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Research Paper

Langerhans Cells Prevent Autoimmunity *via* Expansion of Keratinocyte Antigen-Specific Regulatory T Cells

Daniela Y. Kitashima [a,1], Tetsuro Kobayashi [b,1,2], Therese Woodring [b], Kacey Idouchi [b], Thomas Doebel [b,2], Benjamin Voisin [b,2], Takeya Adachi [a], Takeshi Ouchi [a], Hayato Takahashi [a], Koji Nishifuji [c], Daniel H. Kaplan [d], Björn E. Clausen [e], Masayuki Amagai [a], Keisuke Nagao [b,*,2]

[a] *Department of Dermatology, Keio University School of Medicine, Tokyo, Japan*
[b] *Dermatology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA*
[c] *Division of Animal Life Science, Tokyo University of Agriculture and Technology, Tokyo, Japan*
[d] *Departments of Dermatology and Immunology, University of Pittsburgh, PA, USA*
[e] *Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz 55131, Germany*

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ABSTRACT

Langerhans cells (LCs) are antigen-presenting cells in the epidermis whose roles in antigen-specific immune regulation remain incompletely understood. Desmoglein 3 (Dsg3) is a keratinocyte cell-cell adhesion molecule critical for epidermal integrity and an autoantigen in the autoimmune blistering disease pemphigus. Although antibody-mediated disease mechanisms in pemphigus are extensively characterized, the T cell aspect of this autoimmune disease still remains poorly understood. Herein, we utilized a mouse model of $CD4^+$ T cell-mediated autoimmunity against Dsg3 to show that acquisition of Dsg3 and subsequent presentation to T cells by LCs depended on the C-type lectin langerin. The lack of LCs led to enhanced autoimmunity with impaired Dsg3-specific regulatory T cell expansion. LCs expressed the IL-2 receptor complex and the disruption of IL-2 signaling in LCs attenuated LC-mediated regulatory T cell expansion *in vitro*, demonstrating that direct IL-2 signaling shapes LC function. These data establish that LCs mediate peripheral tolerance against an epidermal autoantigen and point to langerin and IL-2 signaling pathways as attractive targets for achieving tolerogenic responses particularly in autoimmune blistering diseases such as pemphigus.

1. Introduction

Langerhans cells (LCs) are unique antigen-presenting cells (APC) in the epidermis whose roles in host defense have become clearer over the last few years. We previously demonstrated that LCs take up protein antigens and bacterial toxins by extending their dendrites through intact tight junction barriers (Kubo et al., 2009) to initiate protective humoral responses (Nagao et al., 2009; Ouchi et al., 2011). This non-redundant function for LCs *in vivo* was observed by controlling the distribution of antigens and utilizing protein antigens that do not penetrate epidermal tight junctions and thus are accessible to only LCs (Ouchi et al., 2011). On the other hand, when epidermal barriers are breached during epicutaneous *Candida albicans* infection, LCs are capable of inducing Th17-mediated cellular responses (Igyártó et al., 2011). The route of antigen delivery that allows for natural antigen uptake by LCs was an important factor in determining LC function in these studies.

Langerin is a C-type lectin required for the formation of Birbeck granules (Kissenpfennig et al., 2005a; Valladeau et al., 2000) and was demonstrated to be an endocytic receptor in *in vitro* propagated LCs and in fibroblasts transfected with *Langerin* (Valladeau et al., 2000). However, the genomic ablation of langerin did not result in any obvious immune phenotypes (Kissenpfennig et al., 2005a), and its function(s) *in vivo* had remained elusive. Human LCs have been shown to scavenge HIV *via* langerin (de Witte et al., 2007), but functional contributions of langerin during immune responses have not been demonstrated.

Whether LCs are capable of suppressing immunity has been a topic of debate. Loss of LCs leads to attenuated disease in leishmaniasis with decreased numbers of regulatory T (T_{reg}) cells (Kautz-Neu et al., 2011), and the treatment of mice with antigen-conjugated anti-langerin antibodies results in enhanced T_{reg} cell expansion (Flacher et al., 2014; Idoyaga et al., 2013). LCs have also recently been shown to induce the expansion of T_{reg} cells in response to ionizing irradiation (Price et al., 2015). However, the physiological setting in which LCs mediate

* Corresponding author at: Dermatology Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bldg 10, Rm 12N240B, 9000 Rockville Pike, Bethesda, MD 20892, USA.
E-mail address: keisuke.nagao@nih.gov (K. Nagao).
[1] These authors contributed equally to this work.
[2] Current affiliation: Dermatology Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, U.S.A.

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immuno-regulatory responses and whether this occurs in an antigen-specific manner has yet to be clearly demonstrated.

Past models including contact hypersensitivity responses, intradermal injection of pathogens and transgenic mice expressing neo-autoantigens have been utilized to explore LC function. However, the route of antigen delivery or the superphysiological load of antigens may lead to experimental outcomes that do not reflect physiological LC function. This issue may be avoided by studying immune responses against keratinocyte-associated autoantigens that are physiologically expressed.

Desmoglein 3 (Dsg3) is a classic cadherin family cell adhesion molecule and a major desmosomal glycoprotein that is expressed by keratinocytes (Amagai et al., 1991). Dsg3 is not only critical for maintaining epidermal integrity, it is also a *bona fide* autoantigen that is targeted in pemphigus vulgaris, an autoimmune blistering disease (Amagai et al., 1991). While mechanisms regarding T cell immunity against Dsg3 remain incompletely characterized, a mouse model has helped provide some insight (Takahashi et al., 2009). Experimental autoimmune dermatitis (EAD) is a mouse model in which $CD4^+$ T cells target Dsg3 to mediate autoimmune skin inflammation (Takahashi et al., 2011) and represents a unique model in which autoimmunity against a physiologically expressed, functional self-antigen can be studied. Herein, we utilized *in vitro* and *in vivo* systems and determined that langerin-mediated acquisition of Dsg3 by LCs leads to the expansion of antigen-specific T_{reg} cells. We also demonstrate that LCs expanded T_{reg} cells *via* a mechanism that involves direct IL-2 signaling in LCs.

2. Materials and Methods

2.1. Mice

C57BL/6J and C57BL/6J *Rag2*$^{-/-}$ mice were purchased from CLEA Japan, Inc. and the Central Institute for Experimental Animals (Tokyo, Japan), respectively. Langerin-DTA mice (Kaplan et al., 2005) were crossed with the C57BL/6J *Rag2*$^{-/-}$ background. Homozygous or heterozygous Langerin-DTR mice (Bennett et al., 2005) in the C57BL/6SJL (CD45.1) background were obtained by breeding homozygous Langerin-DTR mice with each other or with C57BL/6SJL wild-type mice. *Dsg3*$^{-/-}$ mice were crossed with K5-Dsg1 transgenic mice, which express another desmosomal protein, Dsg1, in keratinocytes (Hata et al., 2011). The Dsg1 expression driven by the K5 promoter compensates for the loss of Dsg3 and ameliorates the mucosal erosions that *Dsg3*$^{-/-}$ mice are prone to develop, thereby enhancing the viability of *Dsg3*$^{-/-}$ mice (Hata et al., 2011). For the sake of simplicity, the K5-Dsg1 transgenic *Dsg3*$^{-/-}$ mice have been referred to as *Dsg3*$^{-/-}$ mice throughout this manuscript. The Dsg3-specific T cell receptor transgenic mice (H1 mice) in the C57BL/6J background were previously generated (Takahashi et al., 2011). *CD25*$^{-/-}$ mice were kindly provided by Tai Xuguang and Alfred Singer (National Cancer Institute, National Insitutes of Health, Bethesda).

To generate K5-Dsg3-eGFP mice, a transgene vector pGEM3Z-hK5-mDsg3-EGFP containing the human keratin 5 (K5) promoter [which was kindly provided by Dr. Junji Takeda (Osaka University)], a full length mouse Dsg3 (mDsg3) and enhanced GFP (eGFP) were constructed. Full length mDsg3 fused with eGFP was subcloned between the β-globin cassette and BGHpA of the modified K14pNotIpGEM3Z vector (Hata et al., 2011). Then, the K14 promoter was replaced with the K5 promoter as previously described (Hata et al., 2011). The nucleotide region from the K5 promotor to BGHpA was excised and microinjected into the pronuclei of the C57BL/6J mice zygotes. The zygotes were implanted into pseudopregnant foster C57BL/6J mice to generate mDsg3-eGFP transgenic mice.

All mice were bred and housed in specific pathogen-free facilities. All animal procedures and study protocols were approved by the Keio University Ethics Committee for Animal Experiments.

2.2. Antibodies

Anti-mouse langerin (clone L31, eBioscience) was used either purified or in conjugated forms labeled in house with Alexa Fluor 647 (Invitrogen). Additional anti-mouse mAbs obtained from BioLegend (except otherwise noted) were used for flow cytometry or immunofluorescence staining: CD122 (TM-β1), CD132 (TUGm2), CD25 (PC61), CD3ε (145-2C11), CD4 (GK1.5), CD45 (30F-11), CD80 (16-10A1), CD86 (GL-1), EpCAM (G8.8), Foxp3 (FJK-16s; eBioscience), IL-2 (JES6-1A12), MHC II (anti-IA/IE; M5/114.15.2), Vβ6 (RR4-7; BD Bioscience), CD11b (M1/70; eBioscience) and pSTAT5 (pY694: BD Biosciences).

LEAF™ Purified anti-mouse I-A/I-E (M5/114.15.2, BioLegend) and the appropriate isotypes were used for functional assays. Mouse CD4 MicroBeads (L3T4, Miltenyi Biotec) were used for $CD4^+$ T cell isolation. Anti-Cy7 and anti-Biotin MicroBeads were used for the $CD11b^+$ dermal DC isolation *via* a MACS cell separation system.

Primary antibodies were detected with Alexa Fluor-labeled secondary antibodies (Invitrogen). Cell nuclei were stained with Hoechst 33258 dye (Invitrogen). NAK5 (anti-Dsg3 IgG1 mAb) was used to stain epidermal sheets.

For *in vitro* blocking assays, anti-mouse antibodies against I-A/I-E (M5/114.15.2), CD25 (PC61), CD80 (16-10A1), CD86 (GL-1), CD275 (HK5.3), PD-1 (29F.1A12 and RMP1-14), PD-L1 (10F.9G2), IL-2 (JES6-1A12), IL-6 (MP5-20F3) and IL-10 (JES5-16E3) from BioLegend, IL-15 (AIO.3) from eBioscience and TGF-beta (1D11) from R&D were used.

2.3. Epidermal Sheet and Cryosection Preparation

Epidermal sheets were prepared as previously described (Nagao et al., 2009). Briefly, mouse ears were divided into dorsal and ventral halves with forceps and incubated for 15 min at 37 °C on 3.8% ammonium thiocyanate (Wako Pure Chemical Industries) in phosphate buffer (pH 7.0). The epidermal sheets were manually detached from the dermis under a dissecting microscope (Olympus). Tissue samples for cryosections were embedded in O.C.T. compound (Tissue Tek) and frozen at −80 °C.

2.4. Immunofluorescence Microscopy

Staining of the epidermal sheets and cryosections was performed as previously described. Briefly, the epidermal sheets or cryosections were fixed in acetone at −20 °C for 15 min and were rehydrated in PBS for at least 5 min (3 times). The samples were blocked with 3% dry milk (Morinaga) and 5% goat serum (Dako) in PBS for an hour at room temperature. The primary antibodies were incubated overnight at 4 °C and washed and detected with appropriate secondary antibodies. Nuclei were stained with Hoechst 33258 (Invitrogen). Image acquisition was performed with an Axio Observer.Z1 (Zeiss) and the AxioVision software (ver. 4.8), with a Leica confocal microscope (TCS-SP5, Leica) or with a Zeiss confocal microscope (LSM880, Zeiss) and the Zeiss Zen (v2.1) software. The levels of the images were linearly adjusted using Photoshop CS5 (Adobe) when necessary. 3D reconstruction images were built using sp5 software (Leica) and movies were processed using iMovie (Apple).

2.5. Cell Suspension Preparation

Epidermal cell suspensions were prepared as previously described with slight modifications (Nagao et al., 2009). The shaved mouse trunk skins were floated on RPMI 1640 medium containing 0.15% trypsin and 0.27 mM EDTA at 37 °C for 30 or 45 min. The epidermis was mechanically scraped off in 5% FCS-PBS and washed and filtered through cell strainers (BD Falcon). To obtain the dermal cell suspension, the dermis was minced with scissors or scalpels and incubated in RPMI 1640 medium containing 0.25 mg/ml of Liberase TL Research grade (Roche) and 200 U/ml of DNase I (Sigma) at 37 °C for 60 min. Skin-draining

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