陰性コントロールの%CV は 14.8%であり、Z' factor は 0.77 と良好な値を示した。130 個の化合物について、ルシフェラーゼアッセイを用い、retest screening を施行したところ、約 40%の化合物が再度、強い発光増加を示した。

D．考察

今回のスクリーニングで、%CV、Z' factor ともに良好な値を示し、リードスルー活性測定において精度と質の高いアッセイ系であることを確認した。この良質なアッセイを用い、我々は約 50 個のヒット化合物を同定した。これらの化合物は、強いリードスルー活性を持つことが予想され、今後アトピー性皮膚炎や種々の遺伝性疾患への臨床応用につながることが期待される。また、今後、これらのヒット化合物について、GFP など他のレポータージーンアッセイを用いた評価を進めるとともに、フィラグリン遺伝子にナンセンス変異を持つアトピー性皮膚炎患者由来の培養細胞を治療し、フィラグリンの発現が回復するか検討を行う予定である。また、ナンセンス変異を持つモデル動物への投与により、*in vivo* での効果と副作用についても詳細な検討を行う予定である。

E．結論

我々は、リードスルー活性を検出可能なレポータージーンアッセイシステムを用い、約 2 万種類の化合物をスクリーニングし、約 50 個のリードスルー活性の高い化合物を同定することに成功した。これらの化合物は、アトピー性皮膚炎の治療や予防のみならず、種々の遺伝性疾患の治療に有効である可能性があり、今後、モデル動物を用い、効果と安全性を検討して行く予定である。また、今回我々が確立したスクリーニングシステムはリードスルー化合物の検索に有用であったため、追加で化合物ライブラリーを購入し、さらなる drug screening を行うことも可能である。

F．健康危険情報

特になし。

G．研究発表

特になし。

H．知的財産の出願・登録状況

特になし。

厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）
分担研究報告書

フィラグリン遺伝子変異の有無を指標にしたアトピー性皮膚炎に対するテーラーメイド治療の確立

研究分担者　乃村俊史　北海道大学・北海道大学病院・皮膚科学分野　助教

研究要旨　アトピー性皮膚炎患者の約 30%がフィラグリン遺伝子変異を持つ。フィラグリン関連アトピーとフィラグリン非関連アトピーの間に既存のアトピー治療薬への反応の差異があるかどうかについては、これまで本邦に限らず欧米でも検討されていない。そこで、我々は、フィラグリン遺伝子変異の有無を指標にしたアトピー性皮膚炎に対するテーラーメイド治療の確立を目指すこととし、アトピー性皮膚炎患者をフィラグリン変異保有群と被保有群に分け、アトピー性皮膚炎で頻用される保湿剤、ステロイド外用剤、タクロリムス外用剤の治療効果と、さらに保湿剤のアトピー予防効果を判定する介入研究を企画し、北海道大学病院倫理委員会の承認を得ることに成功した。

A．目的

アトピー性皮膚炎の病因はこれまで不明であったが、最近、我々のグループは、アトピー性皮膚炎患者の約 25-30%がフィラグリン遺伝子に機能喪失変異を有していることを明らかにした。フィラグリンは皮膚バリアにおいて最も重要な角質の形成に必須であり、かつ、皮膚の保水に重要なタンパク質である。従って、フィラグリンが遺伝的に減少すると、皮膚バリア機能不全により、種々のアレルゲンに対して経皮的に易感作性となり、アトピー性皮膚炎や気管支喘息を発症しやすくなると考えられている。事実、欧州で行われた種々の大規模スタディーにより、フィラグリン遺伝子変異を持つ患者は、アトピー性皮膚炎を 2 歳未満という早期に発症しやすいこと、気管支喘息を合併しやすいこと、IgE が高値を示しやすいことなどが明らかにされてきた。しかしながら、フィラグリン関連アトピーとフィラグリン非関連アトピーの間に既存のアトピー治療薬への反応の差異があるかどうかについては、これまで本邦に限らず欧米でも検討されていない。そこで、我々は、フィラグリン遺伝子変異の有無を指標にしたアトピー性皮膚炎に対するテーラーメイド治療の確立を目指すこととし、アトピー性皮膚炎患者をフィラグリン変異保有群と被保有群に分け、アトピー性皮膚炎で頻用される保湿剤、ステロイド外用剤、タクロリムス外用剤の治療効果と、さらに保湿剤のアトピー予防効果を判定する介入研究を企画した。

B．研究方法

対象は 16 歳未満の小児アトピー性皮膚炎患者とした。介入研究の円滑な遂行のため、必要な患者数を減らす目的で、分割実験のデザインを用いることとし、1 人の患者につき、両肘窩、

両膝窩の4箇所を独立した実験箇所として採用した。フィラグリン遺伝子変異の有無で第1段階目の分割をし、それに対して保湿剤の使用の有無で第2段階目の割り付けをし、さらに治療の種類を割りつけた。治療の割り付けは、6種類の塗り分け方法（無外用、保湿剤のみ、ステロイドのみ、タクロリムスのみ、保湿剤＋ステロイド、保湿剤＋タクロリムス）の中から1人の患者につき4種類が、統計解析・デザインソフトのJMP9.01により作成された割り付け表に従って割り振られる。これにより、変異保有群、非保有群各 15人の計120実験箇所で、両群の治療効果の差(スコア改善度%)が10%の時、統計学的に十分な有意差を検出可能である。臨床的重症度の評価には、Severity Scoring of Atopic Dermatitis (SCORAD)で採用される皮疹評価スコアや、経皮的水分喪失量、角質水分量、掻痒の程度（VAS scale）を用い、4週間にわたり、毎週、臨床的重症度の経時的変化（治療効果）を判定する。以上の介入研究についての基本計画を作成し、北海道大学病院倫理委員会に提出、審議が行われた。

C．研究成果

実験デザイン、患者数、統計学的信頼度、安全性などすべての点で基準をクリアし、北海道大学病院倫理委員会から本介入試験の承認を得ることに成功した。

D．考察

我々は、すでに200名を超えるアトピー性皮膚炎患者についてフィラグリン遺伝子変異検索を終了しており、介入試験への参加依頼の可能なフィラグリン遺伝子変異保有患者と非保有患者を多数有している。本介入試験の施行により、これまで全く不明であった、フィラグリン遺伝子変異の有無によるアトピー性皮膚炎の治療効果の差異が明らかになるものと期待される。

E．結論

アトピー性皮膚炎に対するテーラーメイド治療の確立に向けて、小児アトピー患者を対象にした介入試験を企画し、倫理委員会の承認を得ることに成功した。テーラーメイド治療により、従来の画一的な治療と比べて、治療効果の向上や不必要な投薬の減少、それに伴う医療費の削減等が期待される。

F．健康危険情報

特になし。

G．研究発表

1．論文発表

特になし。

2．学会発表

Goto-Ohguchi Y, Nomura T, Miyamura Y, Ono N, Kosuge H, Okamoto H, McLean WHI, Akiyama M, Shimizu H. Prevalent and rare filaggrin gene mutations in the Japanese population. The 36th Annual Meeting of the Japanese Society for Investigative Dermatology, Kyoto, Japan. December 9-11, 2011.

H．知的財産の出願・登録状況

特になし。

Ⅳ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（雑誌）

発表者氏名	論文タイトル名	発表雑誌	巻号	ページ	出版年
Watanabe M, Ujiie H, Iitani MM, Abe R, **Shimizu H**	Psoriatic onycho-pachydermo periostitis that progressed to generalized pustular psoriasis.	**Clin Exp Dermatol**			in press
Ujiie H, Shibaki A, Nishie W, Shinkuma S, Moriuchi R, Qiao H, **Shimizu H**	Noncollagenous 16A domain of type XVII collagen-reactive CD4(+) T cells play a pivotal role in the development of active disease in experimental bullous pemphigoid model.	**Clin Immunol**			in press
Sasaki K, **Akiyama M**, Yanagi T, Sakai K, Miyamura Y, Satok M, **Shimizu H**	CYP4F22 is highly expressed at the site and onset of keratinization during human skin development.	**J Dermatol Sci**			in press
Natsuga K, Shinkuma S, Kanda M, Suzuki Y, Chosa N, Narita Y, Setoyama M, Nishie W, **Akiyama M**, **Shimizu H**	Possible modifier effects of keratin 17 gene mutation on keratitis-ichthyosis-deafness syndrome.	**Br J Dermatol**			in press
Mizuno O, Yanagi T, Baba K, Yamane N, Inokuma D, Ito K, **Akiyama M**, **Shimizu H**	Sweet's syndrome presenting with vegetative nodules on the hands: relationship to neutrophilic dermatosis of the dorsal hands.	**Int J Dermatol**			in press
Koguchi H, **Arita K**, Yamane N, Shinkuma S, **Shimizu H**	Erythema annulare centrifugum-like neutrophilic dermatosis:Effects of potassium iodide.	**Acta Derm Venereol**			in press
Izumi K, Yanagi T, **Akiyama M**, Moriuchi R, **Arita K**, **Shimizu H**	Intractable erythematous plaques on the hands: palmoplantar eosinophilic pustular folliculitis.	**Int J Dermatol**			in press
Homma E, Aoyagi S, Baba K, Iitani M, Hata H, **Shimizu H**	Angiosarcoma on the lower abdominal wall associated with chronic lymphedema in an obese woman.	**Int J Dermatol**			in press
Hirata Y, Abe R, Kikuchi K, Hamasaka A, Shinkuma S, **Nomura T**, Nishie W, **Arita K**, **Shimizu H**	Intraepidermal neutrophilic IgA pemphigus successfully treated with dapson.	**Eur J Dermatol**			in press
Hayashi I, Shinkuma S, Shimizu S, Natsuga K, Ujiie H, Yasui C, Tsuchiya K, Nishie W, **Shimizu H**	Mucous membrane pemphigoid with generalized blisters: IgA and IgG autoantibodies target both laminin-332 and type XVII collagen.	**Br J Dermatol**			in press

Hamade Y, **Arita K**, Toyonaga E, Inokuma D, Hamasaka K, **Shimizu H** .	Lichen Planus in Childhood Showing Various Cutaneous Features.	**Acta Derm Venereol**			in press
Fujita Y, Inokuma D, Abe R, Sasaki M, Nakamura H, Shimizu T, **Shimizu H**	Conversion from human haematopoietic stem cells to keratinocytes requires keratinocyte secretory factors.	**Clin Exp Dermatol**			in press
Murrell DF, Daniel BS, Joly P, Borradori L, Amagai M, Hashimoto T, Caux F, Marinovic B, Sinha AA, Hertl M, Bernard P, Sirois D, Cianchini G, Fairley JA, Jonkman MF, Pandya AG, Rubenstein D, Zillikens D, Payne AS, Woodley D, Zambruno G, Aoki V, Pincelli C, Diaz L, Hall RP, Meurer M, Mascaro JM, Jr., Schmidt E, **Shimizu H**, Zone J, Swerlick R, Mimouni D, Culton D, Lipozencic J, Bince B, Bystryn JC, Werth VP	Definitions and outcome measures for bullous pemphigoid: Recommendations by an international panel of experts.	**J Am Acad Dermatol**			in press (Published online Nov.5)
Yoshihisa Y, Makino T, Matsunaga K, Honda A, Norisugi O, Abe R, **Shimizu H**, Shimizu T	Macrophage migration inhibitory factor is essential for eosinophil recruitment in allergen-induced skin inflammation.	**J Invest Dermatol**	131	925-931	2011
Yanagi T, **Akiyama M**, Nishihara H, Miyamura Y, Sakai K, Tanaka S, **Shimizu H**	AKT has an anti-apoptotic role in ABCA12-deficient keratinocytes.	**J Invest Dermatol**	131	1942-1945	2011
Umemoto H, **Akiyama M**, Yanagi T, Sakai K, Aoyama Y, Oizumi A, Suga Y, Kitagawa Y, **Shimizu H**	New insight into genotype/phenotype correlations in ABCA12 mutations in harlequin ichthyosis.	**J Dermatol Sci**	61	136-138	2011
Tanimura S, Tadokoro Y, Inomata K, Binh NT, Nishie W, Yamazaki S, Nakauchi H, Tanaka Y, McMillan JR, Sawamura D, Yancey K, **Shimizu H**, Nishimura EK	Hair follicle stem cells provide a functional niche for melanocyte stem cells	**Cell Stem Cell**	8	177-187	2011

Suga H, Tsunemi Y, Sugaya M, Shinkuma S, **Akiyama M**, **Shimizu H**, Sato S	Hair shaft abnormalities in localized autosomal recessive hypotrichosis 2 and a review of other non-syndromic human alopecias.	**Acta Derm Venereol**	91	486-488	2011
Shinkuma S, McMillan JR, **Shimizu H**	Ultrastructure and molecular pathogenesis of epidermolysis bullosa.	**Clin Dermatol**	29	412-419	2011
Osawa R, **Akiyama M**, Izumi K, Ujiie H, Sakai K, Nemoto-Hasebe I, Yanagi T, Koizumi H, **Shimizu H**	Extremely severe palmoplantar hyperkeratosis in a generalized epidermolytic hyperkeratosis patient with a keratin 1 gene mutation.	**J Am Acad Dermatol**	64	991-993	2011
Natsuga K, Nishie W, Shinkuma S, Nakamura H, Matsushima Y, Tatsuta A, Komine M, **Shimizu H**	Expression of exon-8-skipped kindlin-1 does not compensate for defects of Kindler syndrome.	**J Dermatol Sci**	61	38-44	2011
Natsuga K, Nishie W, Shinkuma S, Nakamura H, **Arita K**, Yoneda K, Kusaka T, Yanagihara T, Kosaki R, Sago H, **Akiyama M**, **Shimizu H**	A founder effect of c.1938delC in ITGB4 underlies junctional epidermolysis bullosa and its application for prenatal testing.	**Exp Dermatol**	20	74-76	2011
Nakamura H, Natsuga K, Nishie W, McMillan JR, Sawamura D, **Akiyama M**, **Shimizu H**	DNA-based prenatal diagnosis of plectin-deficient epidermolysis bullosa simplex associated with pyloric atresia.	**Int J Dermatol**	50	439-442	2011
Nakajima K, Sano S, Uchida Y, **Akiyama M**, Morita Y, **Shimizu H**	Altered lipid profiles in the stratum corneum of Sjogren-Larsson syndrome.	**J Dermatol Sci**	63	64-66	2011
Lin HY, Yanagi T, **Akiyama M**, Iitani MM, Moriuchi R, Natsuga K, Shinkuma S, Yamane N, Inokuma D, **Arita K**, **Shimizu H**	Childhood subepidermal blistering disease with autoantibodies to type VII collagen and laminin-332.	**Br J Dermatol**	164	452-454	2011
Li Q, Frank M, **Akiyama M**, **Shimizu H**, Ho SY, Thisse C, Thisse B, Sprecher E, Uitto J	Abca12-mediated lipid transport and Snap29-dependent trafficking of lamellar granules are crucial for epidermal morphogenesis in a zebrafish model of ichthyosis.	**Dis Model Mech**	4	777-785	2011
Kusajima E, **Akiyama M**, Sato M, Natsuga K, **Shimizu H**	Type XVII collagen ELISA indices significantly decreased after bullous pemphigoid remission.	**Int J Dermatol**	50	238-240	2011

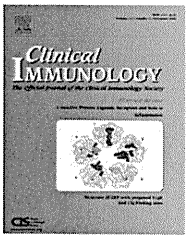
Kikuchi K, Natsuga K, Shinkuma S, Nishie W, Kajita S, Sato H, **Shimizu H**	Subepidermal blistering disease with 3 distinct autoantibodies: Anti-BP230, anti-laminin gamma-1, and anti-laminin-332.	**J Am Acad Dermatol**	65	878-880	2011
Kikuchi K, **Arita K**, Tateishi Y, Onozawa M, **Akiyama M**, **Shimizu H**	Recurrence of hydroxyurea-induced leg ulcer after discontinuation of treatment.	**Acta Derm Venereol**	91	373-374	2011
Kikuchi K, Abe R, Shinkuma S, Hamasaka H, Natsuga K, Hata H, Tateishi Y, Shibata M, Tomita Y, Abe A, Aoyagi A, Mukai M, **Shimizu H**	Spontaneous Remission of Solitary-Type Infantile Myofibromatosis.	**Case Rep Dermatol**	3	181-185	2011
Kempf M, Miyamura Y, Liu PY, Chen AC, Nakamura H, **Shimizu H**, Tabata Y, Kimble RM, McMillan JR	A denatured collagen microfiber scaffold seeded with human fibroblasts and keratinocytes for skin grafting.	**Biomaterials**	32	4782-4792	2011
Fujita Y, Yoshioka N, Abe R, Murata J, Hoshina D, Mae H, **Shimizu H**	Rapid immunochromatographic test for serum granulysin is useful for the prediction of Stevens-Johnson syndrome and toxic epidermal necrolysis.	**J Am Acad Dermatol**	65	65-68	2011
Fujita Y, Abe R, Nishie W, **Shimizu H**	Regenerative medicine for severe congenital skin disorders: restoration of deficient skin component proteins by stem cell therapy.	**Inflammation and Regeneration**	31	282-289	2011
Frew J, Lim SW, Klausseger A, Chow CW, Tran K, Su J, Orchard D, Varigos G, Sawamura D, Nishie W, **Shimizu H**, Murrell DF	Autosomal dominant bullous dermolysis of the newborn associated with a heterozygous missense mutation p.G1673R in type VII collagen.	**Australas J Dermatol**	52	e1-4	2011
Chen AC, McNeilly C, Liu AP, Flaim CJ, Cuttle L, Kendall M, Kimble RM, **Shimizu H**, McMillan JR	Second harmonic generation and multiphoton microscopic detection of collagen without the need for species specific antibodies.	**Burns**	37	1001-1009	2011

Fukamachi S, Mori T, Sakabe J-I, Shiraishi N, Kuroda E, Kobayashi M, Bito T, Kabashima K, Nakamura M, **Tokura Y**	Topical Cholecystokinin Depresses Itch-Associated Scratching Behavior in Mice	**J Invest Dermatol**	131	956-961	2011
Sawada Y, Hino R, Hama K, Ohmori S, Fueki H, Yamada S, Fukamachi S, Tajiri M, Kumo R, Yoshioka M, Nakashima D, Sugita K, Yoshiki R, Shimauchi T, Mori T, Izu K, Kobayashi M, Nakamura M, **Tokura Y**	The type of skin eruptions is an independent prognostic indicator for adult T-cell leukemia/lymphoma.	**Blood**	117	3161-3967	2011
Fukamachi S, Bito T, Shiraishi N, Kobayashi M, Kabashima K, Nakamura M, **Tokura Y**	Modulation of semaphorin 3A expression by calcium concentration and histamine in human keratinocytes and fibroblasts.	**J Dermatol Sci**	62	118-123	2011

Ⅴ．研究成果の刊行物・別刷

ARTICLE IN PRESS YCLIM-06920; No. of pages: 9; 4C:
Clinical Immunology (2011) xx, xxx-xxx

available at www.sciencedirect.com
Clinical Immunology
www.elsevier.com/locate/yclim



Noncollagenous 16A domain of type XVII collagen-reactive CD4$^+$ T cells play a pivotal role in the development of active disease in experimental bullous pemphigoid model

Hideyuki Ujiie*, [1], Akihiko Shibaki, Wataru Nishie, Satoru Shinkuma, Reine Moriuchi, Hongjiang Qiao, Hiroshi Shimizu*

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

Received 8 September 2011; accepted with revision 7 October 2011

KEYWORDS
Autoimmune disease;
Autoreactive T cells;
CD40 ligand;
Pathomechanism

Abstract Bullous pemphigoid (BP), the most common autoimmune blistering disease, is caused by autoantibodies against type XVII collagen (COL17). We recently demonstrated that CD4$^+$ T cells were crucial for the production of anti-COL17 IgG and for the development of the BP phenotype by using a novel active BP mouse model by adoptively transferring immunized splenocytes into immunodeficient COL17-humanized mice. Noncollagenous 16A (NC16A) domain of COL17 is considered to contain the main pathogenic epitopes of BP, however, the pathogenicity of COL17 NC16A-reactive CD4$^+$ T cells has never been elucidated. To address this issue, we modulated the immune responses against COL17 in active BP model by using anti-CD40 ligand (CD40L) monoclonal antibody MR1, an inhibitor of the CD40–CD40L interaction, in various ways. First, we show the essential role of CD4$^+$ T cells in the model by showing that CD4$^+$ T cells isolated from wild-type mice immunized with human COL17 enabled naïve B cells to produce anti-COL17 NC16A IgG in vivo. Second, we show that the activation of anti-COL17 NC16A IgG-producing B cells via CD40–CD40L interaction was completed within 5 days after the adoptive transfer of immunized splenocytes. Notably, a single administration of MR1 at day 0 was enough to inhibit the production of anti-COL17 NC16A IgG and to diminish skin lesions despite the presence of restored anti-COL17 IgG at the later stage. In contrast, the delayed administration of MR1 failed to inhibit the production of anti-COL17 NC16A IgG and the development of the BP phenotype. These results

Abbreviations: BP, bullous pemphigoid; COL17, type XVII collagen; BMZ, basement membrane zone; NC16A, noncollagenous 16A domain; WT, wild type; hCOL17, human COL17; Tg, transgenic; CD40L, CD40 ligand; IF, immunofluorescence; OD, optimal density.

* Corresponding authors at: Department of Dermatology, Hokkaido University Graduate School of Medicine, N.15 W.7, Kita-ku, Sapporo 060-8638, Japan. Fax: +81 11 706 7820.
E-mail address: h-ujiie@med.hokudai.ac.jp (H. Ujiie).
[1] Designated author to communicate with the Editorial and Production offices.

1521-6616/$ - see front matter
doi:10.1016/j.clim.2011.10.002

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive CD4$^+$ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

strongly suggest that COL17 NC16A-reactive CD4+ T cells play a pivotal role in the production of pathogenic autoantibodies and in the development of active disease in experimental BP model.

1. Introduction

Bullous pemphigoid (BP) is the most common autoimmune blistering disorder. Clinically, tense blisters, erosions and crusts with itchy urticarial plaques and erythema develop on the entire body. Histologically, subepidermal blisters associated with inflammatory cell infiltration in the dermis are observed. BP is induced by autoantibodies against type XVII collagen (COL17, also called BP180 or BPAG2), a hemidesmosomal protein which spans the lamina lucida and projects into the lamina densa of the epidermal basement membrane zone (BMZ) [1–6]. The juxtamembranous noncollagenous 16A (NC16A) domain is considered to contain the main pathogenic epitopes on COL17, although BP patients' sera can also react with other parts [7–9].

Recently, we developed a novel active BP mouse model by adoptively transferring wild-type (WT) splenocytes immunized by human COL17 (hCOL17)-expressing transgenic (Tg) skin-grafting into *Rag-2*$^{-/-}$/*COL17*$^{m-/-,h+}$ (*Rag-2*$^{-/-}$/COL17-humanized) mice that express hCOL17 in the skin and lack both T and B cells [10]. The recipient mice accepted transferred splenocytes and produced high titers of anti-hCOL17 IgG in vivo for more than 10 weeks after the adoptive transfer, while circulating anti-hCOL17 NC16A IgG titer decreased in a short period for unknown reasons [10]. They developed blisters and erosions corresponding to clinical, histological and immunopathological features of BP [10]. This new active BP model enables us to observe the dynamic immune reactions induced by pathogenic antibodies against hCOL17 molecule.

In BP, the presence of autoreactive CD4+ T cells has been reported [11–13]. Particular MHC class II alleles occur more frequently in BP patients [14]. These findings indicated the contribution of CD4+ T cells to the pathogenesis of BP. Generally, the production of IgG by B cells requires the help of CD4+ T cells [15–17]. Our previous study demonstrated that CD4+ T cells were crucial for the production of anti-hCOL17 IgG and for the development of the BP phenotype because both the depletion of CD4+ T cells from immunized splenocytes, and the administration of cyclosporin A significantly suppressed the pathogenic IgG production and diminished the disease severity [10]. However, the pathogenicity of COL17 NC16A-reactive CD4+ T cells has never been elucidated. To address this issue, we modulated the CD4+ T cell function in active BP model by administering anti-CD40L monoclonal antibody MR1 [18] in various ways, and observed the phenotypic changes of the treated mice.

CD40 ligand (CD40L) is a costimulatory molecule which is transiently expressed on the surface of activated CD4+ T cells and which binds to CD40 on antigen-presenting cells including B cells. CD40–CD40L interaction is crucial for the proliferation and differentiation of B cells into immunoglobulin-secreting plasma cells and for the formation of humoral memory [19]. Immunosuppressive effects of anti-CD40L monoclonal antibody have been shown in some T-cell-mediated antibody-induced autoimmune animal models, such as experimental autoimmune myasthenia gravis [20], and pemphigus vulgaris [21, 22]. In this study, we demonstrate that COL17 NC16A-reactive CD4+ T cells play a pivotal role in the development of BP through the CD40–CD40L interaction at an early stage of the disease in active BP model, which suggests that COL17 NC16A-reactive CD4+ T cell is a promising therapeutic target for BP.

2. Materials and methods

2.1. Mice

C57BL/6J mice were purchased from Clea Japan. *Rag-2*$^{-/-}$/*COL17*$^{m-/-,h+}$ mice which carry the homozygous null mutations of both the *Rag-2* and *mouse Col17* genes and the transgene of *human COL17* were generated by crossing *Rag-2*$^{-/-}$ mice (C57BL/6 background) with *COL17*$^{m-/-,h+}$ (COL17-humanized) mice (C57BL/6 background) as described previously [10]. All animal procedures were conducted according to guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

2.2. Induction of active BP by adoptive transfer of immunized splenocytes

Immunization of WT mice by *hCOL17*-expressing Tg skin graft was performed according to the method reported previously [10, 23]. After the confirmation of anti-hCOL17 IgG production at 5 weeks after skin grafting by indirect immunofluorescence (IF) analysis using normal human skin, splenocytes were isolated and pooled from several Tg skin-grafted immunized WT mice and administered into *Rag-2*$^{-/-}$/COL17-humanized mice by intravenous injection into the tail vain at $1.5–2.0 \times 10^8$ splenocytes in 500 μL PBS per mouse [10, 24].

2.3. Evaluation of active BP model mice

Weekly, the recipient mice were examined for general condition and cutaneous lesions (i.e., erythema, blisters, erosions, crusts and hair loss). Extent of skin disease was scored as follows: 0, no lesions; 1, lesions on less than 10% of the skin surface; 2, lesions on 10–20% of the skin surface; 3, lesions on 20–40% of the skin surface; 4, lesions on 40–60% of the skin surface; 5, lesions on more than 60% of the skin surface, as previously described [10]. Serum samples were also obtained from recipient mice weekly and assayed by indirect IF microscopy and hCOL17 NC16A ELISA as previously described [10]. The ELISA index value was defined by the following formula: index = (OD_{450} of tested serum − OD_{450} of negative control) / (OD_{450} of positive control − OD_{450} of

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive CD4+ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

negative control)×100 [10]. Biopsies of lesional skin were obtained for light microscopy (H&E), and for direct IF using FITC-conjugated antibody against mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and C3 (Cappel; Valeant Pharmaceuticals, Costa Mesa, CA).

2.4. Isolation of CD4+ T cells or CD45R+ B cells from splenocytes in mice

To examine the pathogenic role of CD4+ T cells in active BP model, we isolated CD4+ T cells from splenocytes of Tg skin-grafted WT mice by using a CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Galdbach, Germany). 0.5 to 8×10^7 CD4+ T cells were mixed with 2.0×10^8 naïve splenocytes from WT mice and adoptively transferred to *Rag*-$2^{-/-}$/COL17-humanized mice. In another experiment, CD45R+ B cells were isolated from Tg skin-grafted WT mice by using CD45R MicroBeads (Miltenyi Biotec). 0.4×10^8 of CD45R+ B cells were transferred to *Rag*-$2^{-/-}$/COL17-humanized mice. The isolation of CD4+ T cells and CD45R+ B cells was confirmed by flow cytometric analysis on FACSAria (BD Bioscience Pharmingen) using monoclonal antibodies purchased from BD Biosciences Pharmingen: H129.19-FITC (anti-CD4) and RA3-6B2-PE (anti-CD45R/B220).

2.5. In vivo monoclonal antibody treatment

Rag-$2^{-/-}$/COL17-humanized recipients that were adoptively transferred with immunized splenocytes were intraperitoneally injected with 500 μg hamster monoclonal antibody MR1 specific to mouse CD40L (Taconic Farms, Hudson, NY) or an equivalent amount of control hamster IgG (Rockland Immunochemicals, Gilbertsville, PA) at days 0, 2 and 6 after the adoptive transfer of immunized splenocytes as previously described [21], with some minor modifications. In a delayed treatment experiment, MR1 was injected at days 13, 16 and19 after the adoptive transfer. Some recipient mice were injected with 500 μg of MR1 just once on one of days 1 to 5 after the adoptive transfer, respectively. To investigate the immune responses in active BP model modulated by early single administration of MR1, 1000 μg of MR1 was injected into recipient mice at day 0 soon after the adoptive transfer. All treated mice were carefully observed for at least ten weeks after the adoptive transfer.

2.6. ELISPOT assay

ELISPOT assay was performed as previously described [10, 24]. Polyvinylidene-difluoride-bottomed 96-well multiscreen plates (Millipore) were coated with 30 μg/mL of recombinant hCOL17 NC16A protein. Splenocytes isolated from the *Rag*-$2^{-/-}$/COL17-humanized recipients were incubated on the plate at 37 °C in a 5% CO_2 incubator for 4 h. IgG bound to the membrane was visualized as spots, using alkaline-phosphatase-conjugated anti-mouse IgG antibody. The number of spots was counted using the ImmunoSpot S5 Versa Analyzer (Cellular Technology Ltd., Shaker Heights, OH), and the frequency of anti-hCOL17 NC16A IgG-producing B cells was defined as the number of spots in 10^5 mononuclear cells.

2.7. Statistical analysis

Data expressed as mean ± standard error of means were analyzed using Student's *t*-test. We considered *P* values of less than 0.05 as significant.

3. Results

3.1. CD4+ T cells are required for the production of pathogenic antibody in active BP model

We previously reported that CD4+ — but not CD8+ — T cells are crucial for the production of anti-hCOL17 IgG and for the development of the BP phenotype in active BP model [10]. To further analyze the contribution of CD4+ T cells, we additionally conducted two experiments. First, mixed transfer into *Rag*-$2^{-/-}$/COL17-humanized mice of 4 or 8×10^7 CD4+ T cells from WT splenocytes immunized by hCOL17-expressing Tg skin-grafting and 2×10^8 naïve splenocytes from unimmunized WT mice produced high titers of anti-hCOL17 NC16A IgG and severe BP skin changes associated with linear deposition of IgG at the BMZ. In contrast, reducing the number of CD4+ T cells (0.5×10^7) failed to produce such titers and skin changes (n=3, respectively; Fig. 1). Second, we isolated CD45R+ B cells from immunized splenocytes and adoptively transferred 0.4×10^8 of those cells into *Rag*-$2^{-/-}$/COL17-humanized recipients (n=3), which produced quite low levels of anti-hCOL17 NC16A IgG (mean index value of ELISA at day 9: 3.28) and no skin changes (not shown). These results show that the production of anti-hCOL17 NC16A IgG by B cells and the development of BP skin changes in active BP model depend heavily on immunized CD4+ T cells.

3.2. Anti-CD40L monoclonal antibody suppresses the production of anti-hCOL17 IgG and skin changes in active BP model

To investigate the precise mechanism of the activation of B cells by immunized CD4+ T cells in active BP model, we assessed the role of CD40–CD40L interaction. *Rag*-$2^{-/-}$/COL17-humanized recipients were injected intraperitoneally with 500 μg of monoclonal antibody MR1 specific to mouse CD40L or an equivalent dose of hamster IgG as a control on days 0, 2 and 6 after the adoptive transfer of immunized splenocytes (n=6, respectively). All the control *Rag*-$2^{-/-}$/COL17-humanized recipients produced high titers of IgG against BMZ of normal human skin, which reflects the presence of anti-hCOL17 IgG, and those against hCOL17 NC16A, as previously reported [10]. In contrast, the production of those antibodies was almost completely inhibited in all the mice that were injected with MR1, and the inhibitory effect persisted for more than 10 weeks (Figs. 2A, B). The control mice developed patchy hair loss associated with erythema around day 14 after the adoptive transfer. Then, blisters and erosions spontaneously developed in the depilated areas on the trunk (Fig. 3A). Disease severity, scored by the percent of skin surface with the BP phenotype [10, 25], gradually increased, plateauing 7 weeks after the transfer in the control mice (Fig. 3G). In contrast, none of the MR1-

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive CD4+ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

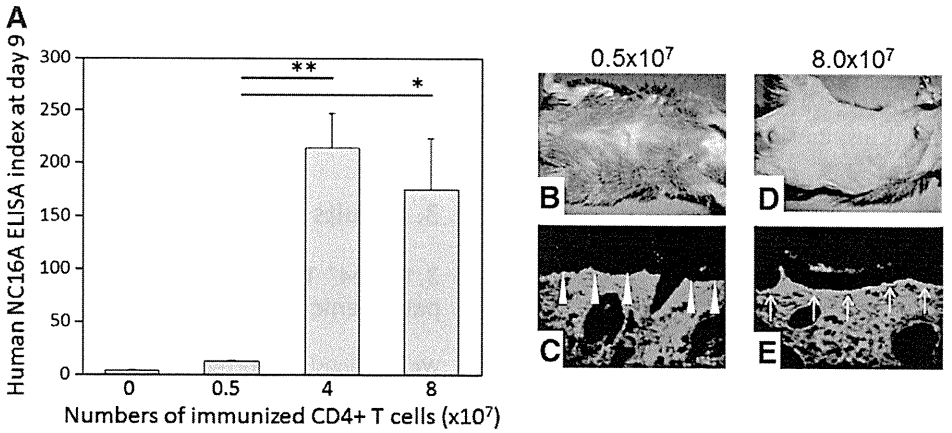


Fig. 1 Immunized $CD4^+$ T cells can activate naïve B cells to produce anti-hCOL17 NC16A IgG in vivo. (A) $CD4^+$ T cells isolated from WT splenocytes immunized by hCOL17-expressing Tg skin-grafting were mixed with naïve splenocytes from untreated WT mice, and were adoptively transferred into *Rag*-$2^{-/-}$/COL17-humanized mice (n=3, respectively). Mice transferred with 4 or 8×10^7 immunized $CD4^+$ T cells mixed with naïve splenocytes produce significantly higher levels of anti-hCOL17 NC16A IgG than with 0.5×10^7 $CD4^+$ T cells mixed with naïve splenocytes (*$P<0.05$, **$P<0.01$). Mice transferred with 0.5×10^7 of immunized $CD4^+$ T cells and naïve splenocytes show no skin changes (B) or deposition of IgG (C). In contrast, mice transferred with 8×10^7 immunized $CD4^+$ T cells and naïve splenocytes develop severe BP skin changes (D) associated with linear deposition of IgG at the BMZ (E).

treated mice developed any skin lesions (Figs. 3D, G). Histopathological analysis of the skin revealed the dermal–epidermal separation that is associated with mild inflammatory cell infiltration in control mice (Fig. 3B), whereas there were no histopathological changes in MR1-treated mice (Fig. 3E). Direct IF analysis of lesional skin revealed linear deposition of IgG (Fig. 3C) at the BMZ in the control mice, whereas IgG deposition was absent or faint in the MR1-treated mice (Fig. 3F). We also examined the number of splenocytes which produced anti-hCOL17 NC16A IgG by enzyme-linked immunospot assay at day 9. In the control, 226.5±25.0 cells in 10^5 splenocytes produced anti-hCOL17 NC16A IgG, whereas only 9.0±3.0 cells in 10^5 splenocytes produced them in the mice treated with MR1 (n=3, respectively; Fig. 3H). Thus, preventive and repetitive administration of MR1 can continuously suppress the production of anti-hCOL17 IgG and skin changes in active BP model.

3.3. Anti-CD40L monoclonal antibody shows no effects in mice with established active BP

To examine the effect of MR1 in mice with producing IgG against hCOL17 and hCOL17 NC16A, 500 μg of MR1 or the equivalent dose of normal hamster IgG were administered into active BP model at days 13, 16 and 19 after the adoptive transfer of splenocytes (n=4, respectively). There were no significant differences in the titers of anti-hCOL17 or anti-hCOL17 NC16A IgG, nor in disease severity in both groups at more than 10 weeks after the adoptive transfer (Fig. 4).

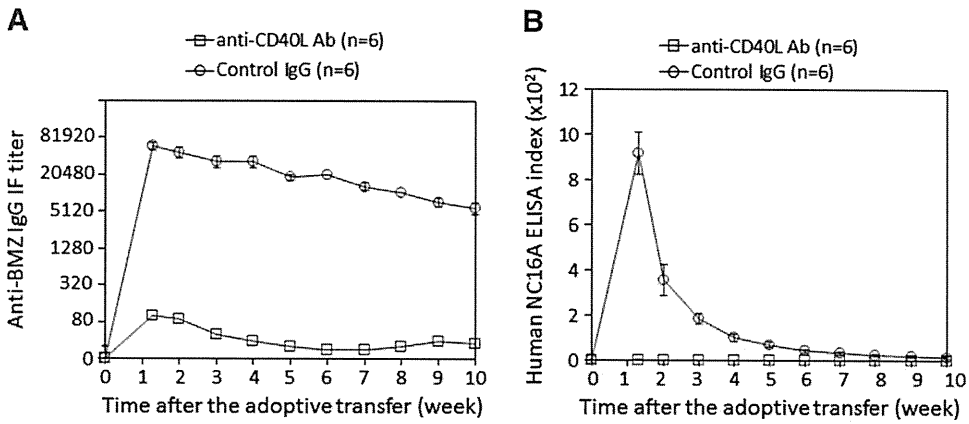


Fig. 2 Anti-CD40L monoclonal antibody strongly suppresses the production of anti-hCOL17 and anti-hCOL17 NC16A IgG and in active BP model. *Rag*-$2^{-/-}$/COL17-humanized recipients were injected intraperitoneally with monoclonal antibody specific to mouse CD40L (MR1) or the equivalent dose of control hamster IgG on day 0, 2 and 6 after the adoptive transfer of immunized splenocytes (n=6, respectively). All the *Rag*-$2^{-/-}$/COL17-humanized recipients that were injected with control IgG produce significantly high titers of IgG against hCOL17 (BMZ of normal human skin) and hCOL17 NC16A, while the production of those antibodies is almost completely inhibited in all mice injected with MR1 (A, B) $P<0.01$ from day 9 to day 70 in both graphs.

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive $CD4^+$ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

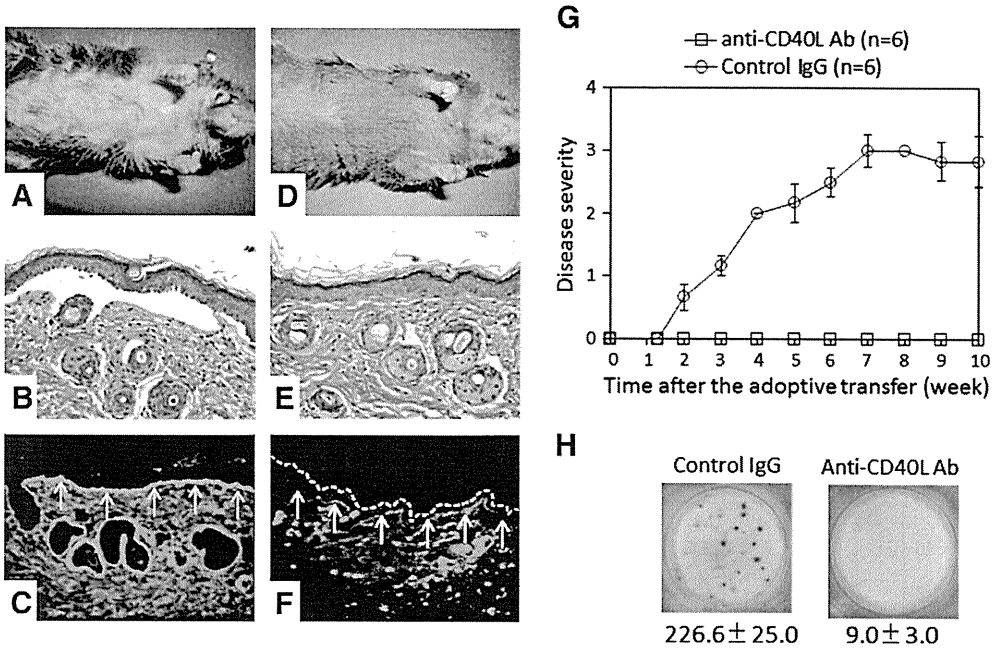


Fig. 3 Skin changes are completely inhibited in the MR1-treated mice. (A) Control *Rag*-$2^{-/-}$/COL17-humanized recipients develop blisters and erosions spontaneously develop in the depilated areas on the trunk (n=6). (B) Histopathologic analysis of the skin reveals the dermal–epidermal separation associated with mild inflammatory cell infiltration in the control group. (C) Direct IF analysis of lesional skin demonstrates linear deposition of IgG at the BMZ in control mice. None of the MR1-treated mice demonstrate any skin lesions (D) or histopathologic changes (E) (n=6). (F) No or faint IgG deposition is detected in the treated mice. (G) Disease severity, which was scored by the percentage of affected skin surface area, gradually increases and plateaus at 7 weeks after the adoptive transfer in the control mice, whereas that is stably zero in the MR1-treated mice ($P<0.05$ at day 14, $P<0.01$ from day 21 to day 70) (H) Enzyme-linked immunospot assay using recombinant hCOL17 NC16A protein at day 9 after the adoptive transfer. In contrast to the control, very few spots are seen in the well of the MR1-treated splenocytes. The number of anti-hCOL17 NC16A IgG-producing B cells is displayed per 10^5 cells in the spleen (n=3, respectively).

These findings show that delayed administration of MR1 fails to diminish the disease activity in established active BP mice.

3.4. Activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction is completed within five days after the adoptive transfer of immunized splenocytes

Since the delayed administration of MR1 failed to diminish the disease activity, we considered that the timing of T–B interaction via the CD40–CD40L pathway after the adoptive transfer needed to be elucidated. Single injections of 1000 μg of MR1 at days 1 to 5 after the adoptive transfer of immunized splenocytes into the *Rag*-$2^{-/-}$/COL17-humanized recipients were administered (n=4, respectively). Injection of MR1 at day 1, day 2 or day 3 strongly inhibited the production of anti-h COL17 NC16A IgG in recipients (Fig. 5A). The effects of MR1 successively decreased if the treatment was initiated at day 4 or day 5. Anti-hCOL17 NC16A IgG titer and disease severity of the recipients treated at day 5 were similar to those in active BP model without MR1 treatment (mean index value of anti-hCOL17 NC16A IgG at day 9: 765.3 vs. 918.97, $P>0.05$; mean disease severity at day 35: 3.00 vs. 2.16, $P>0.05$) (Figs. 2B, 3G and 5). Thus, the activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction is completed within 5 days after the adoptive transfer of immunized splenocytes in active BP model.

3.5. Anti-hCOL17 IgG restored after the early single administration of anti-CD40L monoclonal antibody do not contain anti-hCOL17 NC16A IgG, and only weak pathogenicity is shown

The results above suggested that the early short-term effect of MR1 was sufficient to inhibit the production of anti-hCOL17 NC16A IgG. To observe the phenotypic changes in active BP model without the presence of anti-hCOL17 NC16A IgG, we induced the transient immunosuppressive condition in *Rag*-$2^{-/-}$/COL17-humanized recipients by single injections of 1000 μg of MR1 at day 0 (n=6). The production of anti-hCOL17 IgG in treated mice gradually recovered to levels similar to those in the control mice without MR1-treatment at 7 weeks after the adoptive transfer (Fig. 6A), but the restored IgG did not contain anti-hCOL17 NC16A IgG (Fig. 6B). The disease severity of the treated mice slowly increased but was significantly lower than that of the controls (Fig. 6C). Each of the IgG subclasses (IgG1, IgG2b, IgG2c, IgG3) against hCOL17 showed similar titers between an MR1-treated group and an untreated group at 10 weeks after the adoptive transfer (not shown). Although 3 out of 6 treated mice showed distinct deposition of C3, they

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive CD4+ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

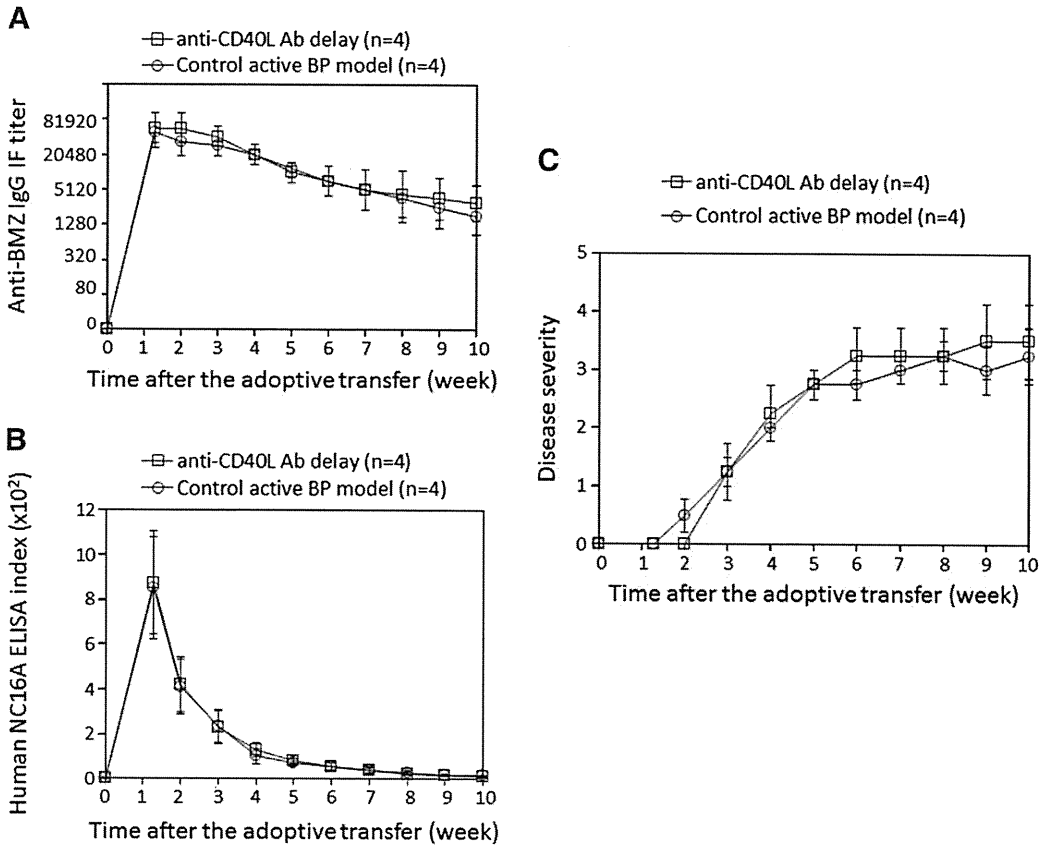


Fig. 4 Delayed treatment with anti-CD40L monoclonal antibody shows no effects in mice with established active BP. MR1 or control hamster IgG was injected into active BP model at days 13, 16 and 19 after the adoptive transfer of immunized splenocytes (n=4, respectively). There are no significant differences in the titers of anti-hCOL17 IgG (A) or anti-hCOL17 NC16A IgG (B), and in disease severity (C) between the groups. $P > 0.05$.

developed only mild skin changes (Fig. 6D). Thus, anti-hCOL17 IgG restored after the transient blockade of CD40–CD40L interaction contain no anti-hCOL17 NC16A IgG and show only weak pathogenicity. This strongly suggests that hCOL17 NC16A-reactive $CD4^+$ T cells play a crucial role in the development of BP lesions in active mouse model.

4. Discussion

This study has demonstrated the pivotal role of COL17 NC16A-reactive $CD4^+$ T cells in BP induction for the first time by using active BP mouse model. We first demonstrated the pathogenic role of $CD4^+$ T cells in active BP model by showing that $CD4^+$ T cells immunized by hCOL17-expressing Tg-skin grafting could activate unimmunized B cells to produce anti-hCOL17 NC16A IgG. We also showed that immunized $CD45R^+$ B cells needed the coexistence of activated $CD4^+$ T cells to produce those IgG. These results suggest that the interaction between activated hCOL17-reactive T cells and B cells is essential for the production of anti-hCOL17 IgG. Administrations of anti-CD40L monoclonal antibody have previously demonstrated the strong suppression of humoral immune responses against autoantigens in some T-cell-mediated antibody-induced autoimmune animal models [20–22, 26]. Therefore, we considered that anti-CD40L monoclonal antibody may be utilized for the modulation of immune responses in active BP model.

Blockade of CD40–CD40L interaction by anti-CD40L monoclonal antibody (MR1) continuously suppressed the production of anti-hCOL17 NC16A IgG and the development of the BP phenotype in active BP model when MR1 was repetitively administered close to the time of adoptive transfer of immunized splenocytes. Although the production of anti-hCOL17 IgG detected by indirect IF study using normal human skin was not completely suppressed by MR1 treatment, ELISA revealed an absence of anti-hCOL17 NC16A IgG, resulting in the prevention of BP skin changes. Enzyme-linked immunospot assay demonstrated quite a small number of anti-hCOL17 NC16A IgG-producing B cells in the spleens of the MR1-treated mice.

Because the crucial role of B cell activation via CD40–CD40L interaction was elucidated at the initial stage of active BP model, we then tried to examine the effects of MR1 at the late stage of active BP model. Since the model starts to produce anti-hCOL17 and anti-hCOL17 NC16A IgG within a week after the adoptive transfer if no immunosuppressive treatment is added [10], we injected MR1 at days 13, 16

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive $CD4^+$ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

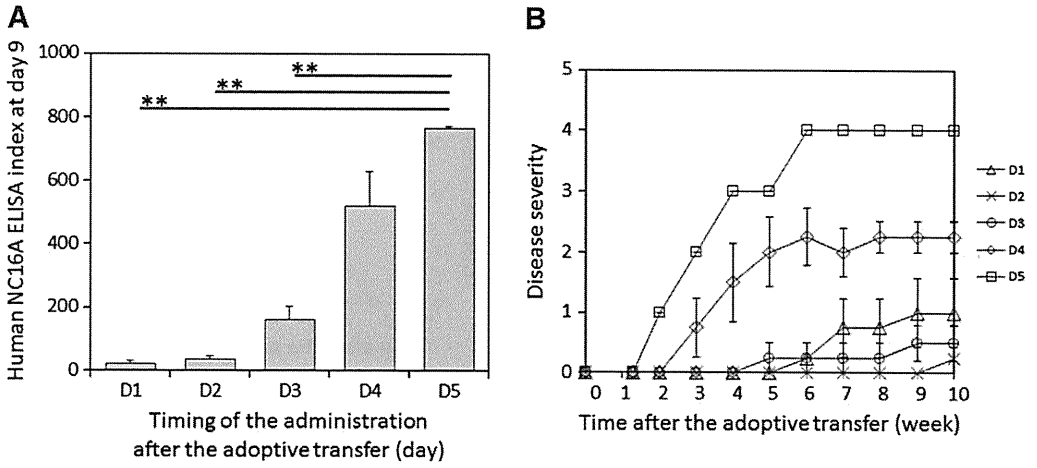


Fig. 5 Activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction is established within 5 days after the adoptive transfer of immunized splenocytes. *Rag-2*$^{-/-}$/COL17-humanized recipients were injected with MR1 just once between days 1 and 5 after the adoptive transfer of immunized splenocytes (n=4, respectively). (A) MR1-treatments at day 1, day 2 or day 3 significantly suppress the titers of anti-hCOL17 NC16A IgG at day 9 compared with those at day 5 (**$P<0.01$). The effect of MR1 gradually decreases if the treatment is initiated late. The IgG titers at day 9 of the mice treated at day 5 are similar to those in active BP model without MR1 treatment (Fig. 2B) (mean index value: 765.3 vs. 918.97, $P>0.05$). (B) Skin changes are strongly suppressed if MR1-treatment is initiated before day 3 after the adoptive transfer. Disease severity of the recipients treated at day 5 is similar to those in active BP model without MR1 treatment (Fig. 3G) (mean disease severity at day 35: 3.00 vs. 2.16, $P>0.05$).

and 19 after the adoptive transfer (delayed treatment). No therapeutic effects were observed in mice with delayed treatment. This result indicates that the CD40–CD40L interaction is not required once the disease is established in active BP model. Similarly, delayed MR1-treatment was unable to suppress the titer of pathogenic antibody in an established pemphigus vulgaris model [21]. Meanwhile, delayed treatment can prevent relapses of ongoing diseases or can halt disease progression in models of multiple sclerosis [27], lupus nephritis [28, 29] and myasthenia gravis [20]. A possible mechanism of those therapeutic effects is the inhibition of epitope spreading. In experimental autoimmune encephalomyelitis, anti-CD40L monoclonal antibody treatment acts in part by inhibiting the expansion and/or differentiation of Th1 effector cells specific to relapse-associated epitopes [27]. Epitope spreading has also been reported in BP patients [30–32] and in an hCOL17-expressing Tg skin-grafting mouse model [33] although it is still unclear whether antibodies against hCOL17 – other than those against the NC16A domain – are pathogenic. Hence, the efficacy of anti-CD40L antibody treatment on epitope spreading in BP seems an interesting line of investigation.

Furthermore, we revealed that the activation of anti-hCOL17 NC16A IgG-producing B cells via CD40–CD40L interaction was completed within 5 days after the adoptive transfer of immunized splenocytes. This suggests that the short-term effect of MR1 at the early stage of active BP is sufficient to inhibit the production of anti-hCOL17 NC16A IgG. Therefore, we tried to investigate the immune responses at the late stage of active BP model under the condition of no anti-hCOL17 IgG by means of early administration of a single dose of MR1. As shown in Figs. 6A and B, the production of anti-hCOL17 NC16A IgG was durably suppressed by the early single MR1-treatment, while the production of anti-hCOL17 IgG gradually recovered. Previous study using active pemphigus vulgaris model demonstrated that MR1-treatment could induce tolerance to desmoglein 3 in the treated mice and the tolerance was transferable [21]. Our results suggest that the MR1-treatment induced immune tolerance to some antigens including hCOL17 NC16A in the treated mice, which induced the durable suppression of the anti-hCOL17 NC16A IgG production. Some other hCOL17-reactive CD4+ T cells which escaped the tolerance-induction might activate B cells as the effect of the MR1-treatment wore off. Of note, the treated mice developed only mild skin changes despite the high titers of restored anti-hCOL17 IgG in the late stage. In this setting, some mice showed the distinct deposition of complements as well as IgG at the BMZ but developed only mild skin changes. Complement activation is considered important in the pathogenesis of BP [34–36], while anti-hCOL17 IgG from BP patients has been proven to reduce the content of hemidesmosomal COL17 and weaken the adhesion of hemidesmosomes to the lamina densa without complements [37]. Thus, the significance of complement activation in the pathogenesis of BP remains controversial. As we reported previously [10], untreated active BP model demonstrates a trend in which the disease severity starts to decrease around 12 weeks after the adoptive transfer. The results shown in Fig. 6 demonstrate that anti-hCOL17 NC16A IgG is the major pathogenic antibody and able to cause severe skin changes for more than 10 weeks after the adoptive transfer. In addition, they indicate that some antibodies against hCOL17 other than against the NC16A domain have weak pathogenicity and partially sustain the disease activity in the late stage of active BP

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive CD4+ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

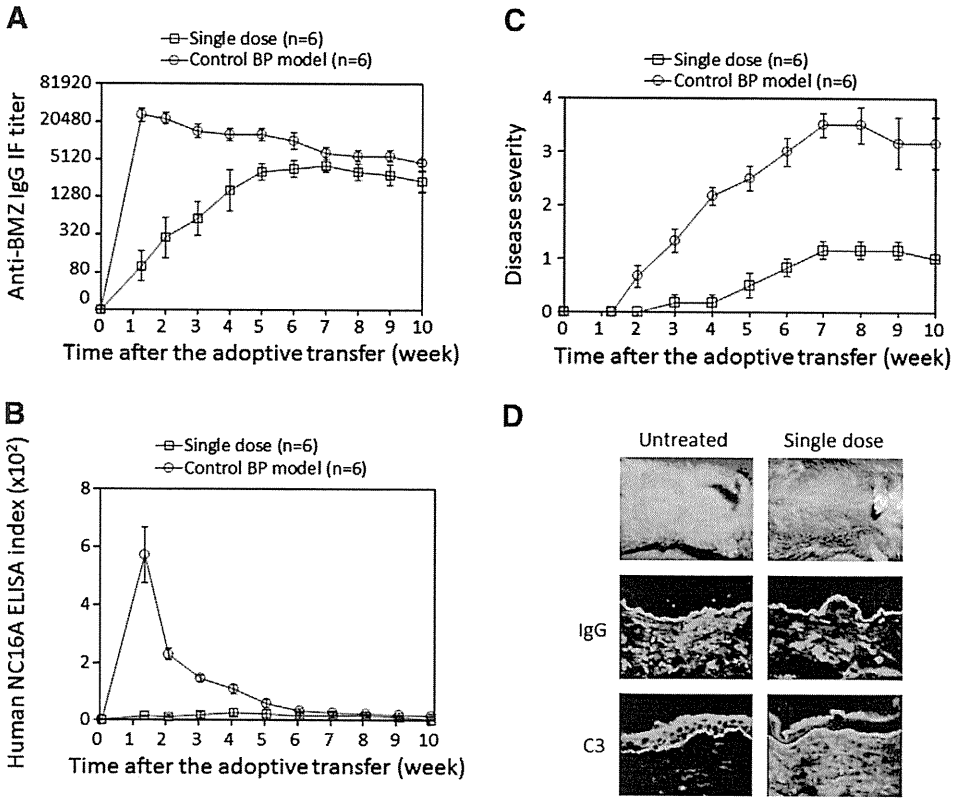


Fig. 6 Early single dose of anti-CD40L monoclonal antibody inhibits the production of anti-hCOL17 NC16A IgG, while the production of anti-hCOL17 IgG is recovered in the late stage. 1000 μg of MR1 was injected into *Rag-2*$^{-/-}$/COL17-humanized recipients at day 0 just once (n=6). (A) Anti-hCOL17 IgG titer gradually increases and reaches to a level similar to that of control active BP model at 7 weeks after the adoptive transfer ($P<0.01$ at days 9, 14 and 21; $P<0.05$ at days 28, 35 and 42; $P>0.05$ at days 0, 49, 56, 63 and 70). (B) Anti-hCOL17 NC16A IgG titers are significantly lower in the treated mice than those in the controls ($P<0.01$ at days 9, 14, 21 and 28). (C) Disease severity of the treated mice slowly increases but is significantly lower than that of the controls ($P<0.05$ at day 14; $P<0.01$ from day 21 to 70). (D) Some of the treated mice show the distinct deposition of C3 and have developed just a mild skin change (Fig. 6D).

model. In conclusion, this study suggests that COL17 NC16A-reactive CD4$^+$ T cells play a pivotal role in the pathogenesis of active BP model via the CD40–CD40L interaction.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

Acknowledgments

The authors wish to thank Ms. Y. Kashima and Ms. M. Tanabe for their technical assistance.

This work was supported in part by Grants-in-Aid for Scientific Research (A) (No. 21249063 to H.S.) and (C) (No. 20591312 to A.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (No. 06–42 to H.S.), and by Grants-in-Aid for Regional R&D Proposal-Based Program from Northern Advancement Center for Science & Technology of Hokkaido Japan (to H.U.). The authors have no conflicts of interest.

References

[1] L.A. Diaz, H. Ratrie III, W.S. Saunders, S. Futamura, H.L. Squiquera, G.J. Anhalt, et al., Isolation of a human epidermal cDNA corresponding to the 180-kD autoantigen recognized by bullous pemphigoid and herpes gestationis sera. Immunolocalization of this protein to the hemidesmosome, J. Clin. Invest. 86 (1990) 1088–1094.

[2] G.J. Giudice, D.J. Emery, L.A. Diaz, Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180, J. Invest. Dermatol. 99 (1992) 243–250.

[3] S.B. Hopkinson, K.S. Riddelle, J.C. Jones, Cytoplasmic domain of the 180-kD bullous pemphigoid antigen, a hemidesmosomal component: molecular and cell biologic characterization, J. Invest. Dermatol. 99 (1992) 264–270.

[4] A. Ishiko, H. Shimizu, A. Kikuchi, T. Ebihara, T. Hashimoto, T. Nishikawa, Human autoantibodies against the 230-kD bullous pemphigoid antigen (BPAG1) bind only to the intracellular domain of the hemidesmosome, whereas those against the 180-kD bullous pemphigoid antigen (BPAG2) bind along the plasma membrane of the hemidesmosome in normal human and swine skin, J. Clin. Invest. 91 (1993) 1608–1615.

[5] C. Bedane, J.R. McMillan, S.D. Balding, P. Bernard, C. Prost, J.M. Bonnetblanc, et al., Bullous pemphigoid and cicatricial pemphigoid autoantibodies react with ultrastructurally separable epitopes on the BP180 ectodomain: evidence that BP180

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive CD4$^+$ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

spans the lamina lucida, J. Invest. Dermatol. 108 (1997) 901–907.
[6] J.R. McMillan, M. Akiyama, H. Shimizu, Epidermal basement membrane zone components: ultrastructural distribution and molecular interactions, J. Dermatol. Sci. 31 (2003) 169–177.
[7] G.J. Giudice, D.J. Emery, B.D. Zelickson, G.J. Anhalt, Z. Liu, L.A. Diaz, Bullous pemphigoid and herpes gestationis autoantibodies recognize a common non-collagenous site on the BP180 ectodomain, J. Immunol. 151 (1993) 5742–5750.
[8] D. Zillikens, P.A. Rose, S.D. Balding, Z. Liu, M. Olague-Marchan, L.A. Diaz, et al., Tight clustering of extracellular BP180 epitopes recognized by bullous pemphigoid autoantibodies, J. Invest. Dermatol. 109 (1997) 573–579.
[9] Y. Tsuji-Abe, M. Akiyama, Y. Yamanaka, T. Kikuchi, K.C. Sato-Matsumura, H. Shimizu, Correlation of clinical severity and ELISA indices for the NC16A domain of BP180 measured using BP180 ELISA kit in bullous pemphigoid, J. Dermatol. Sci. 37 (2005) 145–149.
[10] H. Ujiie, A. Shibaki, W. Nishie, D. Sawamura, G. Wang, Y. Tateishi, et al., A novel active mouse model for bullous pemphigoid targeting humanized pathogenic antigen, J. Immunol. 184 (2010) 2166–2174.
[11] L. Budinger, L. Borradori, C. Yee, R. Eming, S. Ferencik, H. Grosse-Wilde, et al., Identification and characterization of autoreactive T cell responses to bullous pemphigoid antigen 2 in patients and healthy controls, J. Clin. Invest. 102 (1998) 2082–2089.
[12] M.S. Lin, C.L. Fu, G.J. Giudice, M. Olague-Marchan, A.M. Lazaro, P. Stastny, et al., Epitopes targeted by bullous pemphigoid T lymphocytes and autoantibodies map to the same sites on the bullous pemphigoid 180 ectodomain, J. Invest. Dermatol. 115 (2000) 955–961.
[13] S. Thoma-Uszynski, W. Uter, S. Schwietzke, G. Schuler, L. Borradori, M. Hertl, Autoreactive T and B cells from bullous pemphigoid (BP) patients recognize epitopes clustered in distinct regions of BP180 and BP230, J. Immunol. 176 (2006) 2015–2023.
[14] J.C. Delgado, D. Turbay, E.J. Yunis, J.J. Yunis, E.D. Morton, K. Bhol, et al., A common major histocompatibility complex class II allele HLA-DQB1* 0301 is present in clinical variants of pemphigoid, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 8569–8571.
[15] M. Aoki-Ota, K. Tsunoda, T. Ota, T. Iwasaki, S. Koyasu, M. Amagai, et al., A mouse model of pemphigus vulgaris by adoptive transfer of naive splenocytes from desmoglein 3 knockout mice, Br. J. Dermatol. 151 (2004) 346–354.
[16] A.G. Sitaru, A. Sesarman, S. Mihai, M.T. Chiriac, D. Zillikens, P. Hultman, et al., T cells are required for the production of blister-inducing autoantibodies in experimental epidermolysis bullosa acquisita, J. Immunol. 184 (2010) 1596–1603.
[17] G.X. Zhang, B.G. Xiao, M. Bakhiet, P. van der Meide, H. Wigzell, H. Link, et al., Both CD4+ and CD8+ T cells are essential to induce experimental autoimmune myasthenia gravis, J. Exp. Med. 184 (1996) 349–356.
[18] R.J. Noelle, M. Roy, D.M. Shepherd, I. Stamenkovic, J.A. Ledbetter, A. Aruffo, A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 6550–6554.
[19] A.L. Peters, L.L. Stunz, G.A. Bishop, CD40 and autoimmunity: the dark side of a great activator, Semin. Immunol. 21 (2009) 293–300.
[20] S.H. Im, D. Barchan, P.K. Maiti, S. Fuchs, M.C. Souroujon, Blockade of CD40 ligand suppresses chronic experimental myasthenia gravis by down-regulation of Th1 differentiation and up-regulation of CTLA-4, J. Immunol. 166 (2001) 6893–6898.
[21] M. Aoki-Ota, M. Kinoshita, T. Ota, K. Tsunoda, T. Iwasaki, S. Tanaka, et al., Tolerance induction by the blockade of CD40/CD154 interaction in pemphigus vulgaris mouse model, J. Invest. Dermatol. 126 (2006) 105–113.
[22] M. Ohyama, T. Ota, M. Aoki, K. Tsunoda, R. Harada, S. Koyasu, et al., Suppression of the immune response against exogenous desmoglein 3 in desmoglein 3 knockout mice: an implication for gene therapy, J. Invest. Dermatol. 120 (2003) 610–615.
[23] E.B. Olasz, J. Roh, C.L. Yee, K. Arita, M. Akiyama, H. Shimizu, et al., Human bullous pemphigoid antigen 2 transgenic skin elicits specific IgG in wild-type mice, J. Invest. Dermatol. 127 (2007) 2807–2817.
[24] M. Amagai, K. Tsunoda, H. Suzuki, K. Nishifuji, S. Koyasu, T. Nishikawa, Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus, J. Clin. Invest. 105 (2000) 625–631.
[25] C. Sitaru, S. Mihai, C. Otto, M.T. Chiriac, I. Hausser, B. Dotterweich, et al., Induction of dermal-epidermal separation in mice by passive transfer of antibodies specific to type VII collagen, J. Clin. Invest. 115 (2005) 870–878.
[26] C.M. Lanschuetzer, E.B. Olasz, Z. Lazarova, K.B. Yancey, Transient anti-CD40L co-stimulation blockade prevents immune responses against human bullous pemphigoid antigen 2: implications for gene therapy, J. Invest. Dermatol. 129 (2009) 1203–1207.
[27] L.M. Howard, A.J. Miga, C.L. Vanderlugt, M.C. Dal Canto, J.D. Laman, R.J. Noelle, et al., Mechanisms of immunotherapeutic intervention by anti-CD40L (CD154) antibody in an animal model of multiple sclerosis, J. Clin. Invest. 103 (1999) 281–290.
[28] S.L. Kalled, A.H. Cutler, S.K. Datta, D.W. Thomas, Anti-CD40 ligand antibody treatment of SNF1 mice with established nephritis: preservation of kidney function, J. Immunol. 160 (1998) 2158–2165.
[29] S.A. Quezada, M. Eckert, O.A. Adeyi, A.R. Schned, R.J. Noelle, C.M. Burns, Distinct mechanisms of action of anti-CD154 in early versus late treatment of murine lupus nephritis, Arthritis Rheum. 48 (2003) 2541–2554.
[30] G. Di Zenzo, F. Grosso, M. Terracina, F. Mariotti, O. De Pita, K. Owaribe, et al., Characterization of the anti-BP180 autoantibody reactivity profile and epitope mapping in bullous pemphigoid patients, J. Invest. Dermatol. 122 (2004) 103–110.
[31] G. Di Zenzo, S. Thoma-Uszynski, L. Fontao, V. Calabresi, S.C. Hofmann, T. Hellmark, et al., Multicenter prospective study of the humoral autoimmune response in bullous pemphigoid, Clin. Immunol. 128 (2008) 415–426.
[32] G. Di Zenzo, S. Thoma-Uszynski, V. Calabresi, L. Fontao, S.C. Hofmann, J.P. Lacour, et al., Demonstration of epitope-spreading phenomena in bullous pemphigoid: results of a prospective multicenter study, J. Invest. Dermatol. 131 (2011) 2271–2280.
[33] G. Di Zenzo, V. Calabresi, E.B. Olasz, G. Zambruno, K.B. Yancey, Sequential intramolecular epitope spreading of humoral responses to human BPAG2 in a transgenic model, J. Invest. Dermatol. 130 (2010) 1040–1047.
[34] Q. Li, H. Ujiie, A. Shibaki, G. Wang, R. Moriuchi, H.J. Qiao, et al., Human IgG1 monoclonal antibody against human collagen 17 noncollagenous 16A domain induces blisters via complement activation in experimental bullous pemphigoid model, J. Immunol. 185 (2010) 7746–7755.
[35] Z. Liu, G.J. Giudice, S.J. Swartz, J.A. Fairley, G.O. Till, J.L. Troy, et al., The role of complement in experimental bullous pemphigoid, J. Clin. Invest. 95 (1995) 1539–1544.
[36] G. Wang, H. Ujiie, A. Shibaki, W. Nishie, Y. Tateishi, K. Kikuchi, et al., Blockade of autoantibody-initiated tissue damage by using recombinant fab antibody fragments against pathogenic autoantigen, Am. J. Pathol. 176 (2010) 914–925.
[37] H. Iwata, N. Kamio, Y. Aoyama, Y. Yamamoto, Y. Hirako, K. Owaribe, et al., IgG from patients with bullous pemphigoid depletes cultured keratinocytes of the 180-kDa bullous pemphigoid antigen (type XVII collagen) and weakens cell attachment, J. Invest. Dermatol. 129 (2009) 919–926.

Please cite this article as: H. Ujiie, et al., Noncollagenous 16A domain of type XVII collagen-reactive CD4+ T cells plays a pivotal role in the development of active disease..., Clin. Immunol. (2011), doi:10.1016/j.clim.2011.10.002

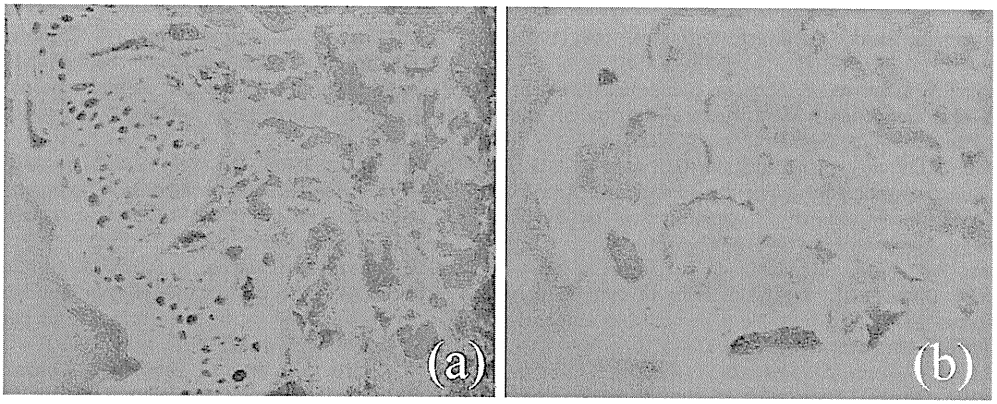


Fig. 2. Indirect immunofluoresence for collagen VII autoantibodies on normal skin (a) and collagen VII deficient skin (b) with serum from EBA patient, 200×.

We agree with the authors that more studies are indicated to determine the use of this test for monitoring disease activity in EBA patients. Similar studies in pemphigus patients with recombinant desmoglein 1 and 3 ELISA's reveal that the sera with identical titers of antibodies by IIF give variable results with ELISA [7]. Unless high titer sera are diluted, saturation of antibody–antigen reactions in ELISA may lead to false low positive ELISA index values to begin with. Such sera may not appear to show a decline in ELISA index values with treatment response [8]. We also have observed, in some pemphigus sera, that even though the IIF titers show a decline, ELISA index values still remain high. Therefore, we may have to use this ELISA with caution to monitor the disease.

References

[1] Saleh MA, Ishii K, Kim YJ, Murakami A, Ishii N, Hashimoto T, et al. Development of NC1 and NC2 domains of Type VII collagen ELISA for the diagnosis and analysis of the time course of epidermolysis bullosa acquisita patients. J Dermatol Sci 2011;62(3):169–75.
[2] Gammon WR, Briggaman RA, Inman III AO, Queen LL, Wheeler CE. Differentiating anti-lamina lucida and anti-sublamina densa anti-BMZ antibodies by indirect immunofluorescence on 1.0 M sodium chloride-separated skin. J Invest Dermatol 1984;82(2):139–44.
[3] Vodegel RM, de Jong MC, Pas HH, Yancey KB, Jonkman MF. Anti-epiligrin cicatricial pemphigoid and epidermolysis bullosa acquisita: differentiation by use of indirect immunofluorescence microscopy. J Am Acad Dermatol 2003;48(4):542–7.
[4] Parker SR, MacKelfresh J. Autoimmune blistering diseases in the elderly. Clin Dermatol 2011;29(1):69–79.
[5] Ishii N, Yoshida M, Hisamatsu Y, Ishida-Yamamoto A, Nakane H, Iizuka H, et al. Epidermolysis bullosa acquisita sera react with distinct epitopes on the NC1 and NC2 domains of type VII collagen: study using immunoblotting of domain-specific recombinant proteins and postembedding immunoelectron microscopy. Br J Dermatol 2004;150(5):843–51.
[6] Ishii N, Yoshida M, Ishida-Yamamoto A, Fritsch A, Elfert S, Bruckner-Tuderman L, et al. Some epidermolysis bullosa acquisita sera react with epitopes within the triple-helical collagenous domain as indicated by immunoelectron microscopy. Br J Dermatol 2009;160(5):1090–3.
[7] Bystryn JC, Akman A, Jiao D. Limitations in enzyme-linked immunosorbent assays for antibodies against desmogleins 1 and 3 in patients with pemphigus. Arch Dermatol 2002;138(9):1252–3.
[8] Cheng SW, Kobayashi M, Kinoshita-Kuroda K, Tanikawa A, Amagai M, Nishikawa T. Monitoring disease activity in pemphigus with enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3. Br J Dermatol 2002;147(2):261–5.

E. Eugene Bain[a], Raminder K. Grover[b,*], Richard W. Plunkett[b], Ernst H. Beutner[a,b]

[a]*Department of Dermatology, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14203, USA;*

[b]*Beutner Laboratories and the Department of Microbiology and Immunology, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14214, USA*

*Corresponding author at: 138 Farber Hall, Beutner Laboratories and the Departments of Microbiology and Immunology, School of Medicine and Biomedical Sciences, University at Buffalo, SUNY, Buffalo, NY 14215, USA. Tel.: +1 716 838 0549; fax: +1 716 838 0798
E-mail address: rgrover2@buffalo.edu (R.K. Grover)

27 July 2011

doi:10.1016/j.jdermsci.2011.12.004

Letter to the Editor

CYP4F22 is highly expressed at the site and timing of onset of keratinization during skin development

Keywords:
Ichthyosis;
Keratinization;
Skin barrier

Autosomal recessive congenital ichthyoses (ARCI) include several subtypes: harlequin ichthyosis (HI), lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE). To date, six causative genes have been identified in ARCI patients: *ABCA12*, *TGM1*, *NIPAL4*, *CYP4F22*, *ALOXE3* and *ALOX12B* [1]. The localization of transglutaminase 1, ABCA12 and 12R-lipoxygenase have been analyzed using samples from patients and model mice [1]. However, as for NIPAL4, CYP4F22, and lipoxygenase-3, neither localization nor function has been fully clarified yet. Herein, we investigate the expression pattern and localization of NIPAL4, CYP4F22 and lipoxygenase-3 in developing human epidermis and primary cultured normal human keratinocytes.

By quantitative reverse transcription (RT)-PCR analysis, at 10 and 14 weeks EGA, mRNA of *NIPAL4*, *CYP4F22* and *ALOXE3* was hardly expressed (Fig. 1A). The *CYP4F22* mRNA expression at 18