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An Epidermotypic Model of Interface Dermatitis Reveals Individual Functions of Fas Ligand and Gamma Interferon in Hypergranulosis, Cytoid Body Formation, and Gene Expression

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Abstract

Analysis of complex cutaneous reactions using animal models allows for the identification of essential or modulatory participants, i.e. cyto- and chemokines, or adhesion molecules. However, complex whole animal modeling is bound to obscure some specific contributions of individual players. Mouse models suggest that expression of Fas ligand (FasL) by donor T cells is essential for the cutaneous acute graft-versus-host reaction (aGvHR), a major complication following allogeneic hematopoietic stem cell transplantation. The role of FasL/Fas in human cutaneous GvHR is not known. To understand the mechanisms of cytotoxicity and inflammation in human cutaneous GvHR, we developed an organotypic model using reconstructed human epidermis (RHE) that was exposed to FasL, gamma-interferon (IFN γ), or both. The model recapitulated key histological hallmarks of cutaneous aGvHR, including interface dermatitis, appearance of cytoid bodies, hypergranulosis, and expression of ICAM-1. Cytoid body formation and expression of ICAM-1 was attributable entirely to IFN γ , whereas hypergranulosis was triggered by FasL. Both FasL and IFN γ triggered vacuolar degeneration of keratinocytes. The validity of the RHE model of GvHR was demonstrated by histological correlation with biopsied skin from aGvHD patients. FasL and IFN γ each elicited potent and specific pro-inflammatory genomic responses in RHE. Inhibition of caspase activity dramatically augmented the FasL-induced pro-inflammatory responses, suggesting an “apoptosis-versus-inflammation” antagonism in cutaneous aGvHR and other lichenoid dermatoses.

Keywords

Apoptosis; inflammation; gene expression; death ligand; death receptor

INTRODUCTION

The lichenoid and interface dermatoses comprise a group of ~30 etiologically diverse inflammatory skin diseases, exemplified by lichen planus, erythema multiforme, toxic

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epidermal necrolysis/Stevens-Johnson syndrome, lupus erythematosus, and acute graft-versus-host disease [1]. Histologically, the term ‘lichenoid’ refers to the presence of a band-like lymphohistiocytic infiltrate in the upper dermis, hugging and often obscuring the epidermodermal interface [1]. Lichen planus is the prototypical lichenoid dermatose. Interface dermatitis refers to the presence of basal cell vacuolization (hydropic degeneration), often accompanied by single cell keratinocyte apoptosis [1]. Most lichenoid infiltrates are accompanied by interface change; however, many dermatoses characterized primarily by interface change such as lupus erythematosus and poikiloderma, do not show a lichenoid infiltrate [1].

Allogeneic hematopoietic stem cell transplantation (HSCT) is used to treat hematological malignancies and non-malignant disorders of the blood, such as sickle-cell anemia and aplastic anemia [2]. Mature CD4+ and CD8+ T cells contained within the graft (donor bone marrow or peripheral-blood stem cells) promote hematopoietic engraftment, reconstitute T-cell immunity, and mediate the beneficial graft-versus-leukemia (GVL) effect. Unfortunately, both of these donor T-cell subsets also cause graft-versus-host disease (GvHD), the broad attack against host tissues by donor T cells [2]. GvHD is the major barrier to successful allogeneic HSCT; most patients who develop the severe manifestations of GvHD succumb to it or to complications of its treatment [3]. Acute GvHD (aGvHD) occurs within the first 100 days after transplantation and affects the skin, liver, and gastrointestinal tract [2-5].

aGvHD proceeds in three phases [2-6]. The first involves damage to host tissues by inflammation from the preparative regimen of chemotherapy and/or radiotherapy. In the second phase, recipient and donor antigen-presenting cells (APCs), as well as inflammatory cytokines, trigger the activation of donor-derived T cells, which expand and differentiate into effector cells. In the third (effector) phase, activated donor T cells mediate cytotoxicity against target host cells through Fas-Fas ligand interactions (see below), perforin-granzyme B, and TNF α . Activated T cells in aGvHD patients produce large amounts of IFN γ [7,8]. IFN γ is involved in several aspects of aGvHD pathophysiology by: (i) upregulating adhesion molecules, chemokines, and major histocompatibility (MHC) class II antigens, thus facilitating antigen presentation and effector T cell recruitment; (ii) mediating the development of pathologic processes in the gastrointestinal tract and skin during GvHD; (iii) upregulating Fas expression in target tissues; (iv) co-operating with bacterial LPS to trigger the production of proinflammatory cytokines and NO from macrophages; and (v) regulating the death of activated donor T cells by enhancing Fas-mediated apoptosis, thus regulating GVHD [9].

Cutaneous aGvHD presents initially an erythematous macular rash, usually on the palms, soles and face. In a few days, the rash becomes deeper in color, and spreads to trunk and limbs. It may progress to erythroderma and is usually followed by desquamation [10]. Histologically [1,10], advanced aGvHD lesions display basal vacuolation (hydropic or liquefactive degeneration), shrunken, apoptotic, keratinocytes as well as eosinophilic, amorphous, keratinocyte remnants (known as cytoid bodies or bodies of Civatte). The apoptotic keratinocytes are often accompanied by “satellite” lymphocytes and this lymphocyte-associated apoptosis is known as “satellite cell necrosis”. Presence of hypergranulosis and the absence of a thickened epidermal basement membrane help in making the differential diagnosis of aGvHD versus several other conditions such as lupus erythematosus and dermatomyositis. The most fulminant lesions of aGvHD show numerous necrotic keratinocytes with frequent subepidermal clefting. This histology resembles erythema multiforme / toxic epidermal necrolysis. However, in erythema multiforme the cornified and granular layer are usually normal, rather than orthoand / or parakeratotic as is seen in aGvHD [10].

The pathophysiology of cutaneous aGvHD has been investigated in murine models. The roles of the two major T-cell cytolytic mechanisms, the Fas/FasL pathway (see below) and the perforin/granzyme pathway, in aGvHD have been investigated with gene-deficient T cells in models of lethal irradiation and bone-marrow rescue. In major histocompatibility antigen (MHC)-matched, multiple minor histocompatibility antigen (miHA)-mismatched transplants, T cells from FasL-deficient mice (gld mice) induced lethal aGvHD; however, cutaneous and hepatic aGvHD was absent [11]. Braun et al. [12] reported a significantly delayed mortality in the recipients of FasL-defective T cells in a MHC-mismatched spleen cell transfer model. Hattori et al. [13] confirmed the role of Fas/FasL in cutaneous aGvHD using FasL-blocking antibodies. All these studies identified the Fas/FasL system as essential for the skin damage in aGvHD, at least in mouse models of the disease. Perforin-deficient T cells induced aGvHD with delayed kinetics, but histological features of cutaneous and hepatic GvHD were similar to those in recipients of wild-type T cells [11].

Whereas the involvement of immunocytes, such as T cells and APCs, in the pathophysiology of aGvHD has been extensively researched ([2-6,14] and references therein), the roles of targeted epithelia within the affected organs (skin, gut, and liver) are still not well understood. The concept of the “vicious cycle” of aGvHD, that is, that the damage to target tissues caused by activated T cells activates more T cells, causing more tissue damage, which activates more T cells, etc., has been appreciated in the literature [3,4]. What is lacking, however, is a detailed mechanistic understanding of the complex interplay between apoptosis and inflammatory responses in host epithelial target cell that drives the vicious cycle. Our recent work has demonstrated that FasL elicits a potent pro-inflammatory response in keratinocytes grown in reconstructed human epidermis [15,16]. This response involves the production of a specific set of cytokines and chemokines, many of which have been implicated in aGvHD. The spectrum of FasL-induced inflammatory genes is modulated by IFN γ . Inhibition of caspase activity dramatically augments the FasL-induced pro-inflammatory response of keratinocytes, demonstrating for the first time a potential “apoptosis-versus-inflammation” antagonism in cutaneous aGvHD. We aim to understand how host keratinocytes propagate the vicious inflammatory cycle in cutaneous aGvHD by elucidating the mechanisms that control “apoptosis-versus-inflammation” decision-making in the lesional epidermis. Understanding these mechanisms could facilitate the development of keratinocyte-targeted strategies to prevent and treat aGvHD with reduced need for systemic immunosuppressants. This, in turn, will allow for better graft-versus-tumor effects and reduce immunosuppression-related infections and cancer relapses.

Currently, GvHD can be studied using either patient-derived material (tissue biopsies or blood) or animal models (murine or non-human primate). A human organotypic model that reproduces faithfully various aspects of cutaneous GvHR in situ will help the elucidation of the specific roles of individual cytokines implicated in the pathogenesis of cutaneous aGvHD (such as FasL and IFN γ , as demonstrated below). An epidermotypic model of aGvHR will also facilitate the understanding of the specific roles and responses of keratinocytes in aGvHD, in the absence of interference from other epidermal cell types (e.g. melanocytes and LCs). Lastly, a human organotypic model of aGvHR would also be useful to test the relevance to human patients of mouse model-generated hypotheses. This is important in view of the fact that, sometimes, mouse genes do not play the same roles as the corresponding human orthologs. For instance, genetic ablation of the mouse Tyk2 kinase, a member of the Jak family of kinases, revealed the non-essential role of Tyk2 for the murine innate and acquired immunity [17,18]. However, a homozygous Tyk2 null-mutation in a human patient was associated with severe hyper-IgE syndrome, including atopic dermatitis, and inability to mount efficient defenses against viral, fungal, and mycobacterial infections [19], suggesting a rather essential role of Tyk2 in human immunity.

MATERIALS AND METHODS

Patient material and antibodies

Cutaneous biopsies from 6 acute GvHD (aGvHD) cases and 3 three normal controls (all anonymous to the authors and after obtaining written informed consent) were obtained from the Department of Dermatology of Oregon Health & Science University. All cases of aGvHD were diagnosed by Dr. Clifton R. White, Jr., M.D., director of dermatopathology at OHSU. Five μ m thick paraffin sections were processed for immunohistochemical analysis following standard procedures. Antigen retrieval, unless specified otherwise below for specific antibodies, was performed by microwave heating in 10 mM Na-citrate buffer, pH 6.0. The following primary antibodies were used: anti-active caspase 3 (Cell Signaling Technology #9961; antigen retrieval – pressure cooking in Na-citrate buffer), anti-FasL (Santa Cruz Biotechnology, N-20, #sc-834), anti-T-bet (Santa Cruz Biotechnology, #sc-21749), anti-ICAM1 (Santa Cruz Biotechnology, #sc-107), anti-Filaggrin (Novocastra, 15C10; antigen retrieval in 1 mM EDTA, pH 8.0), anti-CD4 (Novocastra; antigen retrieval in 1 mM EDTA, pH 8.0), anti-CD8 (Novocastra; antigen retrieval in 1 mM EDTA, pH 8.0 and pressure cooking), and anti-CCR7 (R&D Systems, MAB197).

Reconstructed human epidermis (RHE)

Primary human epidermal keratinocytes (neonatal) (HEKn) were propagated as described [20,21]. For RHE, we employed the method of Poumay *et al.* [22], with modifications as described [15,16]. RHEs display normal epidermal morphology including a pronounced stratum corneum and proper spatial distribution of epidermal marker proteins, such as keratin 5, keratin 1, and filaggrin [[15,16] and Fig. 2E for filaggrin)]. RHEs were fixed in freshly-made 4% *p*-formaldehyde, embedded in paraffin, and, after sectioning, processed for immunohistochemistry following the specific protocols recommended by the respective manufacturers for each antibody.

Chemicals and biologicals

All commonly used chemicals were from Sigma Chemical Company. The pan-caspase inhibitor zVADfmk was from Calbiochem (San Diego, CA). Fc:FasL has been described previously [15,23]. Gamma-Interferon was from R&D Systems.

RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

These techniques were performed as previously described [15,16].

RESULTS AND DISCUSSION

Interface dermatitis, lichenoid tissue reaction, and expression of FasL and IFN γ in cutaneous acute graft-versus-host disease

All cutaneous aGvHD biopsies (n=6) displayed ID manifested by basal vacuolation with shrunken, apoptotic, keratinocytes, cytooid bodies, and pronounced hypergranulosis, a differential diagnostic hallmark of aGvHD [10] (Fig. 1, panels D, E, and F). In the areas of interface dermatitis, activation of caspase-3 was detectable by immunohistochemistry (Fig. 1, panels I and J). The areas of hypergranulosis expressed strongly filaggrin (FLG), a major protein component of the keratohyalin granules [24] (Fig. 1, panels W and X). Consistent with the mouse models demonstrating the essential role of FasL in the skin manifestations of aGvHD [11-13], all human aGvHD (but not control) biopsies demonstrated infiltration with FasL⁺ leukocytes, both in the dermis and at the dermal-epidermal junction, often in areas of vacuolar keratinocyte degeneration (Fig. 1, panels M and N). To detect IFN γ expression in

situ, we performed immunohistochemical detection of the transcription factor T-bet. T-bet is required for IFN γ expression and Th1 lineage commitment, both in the mouse and in man, and is a surrogate marker for IFN γ expression [25-27]. T-bet was expressed in numerous infiltrating leukocytes in aGvHD, but not in control biopsies (Fig. 1, panel P). The leukocytic inflammatory infiltrate consisted predominantly of CD4+ or CD8+ T-lymphocytes localized either in the dermis or at the dermal-epidermal junction, especially in the areas of vacuolar keratinocyte degeneration (not shown). Consistent with the inflammatory state of aGvHD, we observed expression of ICAM-1 in the aGvHD epidermis and on the endothelium of dermal blood vessels (Fig. 1, panels S and T), but not in the control biopsies.

Reproduction of the histopathology of cutaneous aGvHR in an epidermotypic model

In an attempt to develop epidermotypic model consistent with cutaneous aGvHR, we exposed RHEs to FasL (32 ng/ml), IFN γ (50 ng/ml), or both agents, and assessed the morphological changes 24 hrs later. RHEs displayed an interface dermatitis-like reaction, manifested by vacuolar keratinocyte degeneration (Fig. 2, panel D, asterisk), apoptotic keratinocytes (Fig. 2, panels BD), Civatte (cytoid) bodies (Fig. 2, panels B and J, arrows), and hypergranulosis (Fig. 2, panels G and H). Both FasL and IFN γ triggered caspase-3 activation (Fig. 2, panels J-L). As in cutaneous aGvHR, ICAM-1 was expressed in the involved RHEs (Fig. 2, panels N and P). Interestingly, cytoid body formation (Fig. 2, panels B and J, arrows) and expression of ICAM-1 (Fig. 2, panels N and P), were induced exclusively by IFN γ (either with or without FasL), but not by FasL alone. In contrast, hypergranulosis, detected by filaggrin expression, was triggered by FasL (either with or without IFN γ), but not by IFN γ alone (Fig. 2, panels G and H).

Thus, we have developed an epidermotypic model of cutaneous aGvHR using RHE treated with FasL, IFN γ , or both. Individual hallmarks of cutaneous aGvHD morphology could be attributed to the actions of FasL (apoptosis and hypergranulosis), IFN γ (apoptosis, bodies of Civatte, and expression of ICAM-1) or both (vacuolar degeneration). To the best of our knowledge, the first suggestion that cytoid bodies (a.k.a. colloid bodies/bodies of Civatte) are remnants of apoptotic keratinocytes was made in 1979 by Weedon, Searle, and Kerr in their seminal publication "Apoptosis. Its nature and implications for dermatopathology" [28]. The results presented here (Fig. 2, panels B and J), however, argue that cytoid bodies represent a specific morphologic subtype of keratinocyte apoptosis induced by IFN γ . In contrast, keratinocyte apoptosis triggered by FasL (Fig. 2, panels C and K) is not consistent with cytoid body morphology. Consistent with such conclusion is also our failure to detect cytoid body morphology in RHEs undergoing massive keratinocyte apoptosis in response to UV radiation, TNF α , IL-1 β , or combinations thereof (not shown).

FasL-and IFN γ -specific patterns of gene and protein expression in the epidermotypic model of interface dermatitis

We investigated the effects of FasL and IFN γ , either singly or together, on the expression of 16 inflammatory genes in HEKn-derived RHE (15 cytokines and chemokines and 1 adhesion molecule, ICAM-1). To determine the effects of suppressed apoptosis on the genomic responses to FasL and IFN γ , the treatments were also done in the presence or in the absence of the caspase inhibitor zVADfmk. The secretion of the corresponding cytokines and chemokines was also determined by a Luminex-100 System Version IS (Luminex Inc.) and a Human Cytokine/Chemokine LINCoplex Kit (LINCO Research Inc.). The results of these analyses are exemplified by CXCL8/IL-8, CXCL10/IP-10, and CXCL14/BRACK in Fig. 3, while all the qRT-PCR data are summarized as a "log-scale heat map" in Table 1. The pattern of gene expression derived from our RHE model (Table 1) overlapped significantly with the pattern of gene expression in a murine model of cutaneous aGvHD

[29]. Specifically, the following genes were common between our model and the study of Sugerman et al. [29]: CCL2, CCL5, CCL19, CXCL1, CXCL10, IL-1 β , and ICAM-1. The importance of these findings is underscored by implications in human aGvHD. For instance, CXCL10 expression has been detected in the basal keratinocyte layer of aGvHD patients, together with skin-homing donor T cells expressing CXCR3, the receptor for CXCL10 [30]. Furthermore, all (n=6) of our cutaneous aGvHD patients contained dermis-infiltrating cells expressing CCR7, the receptor for CCL19, identified both in our RHE model (Table 1) and by Sugerman et al. [29] (not shown). Whereas Sugerman et al. [29] did not identify CCL20 in their mouse model, we did so in RHE (Table 1). Recently, Varona et al. reported that, in a mouse model, mortality and morbidity due to aGvHD were drastically reduced and delayed when naïve T cells were derived from donor mice deficient in CCR6, the receptor for CCL20 [31]. In summary, we have developed a predictive organotypic model of cutaneous aGvHD.

Interestingly, our model identified two novel candidate genes that have not been previously implicated in GvHD. One is thymic stromal lymphopoietin (TSLP), the most strongly induced gene by FasL ([16] and Table 1). The other is CXCL14/BRACK, a uniquely regulated chemokine whose constitutive expression in ‘healthy’ RHE was strongly suppressed by FasL and IFN γ (Fig. 3 and Table 1). Our previous analysis of FasL-repressed genes in RHE identified CXCL14 as the gene most susceptible to negative regulation by FasL and the only member of the chemokine family to be suppressed in response to FasL ([16], Fig. 3 and Table 1). Interestingly, CXCL14 mRNA expression was also downregulated by IFN γ (Fig. 3 and Table 1). CXCL14 is constitutively expressed by basal keratinocytes in normal human epidermis and its expression is dramatically reduced in basal and squamous carcinomas [32,33]. The biological role of CXCL14 is poorly understood, due in part to the fact that the receptor for CXCL14 has not been identified to date. It has been proposed that this chemokine mediates the epidermal constitutive recruitment of CXCL14-responsive CD14+ dendritic cell (DC) precursors, thereby promoting their in situ differentiation into functional DCs and Langerhans’ cells [34]. Indeed, loss of CXCL14 in tumor tissue was associated with low infiltration by DCs, while restoration of human CXCL14 expression in tumor cells caused attraction of DC both in vitro and in vivo [35]. Interestingly, in lesional skin of patients with atopic dermatitis or psoriasis, the epidermal expression of CXCL14 mRNA was dramatically reduced in areas adjacent to abundant dermal T-cell infiltration [33]. We therefore hypothesize that CXCL14 may play a role as a repulsive (repellent) chemokine for skin-homing T-cells and that downregulation of CXCL14 expression by inflammatory cytokines may be a necessary prerequisite for T cell-keratinocyte interactions in aGvHD.

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Figure 1. Morphology and immunohistochemical (IHC) characteristics of cutaneous aGvHD
Representative images of control (n=3) or aGvHD (n=6) skin biopsies. **(A-F)** Hematoxylin & eosin staining. Panel E: Dermal melanophages (thin double-headed arrow); dyskeratotic KCs (thick black arrow); hypergranulosis (thick white arrow); vacuolar degeneration (asterisk). Panel D: Cytoid body / body of Civatte (thin arrow); vacuolar degeneration (asterisk). **(G-J)** IHC detection of active caspase 3 (brown, arrow), with hematoxylin counterstaining (blue). Panel J: Vacuolar degeneration (asterisk). **(K-N)** IHC detection of FasL (brown, arrow), with hematoxylin counterstaining (blue). Note the nonspecific background in the epidermis. **(O-P)** IHC detection of T-bet (brown), without counterstaining. **(Q-T)** IHC detection of ICAM-1 (brown), with hematoxylin counterstaining (blue). Panel S (inset): magnification of the small rectangular area showing a cross section from a blood vessel. **(U-X)** IHC detection of filaggrin (brown), with hematoxylin counterstaining (blue).

Figure 2. Morphology and immunohistochemical (IHC) characteristics of the epidermotypic model of interface dermatitis

Reconstructed human epidermis (RHE) using primary HEKn and treated, for 24 hrs, as indicated with IFN γ (50 ng/ml), FasL (32 ng/ml), or both. (**A-D**) Hematoxylin & eosin staining. Panels B and J (below): cytooid bodies / bodies of Civatte (arrows); Panel D: vacuolar degeneration (asterisk). (**E-H**) IHC detection of filaggrin (brown), with hematoxylin counterstaining (blue). Panels G and H: hypergranulosis. (**I-L**) IHC detection of active caspase 3 (brown), with hematoxylin counterstaining (blue). (**M-P**) IHC detection of ICAM-1 (brown), with hematoxylin counterstaining (blue).

Figure 3. Selected examples (CXCL8/IL-8, CXCL10/IP-10, and CXCL14/BRACK) of positive and negative gene regulation by FasL, IFN γ , or both, in the presence or in the absence of apoptosis

RHEs were treated with FasL (250 ng/ml, "FL"), IFN γ (50 ng/ml, "IF"), or both, in the presence of a 30 min pretreatment with either DMSO (vehicle, "-") or 50 μ M of zVADfmk ("zV"). qRT-PCR analyses (for mRNA expression) or Luminex-100 (protein expression from collected RHE media) were performed at 4 and 8 hrs post treatments. The data are presented as fold change relative to the corresponding untreated control RHEs (qRT-PCR) or pg/ml (Luminex-100). Error bars, standard deviation from triplicate experimental points.

Table 1
Summarized data for the expression of 16 mRNAs encoding inflammatory mediators from the experiment shown in Figure 3

The heat map indicates the fold change relative to the corresponding untreated control RHEs (logarithmic scale, as indicated on the right-hand side). zV, zVADfmk, FL, FasL. The patterns of expression of CXCL8/IL-8 (strongly induced by FasL) and CXCL10/IP-10 (strongly induced by IFN γ) are traced.