3 PE or PerCP (polyclonal goat IgG, R&D Systems, Minneapolis, MN); and Fixable Viability Stain 450 (BD Biosciences, San Jose, CA). Fluorescence minus one was used as control. Data were acquired on a BD Canto II cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed using FlowJo Software version X. Thymus-derived regulatory T cells, conventional T cells, and type 1 adaptive regulatory T cells were identified as live CD3+CD4+CD25hiCD127lo cells, CD3+CD4+CD25loCD127hi cells, and CD3+CD4+CD45RA−CD49b−LAG3+ cells, respectively.

IL-10 production
CD4 T cells were isolated from freshly isolated peripheral blood mononuclear cells using the CD4 T-cell isolation kit (Miltenyi Biotec). Cell purity was shown to be >95% by flow cytometry. CD4 T cells were plated at 10⁶ cells/ml in complete RPMI 1640 medium and stimulated for 72 hours at 37°C using plate-bound anti-CD3 antibody (1 μg/ml, OKT3, eBioscience) and soluble anti-CD28 antibody (10 μg/ml, CD28.2, BD Biosciences). After 72 hours, cells were washed and assessed for IL-10 production by flow cytometry or further enriched where indicated, using the IL-10 secretion assay (Miltenyi Biotec) according to provider’s recommendations.

Suppression assay
Purified CD4 T cells were labeled with CellTrace CFSE (0.5 μM, Life Technologies, Carlsbad, CA). IL-10—secreting CD4 T cells (suppressor cells) were enriched as described previously and added to autologous carboxyfluorescein succinimidyl ester—labeled CD4 T cells (responder cells) in a 1:1 ratio. IL-10—negative CD4 T cells were used as negative control. Cells were then stimulated for 5 days as described previously and proliferation of CD4 T cells was assessed by carboxyfluorescein succinimidyl ester dilution.

Statistical analyses
Statistical analyses were performed using Prism (GraphPad) for Windows. Medians were compared between two groups using nonparametric Mann-Whitney U tests. P-value < 0.05 was considered significant.

SUPPLEMENTARY REFERENCE
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<th>Characteristic</th>
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Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; cGVHD, chronic graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; MM, multiple myeloma; MMF, mycophenolate mofetil; MPS, myeloproliferative syndrome; MTX, methotrexate; MUD, matched unrelated donor; NA, not applicable; NHL, non-Hodgkin lymphoma; RIC, reduced-intensity conditioning.