Absence of γ-Chain in Keratinocytes Alters Chemokine Secretion, Resulting in Reduced Immune Cell Recruitment

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Loss-of-function mutations in the common gamma (γc) chain cytokine receptor subunit give rise to severe combined immunodeficiency characterized by lack of T and natural killer cells and infant death from infection. Hematopoietic stem cell transplantation or gene therapy offer a cure, but despite successful replacement of lymphoid immune lineages, a long-term risk of severe cutaneous human papilloma virus infections persists, possibly related to persistent γc-deficiency in other cell types. Here we show that keratinocytes, the only cell type directly infected by human papilloma virus, express functional γc and its co-receptors. After stimulation with the γc-ligand IL-15, γc-deficient keratinocytes show significantly impaired secretion of specific chemokines including CXCL1, CXCL8, and CCL20, resulting in reduced chemotaxis of dendritic cells and CD4⁺ T cells. Furthermore, γc-deficient keratinocytes also exhibit defective induction of T-cell chemotaxis in a model of stable human papilloma virus-18 infection. These findings suggest that persistent γc-deficiency in keratinocytes alters immune cell recruitment to the skin, which may contribute to the development and persistence of warts in this condition and would require different treatment approaches.

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INTRODUCTION

The common gamma chain (γc) is the shared signaling subunit for the IL cytokine receptors IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Inherited deficiency of γc results in X-linked severe combined immunodeficiency (X-SCID), characterized by absence of T- and natural killer cells and opportunistic infections (Buckley, 2004; Kovanen and Leonard, 2004). The natural history is death at a very young age, but hematopoietic stem cell transplantation (HSCT) or gene therapy are effective treatments that confer protection from life-threatening infections (Antoine et al., 2003; Gaspar et al., 2004a; Gaspar et al., 2004b; Gaspar et al., 2013). However, despite excellent long-term survival after curative therapy, a persistent susceptibility to human papillomavirus (HPV) infections is well described that does not appear overall to relate to the conditions of transplantation or immune reconstitution (Gaspar et al., 2004a; Laffort et al., 2004). In several independent studies, up to 64% of treated children developed warts, with lesion onset 4 to 19 years after transplantation (Abd Hamid et al., 2017; Gaspar et al., 2004a; Kamili et al., 2014; Laffort et al., 2004).

Original genotype is the main risk factor, suggesting that γc-cytokine signaling is important for host defense against HPV. In support of this, similar HPV infections are seen in patients with SCID caused by deficiency of JAK3, the immediate downstream signaling partner for γc, and to a lesser extent in patients with a defect of IL-7Rα, which selectively abrogates γc-signaling after IL-7R ligation (Gaspar et al., 2004a; Horev et al., 2015; Neven et al., 2009). The observation that warts are milder in IL-7Rα deficiency suggests that other γc-cytokines in addition to IL-7 are likely to play a protective role against HPV.

The main HPV types found in lesions from X-SCID patients were from the α4 clade (e.g., HPV2 and HPV57), which cause cutaneous warts in the general population, and the β1 clade (e.g., HPV5, HPV14, and HPV36), which usually produce lesions only in immunodeficient patients (Laffort et al., 2004). Similar susceptibility to papillomavirus infection has been described in a canine model of γc-deficiency, with severe chronic cutaneous lesions observed in most X-SCID dogs after HSCT despite good immune reconstitution. A high percentage (67%) of dogs with persistent canine papillomavirus infections developed invasive squamous cell carcinoma 3½ years after transplantation (Goldschmidt et al., 2006), highlighting a potential long-term cancer risk for affected X-SCID patients.

Very persistent warts are uncommon in immunocompetent hosts, where most cutaneous HPV lesions spontaneously
regress within 1–5 years (Bruggink et al., 2013; Williams et al., 1993). Although the precise mechanisms of clearance remain to be clarified, evidence of immune activation is associated with lesion regression, including keratinocyte expression of the chemoattractant CCL20 (Mip-3α), the presence of antigen-presenting Langerhans cells in the epidermis, and recruitment of CD4⁺ and CD8⁺ T cells to the dermis (Iwatsuki et al., 1986; Nakayama et al., 2011). In contrast, reduced Langerhans cell numbers in the epidermis and increased regulatory T cells in the dermis are seen in nonregressing cutaneous lesions, suggesting that an immune-suppressed local environment favors persistent warts (Leong et al., 2010; Sperling et al., 2012). The importance of CD4⁺ and CD8⁺ T cells for resolution of established HPV infection is further highlighted by studies of human and animal mucosal HPV infections (reviewed in Hibma, 2012 and Stanley, 2012), where lesion regression is associated with an influx of both subsets, with a prominence of CD4⁺ T cells (Monnier-Benoit et al., 2006; Peng et al., 2007; Tong et al., 2015).

Despite apparently full T-cell correction, patients after HSCT for γc-deficiency present with severe cutaneous infections, mainly located on hands and feet, that are difficult to treat and lead to substantially reduced quality of life. Although it remains feasible that HPV susceptibility in γc-deficient patients is caused by specific defects of hematopoietic immune reconstitution (e.g., in myeloid lineage dermal dendritic cells [DCs] and Langerhans cells), it is also possible that an intrinsic defect in keratinocytes that are not replaced by HSCT is responsible. This is an attractive hypothesis, because keratinocytes are the only cell type directly infected with HPV and have an important role in skin immunity through secretion of a variety of chemokines and cytokines, such as CXCL10 (IP-10), CCL5 (RANTES), and CCL20, that recruit hematopoietic immune cells (Grone, 2002; Tokura et al., 2008; Uchi et al., 2000).

Here, we show that keratinocytes express functional γc and its co-receptors. We also show that secretion of chemokines by keratinocytes, after cytokine stimulation or when harboring HPV genomes, is reduced in γc-deficient keratinocytes and that this leads to changes in lymphoid and myeloid migration. Our data suggest that altered immune cell recruitment as a result of intrinsic keratinocyte dysfunction may contribute to the retained susceptibility of X-SCID patients to HPV-associated disease after HSCT.

RESULTS AND DISCUSSION

Keratinocytes express functional γc and co-receptors

Keratinocyte expressions of γc and some of its co-receptors have been previously reported (Distler et al., 2005; Hong et al., 2015; Kagami et al., 2005; Raingeaud and Pierre, 2005; Zhang et al., 2008). To confirm and extend published findings, we measured expression of γc and all co-receptors in the normal immortalized keratinocytes (NIKS) keratinocyte cell line and primary keratinocytes. Both NIKS and primary keratinocytes expressed γc-mRNA (Figure 1a) and expressed protein at a comparable level to an EDR7 T-cell line engineered to overexpress γc and to primary CD8⁺ T cells (Figure 1b). These data suggest that keratinocytes express γc in physiologically relevant quantities.

In addition, mRNA and protein were detected for the co-receptors IL-2Rβ, IL-4R, IL-7Rα, IL-9R, IL-15Rα, and IL-21R (Figure 1a, 1c, and 1d), whereas protein and mRNA using two sets of primers was absent for IL-2Rα (Figure 1a, 1d), despite detectable expression of IL-2Rβ (Figure 1a, 1c). This indicates that keratinocytes express a specific subset of γc-expressing cytokine receptors that does not include the heterotrimeric, high-affinity IL-2R critical for T-cell survival and proliferation (Sadlack et al., 1995; Wang et al., 2005) but is likely to include the low-affinity IL-2R comprising IL-2Rβ and γc. Presence of a range of γc-containing receptors suggests that their cytokine ligands play an important role in keratinocyte biology. To test whether keratinocyte γc and co-receptors are functional, we measured phosphorylation of the downstream signaling molecules signal transducer and activator of transcription 5 (STAT5) and protein kinase B, after cytokine stimulation. Stimulation with IL-7, IL-9, IL-15, and IL-21 resulted in increased phosphorylation of signal transducer and activator of transcription 5 and phosphorylated protein kinase B (Figure 1e, Supplementary Figure S1 online), confirming intact signaling of multiple γc-receptors in keratinocytes.

To further test the role of γc for keratinocyte function, we generated a cell line using short hairpin RNA technology in which γc-mRNA was reduced by more than 70% (Figure 2a) and γc-protein expression was completely abrogated at the cell surface (Figure 2b). Functional knockdown, resulting in a failure to up-regulate protein kinase B phosphorylation after IL-15 stimulation, was confirmed (Figure 2c).

γc-deficient keratinocytes display impaired chemokine secretion

In hematopoietic lineages, γc-containing receptors regulate numerous cell functions, including secretion of cytokines and chemokines that can act in an autocrine or paracrine manner (Chenoweth et al., 2012; Kotlarz et al., 2013). In the skin, chemokine secretion by both hematopoietic-derived cells and keratinocytes plays a key role in cutaneous immune surveillance, recruiting a variety of immune cell subsets to sites of infection or inflammation (Tan et al., 2015). Although the early immune events in cutaneous HPV infection are poorly understood, micro-wounding of the skin is thought to be a prerequisite for HPV entry and access to basal layers of the skin. Micro-wounding induces keratinocyte release of IL-15 (Kennedy-Crispin et al., 2012), which is distinct among γc-receptor ligands in its ability to be cross-presented to neighboring cells through direct cell-to-cell interaction (Dubois et al., 2002; Olsen et al., 2007). Thus, keratinocyte release of IL-15 is likely to affect neighboring keratinocytes to activate specific cell functions. Therefore, we tested whether IL-15 stimulation was sufficient to induce keratinocyte production of chemokines that may be important for recruitment of other immune cells to the site of HPV infection.

Using semiquantitative array technology to screen a broad range of cytokines and chemokines, we observed that control keratinocytes produced a subset of chemokines that were up-regulated after IL-15 stimulation (see Supplementary Table S1 online). To verify these hits and to specifically test whether keratinocyte γc-deficiency altered chemokine release in response to IL-15, we utilized a quantitative Luminex bead
**Figure 1. Keratinocytes express functional γc and its co-receptors.** (a) RNA of ED-7R cell lines, NIKS, and primary keratinocytes (KCs) was isolated, and reverse transcription PCR was performed with primers specific for γc, its co-receptors, and GAPDH. (b) KC, NIKS, CD8⁺ T cells, and ED-7R cell lines were stained with anti-γc antibody and analyzed by flow cytometry. (c) NIKS and KCs were lysed and analyzed for their expression of IL-7Rα and IL-2Rβ by Western blot. (d) NIKS were stained with antibodies for γc–co-receptors and analyzed by flow cytometry. (e) NIKS were cultured in serum-free medium overnight, stimulated with 100 ng/ml of the indicated cytokines for 10 minutes, and analyzed by immunoblot for the expression of pAKT. γc, common gamma chain; APC, allophycocyanin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KC, keratinocyte; NIKS, normal immortalized keratinocyte cell line; pAKT, phosphorylated protein kinase B.
assay (R&D Systems, Minneapolis, MN) to analyze this subset of seemingly IL-15-responsive chemokines. Additionally, we included CCL20, which was not part of the array screen but which is known to be released by keratinocytes and modified in HPV infection and has been implied to be γc-dependent in other cell types (Kotlarz et al., 2013; Sperling et al., 2012; Tokura et al., 2008). Using the Luminex assay, stimulation with IL-15 was shown to induce a significant increase (1.5- to 2-fold) in the secretion of CXCL1 (Gro-a), CXCL8 (IL-8), and CCL20 from control keratinocytes (Figure 3a). CCL5, IL-1α, and CXCL10, which had been detected at lower levels on the initial semiquantitative array, were detectable but not confirmed to be up-regulated by IL-15 (Figure 3d–f), and tumor necrosis factor-α and GM-CSF remained below the limit of detection (data not shown). Although γc-deficient keratinocytes produced similar basal levels of these chemokines (Figure 3a–f), up-regulation of CXCL8, CCL20, and CXCL1 after IL-15 stimulation was completely abolished, suggesting that γc-deficiency impairs chemokine release from keratinocytes after stimulation with IL-15.

**Impaired immune cell recruitment in response to chemokine secretion by γc-deficient cells**

To test whether the reduced levels of CXCL8, CCL20, and CXCL1 secretion by γc-deficient keratinocytes were sufficient to affect immune cell recruitment, we tested migration of primary human cells in vitro. Because the chemokines identified are best known for their ability to recruit neutrophils, we first analyzed migration of neutrophils purified from peripheral blood (Figure 4a) to a cocktail of CXCL8, CCL20, and CXCL1 combined at the concentrations detected after IL-15 stimulation of control keratinocytes. A significant increase in both average migration speed and directionality (defined in Materials and Methods) was seen in response to the chemokine cocktail (Figure 4b). Analysis of data from multiple donors showed that responses to the chemokine cocktail were comparable to the positive control N-formylmethionyl-leucyl-phenylalanine, a bacterial peptide that acts as a potent chemotactic factor for neutrophils (Figure 4d, 4e). Together, the data show that activation of keratinocytes by IL-15 is sufficient to induce recruitment of neutrophils. Because neutrophils are not thought to be associated with protection against HPV, we tested the response of DCs and CD4+ T cells that are considered to be more relevant during HPV infection (Amador-Molina et al., 2013; Handisurya et al., 2013, 2014; Wang et al., 2015). As seen with neutrophils, the average speed of DC migration in two-dimensional chambers (Figure 5a) was significantly increased using the chemokine cocktail at the concentrations released by IL-15—stimulated control keratinocytes (“scr + IL-15” in Figure 5b–d). In
contrast, lower chemokine concentrations, equivalent to levels secreted from γc-deficient keratinocytes after IL-15 stimulation (“KD + IL-15” in Figure 5), induced no increase in migration speed. Using Transwells (Corning Incorporated, Lowell, MA), CD4+ T-cell migration was also significantly increased toward the higher concentration of chemokines compared with the lower chemokine concentration (Figure 5d, Supplementary Figure S2 online). The increase in migration induced by the higher concentration of chemokines was similar to that observed with CX3CL1 (Figure 5e), a known potent chemoattractant for T cells and monocytes (Bazan et al., 1997). Together, these findings suggest that reduced chemokine secretion after wounding of γc-deficient skin may impair immune cell recruitment, leading to enhanced susceptibility to basal layer keratinocyte infection after HPV entry.

γc-deficient keratinocytes stably harboring HPV18 fail to induce CD4+ T-cell migration

Our initial results suggest that changes in host immunity could increase susceptibility of γc-deficient basal layer keratinocytes to HPV infection. This, however, does not explain why established wart lesions fail to regress in γc-deficiency.
despite restoration of T-cell immunity that is considered the major factor for immunological control of established HPV infection (Coleman et al., 1994; Handisurya et al., 2014; Knowles et al., 1996; Nicholls et al., 2001; Uberoi et al., 2016; Wilgenburg et al., 2005).

Therefore, we set out to test whether defects in T-cell recruitment are seen with γc-deficient keratinocytes stably transfected with HPV18 to mimic established infection (Figure 6a, Supplementary Figure S3 online). HPV18 was chosen because it enables stable transfection of keratinocytes, whereas as low-risk α-types and β-types do not persist in cultured keratinocytes (Doorbar, 2016). In Transwell assays, supernatants from control HPV18-transfected NIKS induced significantly greater CD4+ T-cell migration than supernatants from untransfected cells. This effect was completely abolished when supernatant from γc-deficient keratinocytes was used (Figure 6b), implying that CD4+ T-cell recruitment to sites of established HPV infection could be impaired in γc-deficient patients. In contrast, an increase in CD8+ T-cell migration was observed only with supernatants from γc-deficient cells (Figure 6c), in keeping with dysregulated chemokine secretion by γc-deficient keratinocytes. Our data suggest that, in γc-deficient patients, altered balance between CD4+ and CD8+ T-cell recruitment to skin sites could affect the efficacy of the induced immune response.

Figure 4. Migration of neutrophils. Neutrophils were isolated from whole blood and used for migration experiments using Dunn chambers, with the cells imaged every minute for 1 hour. (a) Cytospin of neutrophils before plating them on coverslips for migration. Scale bar = 25 μm. (b, c) Quantification for migration of one representative donor, where each dot represents one tracked cell. (d, e) Quantification for multiple migration experiments, where each dot represents the mean value for all cells analyzed from one donor. (b–e) “None” contains no chemoattractant; “chemokine cocktail” represents a cocktail of 10 ng/ml CXCL1, 3 ng/ml CXCL8, and 1 ng/ml CCL20. fMLP at 100 nmol/L was used as a positive control. Mean ± standard error of the mean; *P < 0.05, ***P < 0.001; n = 4. Shown are (b, d) migratory speed and (c, d) directionality. fMLP, N-formylmethionyl-leucyl-phenylalanine; ns, not significant.
Together, our results show a role for γc-containing receptors in keratinocyte biology, in particular for chemokine secretion and induction of immune cell recruitment. Our in vitro experiments indicate that γc-deficient keratinocytes are unable to efficiently recruit lymphoid and myeloid immune cells in response to specific stimulation. Of particular relevance for HPV infection, γc-deficient keratinocytes were defective in recruiting CD4+ T cells and DCs after IL-15 stimulation, a condition that mimics micro-wounding during early infection. We propose that this could create a favorable environment for viral infection and subsequent formation of wart lesions by impairing the early immune response. In addition, the ability to induce CD4+ T-cell recruitment, known to be important for wart regression in animal models and humans (Coleman et al., 1994; Handisurya et al., 2014; Nicholls et al., 2001; Peng et al., 2007; Wilgenburg et al., 2005), was impaired in response to established infection in γc-deficient keratinocytes, which could be a factor favoring persistence of warts. We propose a model in which γc-deficiency limited to keratinocytes alters the host response both at the onset and in the persistence of HPV infection (see Supplementary Figure S4 online). Further work is required to show the importance of our findings for the susceptibility to HPV infection in a γc-deficient context.
in vivo and to investigate the contribution of other cell types that also express receptors for CXCL1 and CXCL8 (Inngjerdingen et al., 2001). In particular, analyzing the role of natural killer cells would be of interest because poor natural killer cell engraftment was associated with HPV infection in a small cohort of X-SCID patients (Kamili et al., 2014).

Understanding the role of keratinocytes in persistent HPV in X-SCID is an important goal to inform the design of improved treatment for this intractable complication, including vaccinations that could be administered prophylactically to provide protection against cutaneous HPV types.

**MATERIALS AND METHODS**

Information about the isolation of primary keratinocytes, immunoblot, flow cytometry, reverse transcription PCR, analysis of phosphoproteins, and transfection of NIKS with HPV18 vector can be found in the Supplementary Materials and Methods online.

**Cell lines**

ED-7R cells are a human T-cell line derived from a patient with adult T-cell leukemia. They express IL-2Rα and IL-2Rβ but they lack γc (Arima et al., 1992). The ED-7R + γc line was genetically modified to express the wild-type γc (Ishii et al., 1994). NIKS, a spontaneously immortalized keratinocyte cell line derived from neonatal human foreskin (Allen-Hoffmann et al., 2000), was kindly provided by John Doorbar, Department of Pathology, University of Cambridge.

**Lentiviral preparation and transductions of NIKS**

pGIPZ vectors carrying the short hairpin RNA against γc (TCAG-TAACAAGATCCTCTA) or the scrambled control (TGAACT-CATTITTTCTGCTC) sequences as well as a puromycin resistance cassette and turbo-GFP fluorescent marker were obtained from University College London’s Open Biosystems (London, UK). The lentiviruses containing the short hairpin RNAs were produced, stored, and concentration quantified as described previously using the VSVG packing plasmid (Metelo et al., 2011). NIKS were transduced at a multiplicity of infection of 10. 72 hours after transduction, cells were treated with 2 μg/ml puromycin for a week to select positively transduced cells. The percentage of GFP-positive cells was confirmed by flow cytometry and was greater than 95%.

**Cytokine array and Luminex assays**

NIKS were serum-depleted for 6 hours and then stimulated with 100 ng/ml IL-15 for 24 hours in serum-free E-medium at 37 °C or left...
unstimulated. Supernatants were collected and used for Proteome Profiler Assay or Luminex Bead Assay (R&D Systems, Minneapolis, MN), carried out according to the manufacturer’s instructions.

**Isolating immune cells from whole blood**

Blood samples were obtained from healthy volunteers with authorization from the National Research Ethics Services, Westminster (09/H0715/30), and with informed written consent. Neutrophils were isolated directly from peripheral blood using the Neutrophil Isolation Kit/MACSxpress (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral blood mononuclear cells were collected after centrifugation of whole blood (diluted 1:1 in phosphate buffered saline) over Ficoll (GE Healthcare, Little Chalfont, UK). CD14+ cells and CD4+ and CD8+ T cells were isolated from peripheral blood mononuclear cells using CD14 Microbeads and CD4+ and CD8+ T-cell isolation kits, respectively (Miltenyi Biotec) following the manufacturer’s instructions. Purity of CD8+ and CD4+ T cells was analyzed by flow cytometry and was typically greater than 95%. DCs were generated using our established laboratory protocols. Briefly, CD14+ cells were cultured for 6 days in RPMI supplemented with 100 ng/ml GM-CSF and 25 ng/ml IL-4, which typically yields greater than 95% CD11c+ cells with an immature phenotype (Burns et al., 2004; Metelo et al., 2011) and a characteristic dendritic appearance after adhesion to substrate (Figure 5a).

**Migration experiments using neutrophils**

For migration experiments with neutrophils, Dunn chambers (Hawksley Medical & Laboratory Equipment, Lancing, UK) were used. Migration was carried out as previously described (Record et al., 2015). In brief, 1 x 10^6 neutrophils were seeded onto fibrinogen-coated coverslips. These coverslips were placed on a Dunn chamber (Zicha et al., 1997), where the inner ring was filled with medium only and the outer ring was filled with chemoattractant-containing agarose so that a chemokine gradient formed across the bridge. The bridge was imaged by time-lapse microscopy for 1 hour at 37 °C with a photo taken every minute. Analysis was carried out tracking individual cells using the software Icy (de Chaumont et al., 2012), which enables automatic tracking. For each condition in each experiment, 25–40 cells were analyzed for their migratory speed and the directionality of their movement, defined as the ratio of the direct distance between the start and end position of a cell divided by the total distance of the cell path.

**Migration experiments using DCs**

Migration experiments with DCs were carried out using the µ-Slide Chemotaxis 3D/ Collagen IV coated slides (Ibidi, Martinsried, Germany) according to manufacturer’s instructions. In brief, 6 µl of a 3 x 10^5 cells/ml cell suspension was seeded per slide and cultured at 37 °C at 5% CO2 for 4 hours to allow cells to adhere to the collagen substrate (Burns et al., 2004). The chemoattractant was filled into one of the outer chambers so that a gradient formed along the bridge of the migration chamber where the cells adhered, and then the chamber was sealed to ensure CO2 buffering. The bridge of the slide was imaged for 4 hours at 37 °C with pictures taken every 5 minutes. Migration of cells over the bridge was analyzed using the Manual Tracking plugin from ImageJ (National Institutes of Health, Bethesda, MD) for approximately 10 cells per condition using the same method as previously described (Record et al., 2015).

**Migration experiments using T cells**

Migration experiments using T cells freshly isolated from donor blood were carried out using Transwells with a 5.0-µm polycarbonate membrane in a 24-well Transwell plate. Chemokines diluted in culture medium at concentrations indicated in the figure legends or keratinocyte supernatants were added to the 24-well plate chamber. 1 to 2 x 10^5 T cells were placed into the overlying Transwell. After culturing for 37 °C for 2 hours, Transwells were removed, the bottom of the Transwell was incubated in trypsin to remove cells attached to the underside, and these cells were pooled with the cells that had migrated into chamber of the 24-well plate. The total number of cells collected after migration was determined using a live cell dye (CyQUANT NF cell proliferation assay kit, Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s instructions to stain the T cells at the end of the migration assay. Fluorescence was read using an excitation wave length of 490 nm and an emission detection of 540 nm. Fold-migration was calculated using relative fluorescence levels.

**Statistical analysis**

All graphs were made and statistical analysis carried out using GraphPad Prism (GraphPad, La Jolla, CA). Data were analyzed using statistical tests according to datasets: Kruskal-Wallis test for multiple groups and Mann-Whitney test to compare two groups.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.05.024.

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