臍帯血幹細胞を使用するための倫理委員会の承認手続きは終了しており、ヒト免疫担当細胞群を再構築したモデルマウスの作成を推進するとともに、得られたモデルマウス HLA タイピングを進め、HLA-B51,-DR2, -DR3 など疾患関連 HLA アレルの免疫応答への影響、免疫担当細胞の挙動への影響等の解析を推進する。

E. 研究発表

1. 論文発表

Qin J, Konno H, Ohshima D, Yanai H, Motegi H, Shimo Y, Hirota F, Matsumoto M, Takaki S, Inoue J, Akiyama T. Developmental stage-dependent collabo- ration between the TNF receptor- associated factor 6 and lymphotoxin pathways for B cell follicle organization in secondary lymphoid organs. J Immunol. 2007; 179: 6799-6807.

Yana I, Sagara H, Takaki S, Takatsu K, Nakamura K, Nakao K, Katsuki M, Taniguchi SI, Aoki T, Sato H, Weiss SJ, Seiki M. Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells. J Cell Sci. 2007; 120:1607-1614.

Seita J, Ema H, Ooehara J, Yamazaki S, Tadokoro Y, Yamasaki A, Eto K, Takaki S, Takatsu K, Nakauchi H. Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin- mediated signal transduction. Proc Natl Acad Sci U S A. 2007; 104:2349-2354.

Takizawa S, Takatsu K, Takaki S. Transient blocking of Lnk-mediated pathways as a potential approach to promote engrafting ability of hematopoietic progenitor cells. Inflammation and Regeneration. 2007; 27: 59-64.

2. 学会発表

Takaki S, Iwasaki Y, Kubo C, Takizawa H, Takatsu K. Control of cell adhesion and migration in B lymphocytes and hemato- poietic progenitors by Lnk, a negative regulator of cytokine signaling. The 13th International Congress of Immunology. Rio de Janeiro, Brazil. Aug 2007.

Takizawa H, Eto K, Nakauchi H, Takatsu K, Takaki S. Lnk, An Adaptor Protein, Modulates Signals mediated through integ- rins and cytokine receptors in hemato- poietic progenitors. The 13th International Congress of Immunology. Rio de Janeiro, Brazil. Aug 2007.

Takaki S, Takizawa H, Iwasaki Y, Katayama H, Kubo C, Takatsu K. Dual- functional adaptor Lnk controls lympho- hematopoietic cells by regulating cytokine- and adhesion-dependent signals. 第 36 回 日本免疫学会学術集会. 東京. 2007 年 11 月.

Ikutani M, Kouro T, Miyake K, Takatsu K, Takaki S. A hairy/enhancer of split related transcription factor, a target of Btk- dependent preBCR signal, controls transi- tion from ProB to PreB. 第 36 回 日本免疫学会学術集会. 東京. 2007 年 11 月.

Iwasaki Y, Takizawa H, Yamamoto K, Takatsu K, Takaki S. Control of Dendritic Cell Production by Lnk/SH2B3 adaptor protein, a negative regulator of lympho- hematopoiesis. 第 36 回

日本免疫学会学術集会. 東京. 2007年11月.

Katayama H, Ikutani M, Yoshida N, Takatsu K, Takaki S. Dynamic expression patterns of Lnk, a negative regulatory adaptor in lymphohematopoiesis revealed by gene- rating a GFP knock-in reporter mice. 第36回 日本免疫学会学術集会. 東京. 2007年11月.

F. 知的財産権の出願・登録状況

1. 特許取得

無し

2. 実用新案登録

無し

3. その他

無し

厚生労働科学研究補助金
(難治性疾患克服事業)

HLA多型が寄与する自己免疫疾患の発症機序の解明　分担研究報告書

自己免疫疾患患者HLAと臨床像の関連についての解析

分担研究者　三森明夫　　国立国際医療センター膠原病科（第一病棟部長）
研究協力者　伊藤健司　同　上（リウマチ科医長）

研究要旨

免疫担当細胞群間での調節機能へのHLA多型の関与を、疾患モデル動物を用いて検討する。アセチルコリン受容体で免役することで、マウスの歩行機能が低下する実験的重症筋無力症マウスを用いる。ヒトの治療と同様に、このマウスに大量免疫グロブリンを投与することで、症状が回復することを確認できている。ヒトでの報告と同様に樹状細胞、NK細胞、T細胞間での調節作用が関与していると考えられる。

疾患関連HLAを保有する自己免疫疾患患者の病態、合併症が疾患関連HLAを保有しない患者群と差異を示すかを検討するため、当科の関節リウマチ通院者430人の診療データベース作成を開始した。このデータベースから得られる匿名化された情報は、同施設内の分担研究者と共有され、それぞれの研究結果の解析にも用いられる予定である。基礎資料として、当科のデータベースは有用と考えられる。

A.研究目的

HLAの免疫制御機能という新しい視点で免疫攪乱現象を解析することにより、HLA多型と免疫難病との相関に分子基盤を与え、新たな診断・治療法の開発に資する分子標的を見出すことを目的とする。

免疫反応に関わる細胞群間での機能調節へのHLA多型の関与は不明な点が多いが、MHC受容体が刺激性、抑制性双方のシグナル伝達に関与しているとの報告が増加している。

自己免疫疾患のモデル動物の１つである実験的重症筋無力症マウスを用い、発病期と治療反応期での免疫担当細胞群およびサイトカイン産生の解析、またそれらの反応におけるMHC結合分子関与を解析する。

疾患関連HLAを保有する自己免疫疾患患者の病

態、合併症が疾患関連 HLA を保有しない患者群と差異を示すかを検討する。その基礎となる自施設通院症例のデータベース作成を、本年度から開始した。

B.研究方法

実験的重症筋無力症マウスを用いた解析

免疫担当細胞群間での調節機能への HLA 多型の関与を、株式会社ベネシスとの共同研究で、疾患モデル動物を用いて検討する。

マウスにシビレエイのアセチルコリン受容体（AChR）で免役することで、AChR に対する抗体価が上昇し、歩行機能が低下する実験的重症筋無力症マウスを作成する。ヒトの重症筋無力症の治療と同様に、このマウスに大量免疫グロブリン（400mg/kg/day、5 日間）を投与することで、症状が回復することが分かっている。このマウスを用いて、症状発症期、治療後の免疫担当細胞群それぞれの細胞数、活性化状態、サイトカイン産生能を解析する。

症例データベース解析

本年度から、関節リウマチ通院者の診療記録（治療薬の副作用と効果、合併症）のデータベース作成を開始した。現在までに通院者約 430 人中 240 人につき記入した。今後も記載を追加し時系列化する。

データベース内容：背景（性別、発症連例、家族歴など）、検査値（リウマトイド因子、抗 CCP 抗体を含む）、関節炎所見、使用薬剤（ステロイド薬、メトトレキサート、生物製剤、ほかの抗リウマチ薬、ビスフォスフォネート）、合併症。

（倫理的配慮）

データベース作成時の患者へのインタビュー、および血液検体収集を含む本研究は、国立国際医療センターの倫理審査会で承認された。この申請内容を遵守する。データベースには患者の HLA 情報の追加が必要であるが、次年度での施行を目標に倫理審査予定である。

C.研究結果

実験的重症筋無力症マウスを用いた解析

実験的重症筋無力症マウスに大量免疫グロブリンを投与し、コントロール治療群に比べ、有意な歩行機能の回復を観察した。

症例データベース解析

関節リウマチ症例の途中集計における、メトトレキサート使用者比率は、78%（186/240）だった。

生物製剤の効果と継続率：

現在までに 92 人（Infliximab 76 例, Etanercept 29 例、相互の変更 13 例）に使用した（使用率；92/430=21%）。Infliximab について、継続率 60%であり、副作用による中止 15%、効果不十分 13%、ほかはメトトレキサート中止に伴う中止ないし本人の希望などによる。Etanercept 継続率は未集計である。

慢性下気道病変の合併率および非定型抗酸菌症の検出率：

リウマチ肺のうち、臨床的に慢性咳痰を呈するものは病理的に細気管支炎と推定される。この合併症をもつ患者は関節炎罹患歴が長い傾向があるため生物製剤使用率が高まる可能性があり、慢性下気道病変に伴い非定型抗酸菌（NM）保有率が高い可能性があることから、これらの実態を把握する必要がある。痰を採取できた患者全員に抗酸菌

培養とPCR検査を行なった。その結果、慢性咳・痰は患者の13%にみられ、生物製剤の使用者で、非使用者に比し(27/92,29% vs 30/338, 9%; p<0,0001)有意に高率に認めた。喀痰中のNM陽性率は20%と高値だった。NM症は、画像異常を伴った1例で生物製剤開始後に悪化し、中止と抗菌治療で改善した。この1例以外の生物製剤使用例に画像異常はなく、NM症の発症もなかった。

D.考察

実験的重症筋無力症マウスを大量免疫グロブリンで治療する際の病理、病態については不明である。ヒトでの報告では、NK細胞による樹状細胞への細胞障害の亢進が観察されており、結果として樹状細胞によるT細胞活性化が阻害されることになる。また、治療後長期にわたって、NK細胞のリンパ組織への移行が観察されているが、この現象と免疫抑制効果との関連は不明である。NK細胞はその表面のクラスI MHC結合性抑制性受容体を介し、免疫調節を行っていることが報告されている。今後はこのモデルマウスにおけるクラスI MHC結合性抑制性受容体の発現をスクリーニングし、病態および治療反応性との関連について検索予定である。また同時に他の免疫担当細胞群の数や、表面抗原発現の変化、サイトカイン産生能についても検索予定である。

関節リウマチ患者のデータベースからは、当科の患者集団では、メトトレキサート使用率が高かった。本研究計画の初期の目標である、この薬の副作用と効果の分析が可能になると思われる。

関節リウマチ合併症である慢性下気道炎症(細気管支炎)は13%という高率で検出され、予想どおり生物製剤使用者の中に多かった。また喀痰中の非定型抗酸菌(NM)陽性率は高かった。以上のような集計評価はこれまでに文献報告されていない。我々の集計では、画像異常を伴わないNM陽性のみなら、生物製剤を使用して危険はないことが示唆されたが、今後も集計、追跡が必要である。今後はこのデータベースにHLA情報を加え、HLA多型と臨床像の関連について解析予定である。この情報はヒト細胞を用いた研究を予定している当研究班の分担研究者にとっても有用になると考えられる。

E.結論

実験的重症筋無力症マウスは自己免疫疾患の活動期および治療時における免疫担当細胞群間での相互作用の解析に有用であると考えられた。

関節リウマチの病像、治療効果と副作用、合併症を分析し、そこにHLA情報を加えることで、HLA多型が自己免疫疾患の発症、病態に与える影響を考察するための基礎資料として、当科のデータベースは有用と考えられた。

G.研究発表

1. 論文発表

国内

1. 高橋裕子、森口正人、住永佳久、長汐千秋、狩野俊和、鈴木暁岳、国松淳和、浅尾りん、山下裕之、伊藤健司、三森明夫.:Segmental arterial mediolysis の一例. 日臨免会誌 30(3):193-197, 2007.

海外

1. Nagatani K, Itoh K, Nakajima K, Kuroki H, Katsuragawa Y, Mochizuki, M Aotsuka S, Mimori

A: Rheumatoid arthritis fibroblast-like synoviocytes express BCMA and are stimulated by APRIL. Arthritis Rheum 56(11);3554-3563, 2007.

2. Nakajima K, Itoh1,K, Nagatani K, Okawa M, Fujii T, Kuroki H, Katsuragawa Y, Aotsuka S, Mimori A: Expressionof BAFF and BAFF-R in the synovial tissue of patients with rheumatoid arthritis. Scand J Rheumatol 36:365–372, 2007.
3. Okawa-Takatsuji M, Nagatani K, Nakajima K, Itoh K, Kano T, Nagashio C, Takahashi Y, Aotsuka S, Mimori A: Recruitment of immature neutrophils in peripheral blood following leukocytapheresis therapy for rheumatoid arthritis. J Clin Apheresis 22:323-329, 2007
4. Soejima M, Sugiura T, Kawaguchi Y, Kawamoto M, Katsumata Y, Takagi K, Nakajim A, MitamuraT, Mimori A, Hara M, and Kamatani N: Association of the diplotype configuration at the N-acetyltransferase 2 gene with adverse evnts with co-trioxazole in Japanese patients with systemic lupus erythematosus. Arthritis Res Ther 9(2): R23, 2007

2.学会発表

国内

1. 高橋裕子，国松淳和，浅尾りん，山下裕之，鈴木暁岳、伊藤健司、三森明夫：高安病に伴った難治性反復性多関節炎の 1 例. 第 542 回内科学会関東地方会 東京，2 月，2007
2. 浅尾りん、高橋裕子、國松淳和,山下裕之，鈴木暁岳,伊藤健司, 三森明夫: TNF 遮断治療中の関節リウマチ患者における非定型抗酸菌症. 第 51 回日本リウマチ学会総会，横浜，2007 年 4 月
3. 国松淳和，伊藤健司，浅尾りん，高橋裕子，山下裕之，鈴木暁岳，三森明夫：リウマチ性多発筋痛症と繊維筋痛症における脳血流低下所見. 第 51 回日本リウマチ学会総会，横浜，2007 年 4 月
4. 国松淳和，伊藤健司，浅尾りん，高橋裕子，山下裕之，鈴木暁岳，三森明夫：不明熱の疾患スペクトラムの再評価. 第 51 回日本リウマチ学会総会，横浜，2007 年 4 月
5. 山下裕之，浅尾りん，国松淳和，高橋裕子，鈴木暁岳，伊藤健司，三森明夫：遺伝背景が推定された自己免疫性 TTP 合併シェーグレン症候群の 1 例. 第51回日本リウマチ学会総会, 横浜, 2007 年 4 月
6. 山下裕之，浅尾りん，国松淳和，高橋裕子，鈴木暁岳，伊藤健司，三森明夫：抗 TNF 抗体治療中の関節リウマチ 1 例に生じた，ニューモシステイス肺炎の異例な病像。第 51 回日本リウマチ学会総会，横浜，2007 年 4 月
7. 鈴木暁岳，山下裕之，浅尾りん，国松淳和，高橋裕子，伊藤健司，三森明夫：抗 CD20 抗体とシクロフォスファミドパルス療法（IVCY）により治療に成功した SLE による反応性難治性血球貪食症候群の一例。第 51 回日本リウマチ学会総会，横浜，2007 年 4 月
8. 高橋裕子、杉山温人、狩野俊和、鈴木暁岳、國松淳和、浅尾りん、山下裕之、吉田佳宏、伊藤健司、三村俊英、三森明夫：顕微鏡的多発動脈炎におけるシクロフォスファミドの適応（第 2 報）第 51 回日本リウマチ学会総会，横浜，2007 年 4 月
9. 高橋裕子，鈴木暁岳，國松淳和，山下裕之，浅尾りん，狩野俊和，伊藤健司，三森明夫：ループス腎炎の予後決定因子，病歴データベースによる検討 第 51 回日本リウマチ学会総会，横浜，2007 年 4 月
10. 柳井敦、高橋裕子、山下裕之、鈴木暁岳、伊藤

健司、三森明夫：シクロフォスファミドパルス療法（IVCY）により軽快したSLEによる反応性難治性血球貪食症候群の一例　第18回日本リウマチ学会関東支部会，横浜，12月，2007

11. 高橋裕子、柳井敦、山下裕之、鈴木暁岳、伊藤健司、三森明夫：成人発症Still病による髄膜炎の一例　第18回日本リウマチ学会関東支部会，横浜，12月，2007
12. 柳井敦，高橋裕子，山下裕之，鈴木暁岳，伊藤健司，三森明夫：成人発症Still病に合併した眼瞼炎の一例　第551回内科学会関東地方会，東京，2月2008年

海外

1. Takahashi Y, Suzuki A, Kunimatsu J, Yamashita H, Asao R, Kano T, Itoh K, Mimori A: Prognostic factores of lupus nephritis other than renal pathology. 75th American College of Rheumatology, Annual Scientific Meeting, Boston, November, 2007

H.知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

V. 研究成果の刊行に関する一覧表

Ⅶ. 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tai LH, Goulet ML, Belanger S, Troke AD, St-Laurent AG, Mesci A, Toyama-Sorimachi N*, Carlyle JR*, Makrigiannis AP.* (*These authors were equally contributed.)	Recognition of H-2K(b) by Ly49Q suggests a role for class Ia MHC regulation of plasmacytoid dendritic cell function.	Molecular Immunology	44	2638-2646	2007
Hoshino A, Kawamura YI, Yasuhara M, Toyama-Sorimachi N, Yamamoto K, Matsukawa A, Lira SA, Dohi T.	Inhibition of CCL1-CCR8 interaction prevents aggregation of macrophages and development of peritoneal adhesions.	The Journal of Immunology	178	5296-5304	2007
Mizutani N, Sakurai T, Shibata T, Uchida K, Fujita J, Kawashima R, Kawamura YI, Toyama-Sorimachi N, Imai T, Dohi T.	Dose-dependent differential regulation of cytokine secretion from macrophages by fractalkine.	The Journal of Immunology	179	7478-7487	2007
Gays F, Aust JG, Reid DM, Falconer J, Toyama-Sorimachi N, Taylor PR, Brooks CG.	Ly49B is expressed on multiple subpopulations of myeloid cells.	The Journal of Immunology	177	5840-5851	2007
Oda H, Suzuki H, Sakai K, Kitahara S, Patrick MS, Azuma Y, Sugi K, Kitamura T, Kaye J, Shirai M.	Rac1-mediated Bcl-2 induction is critical in antigen-induced CD4 single-positive differentiation of a CD4+CD8+ immature thymocyte line.	Journal of Leukocyte Biology	81	500-508	2007
Shiroki F, Matsuda S, Doi T, Fujiwara M, Mochizuki Y, Kadowaki T, Suzuki H, Koyasu S.	The p85alpha regulatory subunit of class IA phosphoinositide 3-kinase regulates beta-selection in thymocyte development.	The Journal of Immunology	178	1349-1356	2007

Sakai K, Suzuki H, Oda H, Akaike T, Azuma Y, Murakami T, Sugi K, Ito T, Ichinose H, Koyasu S, Shirai M.	Phosphoinositide 3-kinase in nitric oxide synthesis in macrophage: critical dimerization of inducible nitric-oxide synthase.	The Journal of Biological Chemistry	281	17736-17742	2006
Qin J, Konno H, Ohshima D, Yanai H, Motegi H, Shimo Y, Hirota F, Matsumoto M, Takaki S, Inoue J, Akiyama T.	Developmental stage-dependent collaboration between the TNF receptor-associated factor 6 and lymphotoxin pathways for B cell follicle organization in secondary lymphoid organs.	The Journal of Immunology	179	6799-6807	2007
Yana I, Sagara H, Takaki S, Takatsu K, Nakamura K, Nakao K, Katsuki M, Taniguchi S, Aoki T, Sato H, Weiss SJ, Seiki M.	Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells.	Journal of cell science	120	1607-1614	2007
Seita J, Ema H, Ooehara J, Yamazaki S, Tadokoro Y, Yamasaki A, Eto K, Takaki S, Takatsu K, Nakauchi H.	Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction.	Proceedings of the National Academy of Sciences of the United States of America	104	2349-2354	2007
Takizawa S, Takatsu K, Takaki S	Transient blocking of Lnk-mediated pathways as a potential approach to promote engrafting ability of hematopoietic progenitor cells.	Inflammation and Regeneration	27	59-64	2007
Nagatani K, Itoh K, Nakajima K, Kuroki H, Katsuragawa Y, Mochizuki M, Aotsuka S, Mimori A.	Rheumatoid arthritis fibroblast-like synoviocytes express BCMA and are stimulated by APRIL.	Arthritis and rheumatism	11	3554-3563	2007
Nakajima K, Itoh K, Nagatani K, Okawa-Takatsuji M, Fujii T, Kuroki H, Katsuragawa Y, Aotsuka S, Mimori A.	Expression of BAFF and BAFF-R in the synovial tissue of patients with rheumatoid arthritis.	Scandinavian journal of rheumatology	36	365-372	2007

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okawa-Takatsuji M, Nagatani K, Nakajima K, Itoh K, Kano T, Nagashio C, Takahashi Y, Aotsuka S, Mimori A.	Recruitment of immature neutrophils in peripheral blood following leukocytapheresis therapy for rheumatoid arthritis.	Journal of clinical aphaeresis	22	323-329	2007
Soejima M, Sugiura T, Kawaguchi Y, Kawamoto M, Katsumata Y, Takagi K, Nakajima A, Mitamura T, Mimori A, Hara M, Kamatani N.	Association of the diplotype configuration at the N-acetyltransferase 2 gene with adverse events with co-trimoxazole in Japanese patients with systemic lupus erythematosus.	Arthritis research & therapy	9	R23	2007
高橋裕子, 森口正人, 住永佳久, 長汐千秋, 狩野俊和, 鈴木暁岳, 国松淳和, 浅尾りん, 山下裕之, 伊藤健司, 三森明夫	Segmental arterial mediolysis の一例	日臨免会誌	30	193-197	2007

VI.　研究成果の刊行物・別刷

Available online at www.sciencedirect.com
ScienceDirect
Molecular Immunology 44 (2007) 2638–2646
Molecular Immunology
www.elsevier.com/locate/molimm

Recognition of H-2K^b by Ly49Q suggests a role for class Ia MHC regulation of plasmacytoid dendritic cell function☆

Lee-Hwa Tai[a,b], Marie-Line Goulet[a,b], Simon Belanger[a,b], Angela D. Troke[a], Aaron G. St-Laurent[a,b], Aruz Mesci[c], Noriko Toyama-Sorimachi[d,1], James R. Carlyle[c,1], Andrew P. Makrigiannis[a,b,*,1]

[a] *Institut de Recherches Cliniques de Montréal (IRCM), Laboratory of Molecular Immunology, Université de Montréal, Montréal, Que., Canada*
[b] *Department of Microbiology and Immunology, McGill University, Montréal, Que., Canada*
[c] *Department of Immunology, University of Toronto, Sunnybrook Research Institute, Toronto, Ont., Canada*
[d] *Department of Gastroenterology, Research Institute, International Medical Center of Japan, Tokyo, Japan*

Received 10 November 2006; received in revised form 12 December 2006; accepted 13 December 2006
Available online 22 January 2007

Abstract

Ly49Q is a member of the polymorphic Ly49 family of NK cell receptors that displays both a high degree of conservation and a unique expression pattern restricted to myeloid lineage cells, including plasmacytoid dendritic cells (pDC). The function and ligand specificity of Ly49Q are unknown. Here, we use reporter cell analysis to demonstrate that a high-affinity ligand for Ly49Q is present on H-2^b, but not H-2^d, H-2^k, H-2^q, or H-2^a-derived tumor cells and normal cells *ex vivo*. The ligand is peptide-dependent and MHC Ia-like, as revealed by its functional absence on cells deficient in TAP-1, β_2m, or H-2K^{b}D^b expression. Furthermore, Ly49Q is specific for H-2K^b, as the receptor binds peptide-loaded H-2K^b but not H-2D^b complexes, and Ly49Q recognition can be blocked using anti-K^b but not anti-D^b mAb. Greater soluble H-2K^b binding to ligand-deficient pDC also suggests *cis* interactions of Ly49Q and H-2K^b. These results demonstrate that Ly49Q efficiently binds H-2K^b ligand, and suggest that pDC function, like that of NK cells, is regulated by classical MHC Ia molecules. MHC recognition capability by pDC has important implications for the role of this cell type during innate immune responses.

Keywords: Plasmacytoid dendritic cells; MHC; Ly49Q; H-2K^b; Ligand-identification

1. Introduction

Plasmacytoid dendritic cells (pDC), also known as interferon-producing cells (IPC), are a specialized subset of DC especially suited for initiating immune responses to viruses. Production of abundant IFN-α levels as well as IL-12 and other cytokines by pDC has direct anti-viral effects on infected cells, and further activates effector cells such as natural killer (NK) cells to destroy infected targets. Plasmacytoid DC express TLR7 and TLR9, allowing for recognition of viral single-stranded RNA and unmethylated CpG-containing double-stranded DNA, respectively, in endosomal compartments (Liu, 2005). Likewise, expression of TLR9 by pDC and myeloid dendritic cells (mDC) is necessary for protection against mouse cytomegalovirus (MCMV) challenge (Krug et al., 2004; Tabeta et al., 2004). In addition to activating NK cells via type I interferon, pDC also augment CD8$^+$ T cell cytotoxicity (Salio et al., 2004). Furthermore, activated pDC upregulate T cell costimulatory molecules such as CD86 and class II MHC molecules, allowing them to present antigenic peptides and stimulate CD4$^+$ T cell function (Nakano et al., 2001). In turn, pDC have been

Abbreviations: B6, C57BL/6; NK, natural killer; DC, dendritic cells; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; MHC, major histocompatibility complex

☆ Animal care was provided in accordance with the procedures approved by the IRCM Animal Care Committee.

* Corresponding author at: Institut de Recherches Cliniques de Montréal, Laboratory of Molecular Immunology, Rm. 1340, 110 Avenue des Pins Ouest, Montréal, Que. H2W 1R7, Canada. Tel.: +1 514 987 5630; fax: +1 514 987 5627.
E-mail address: makriga@ircm.qc.ca (A.P. Makrigiannis).

[1] These authors contributed equally to this work.

0161-5890/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2006.12.010

implicated in promoting mDC maturation and B cell differentiation to antibody-producing plasma cells (Liu, 2005).

Plasmacytoid DC are identifiable by their plasma-cell-like morphology and expression of specific markers including Siglec-H (Blasius et al., 2006a), a DAP12-associating transmembrane receptor, and bone marrow stromal cell antigen 2 (BST2), recently shown to be the antigen recognized by the pDC-specific mAbs, 120G8 and mPDCA-1 (Blasius et al., 2006b). Interestingly, pDC also express Ly49Q, a member of the lectin-like Ly49 family of NK cell receptors specific for class I MHC molecules. Ly49Q is not expressed on NK cells or T cells, but is present on adult Gr-1$^+$ splenocytes and bone-marrow cells, CD11b$^+$Gr1$^+$ peripheral blood cells, and most peripheral pDC, where it appears to be a differentiation marker (Kamogawa-Schifter et al., 2005; Toyama-Sorimachi et al., 2004). In contrast to mature pDC in the spleen and lymph nodes, developing Ly49Q$^+$ and Ly49Q$^-$ pDC subsets can be identified in the bone marrow. Here, Ly49Q$^-$ pDC are thought to represent precursor cells and have a more limited ability to produce cytokines in response to TLR stimulation (Omatsu et al., 2005; Toyama-Sorimachi et al., 2005).

Unlike Ly49Q, most other Ly49 proteins are expressed on NK, T, and NKT cells, except perhaps the divergent Ly49B, which is expressed on macrophages (Gays et al., 2006). All known ligands of the mouse inhibitory Ly49 receptors are class Ia MHC molecules (Anderson et al., 2001), while certain rat Ly49 receptors have been shown to recognize class Ib MHC ligands (Naper et al., 2005). *Ly49* genes and alleles show great variation among different inbred mouse strains, unlike the more limited diversity observed for adjacent lectin-related multi-gene clusters, such as the *Nkrp1/Clr* loci (Carlyle et al., 2006). The three sequenced mouse *Ly49* haplotypes, 129S6, B6, and BALB/c, possess 19, 15, and 8 *Ly49* genes, respectively (Anderson et al., 2005). However, despite such variation, *Ly49q* is present in all three haplotypes. *Ly49q* represents one part of three independent pairs of framework genes (*Ly49a* and *g*, *c* and *i*, and *e* and *q*), which are conserved among the known mouse *Ly49* haplotypes (Proteau et al., 2004). The single characterized rat *Ly49* haplotype (BN/SsNHsd/MCW) contains a total of 36 *Ly49* genes, including a cluster of *Ly49q*-related genes (Hao and Nei, 2004; Nylenna et al., 2005; Wilhelm and Mager, 2004). Whether rat *Ly49q*-related genes are also expressed in pDC is unknown. In the mouse, *Ly49q* is extremely well conserved in comparison to the other known *Ly49* genes, which are allelically diverse. In terms of relatedness to other *Ly49* genes, *Ly49q* and *Ly49b* are the most divergent *Ly49* family members and are phylogenetically grouped in their own subfamilies by sequence alignment analysis (Makrigiannis et al., 2005).

Despite this divergence, Ly49Q contains an ITIM in its cytoplasmic domain, predicting it to be an inhibitory receptor by analogy to Ly49 expressed on NK cells. Consistent with this, co-immunoprecipitation experiments have shown that Ly49Q can associate with SHP-1 and SHP-2 (Toyama-Sorimachi et al., 2004). Interestingly, Ly49Q ligation of IFN-γ-treated RAW macrophage-like cells with plate-bound anti-Ly49Q mAb results in cell adherence and elongation, suggesting that Ly49Q may have some stimulatory function. As the identity of the cognate ligand recognized by Ly49Q is unknown, the role of Ly49Q in regulating pDC function has remained elusive to date.

In this study, we utilize a modification of the BWZ reporter cell assay (Carlyle et al., 2004, 2006; Sanderson and Shastri, 1994) to investigate the expression of potential Ly49Q ligands. A series of experiments determined that various C57BL/6 (B6) and 129-derived tumor lines as well as *ex vivo* cell populations express a ligand for Ly49Q. Moreover, ligand expression was found to be peptide- and β_2m-dependent, and absent on cells derived from H-2K^{b}D^b double-deficient mice. Identification of H-2K^b as a ligand for the Ly49Q receptor suggests that classical MHC Ia molecules regulate the function of pDC, in addition to NK and T cells.

2. Materials and methods

2.1. Mice

C57BL/6J (B6), 129S1, 129X1, B6.129P2-*B2m^{tm1Unc}*/J (β2m-KO), and B6.129S2-*Tap1^{tm1Arp}*/J (TAP-KO) were purchased from Jackson Laboratories (Bar Harbour, Maine). *H-2Kb^{tm1}H-2Db^{tm1}* (K^{b}D^b-KO) mice on a B6 background were purchased from Taconic (Hudson, NY). C3H (Dr. Q. Zhu), A/J (Dr. C. Deschepper), and FVB (Dr. A. Veillette) mice were all kind gifts of colleagues from the IRCM (Montréal, Que.). Mice were maintained and bred in the IRCM specific-pathogen free animal facilities.

2.2. Cells

BWZ.36 cells were obtained from Dr. N. Shastri (University of California, Berkeley, CA). B16 (melanoma, H-2^b), RMA (thymoma, H-2^b), RMA-S (MHC-deficient variant of RMA), YAC-1 (lymphoblast, H-2^a), BW5147 (thymoma, H-2^k) and A20 (B lymphoma, H-2^d) were generously provided by Dr. A. Veillette (IRCM). P815 (mastocytoma, H-2^d), EL-4 (thymoma, H-2^b), L929 (fibroblast, H-2^k), RAW309 (macrophage, H-2$^{b/d}$), and NIH/3T3 (fibroblast, H-2^q) were obtained from Dr. Stephen Anderson (SAIC-Frederick, MD). RMA-S-CD1d1 was a kind gift of Dr. Kevin Kane (University of Alberta, Edmonton, AB). C1498 (acute myeloid leukemia, H-2^b) was obtained from Dr. James Ryan (University of California, CA) and MC57G (fibrosarcoma, H-2^b) was obtained from Dr. W-K. Suh (IRCM). DC2.4 (dendritic cell, H-2^b) was a kind gift of Dr. K. Rock (Dana-Farber Cancer Institute, Boston, MA). T2 (human T–B lymphoblast hybrid) cells and the Qa1^b-transfected T2g37 (T2-Qa1^b) subline were a kind gift of Dr. M. Soloski (Johns Hopkins University School of Medicine, Baltimore, MD). The B78H1TAP (B16 melanoma-derived, H-2^b) parental (TAP) and Q9TAP.13 Q9-expressing subline (TAP-Q9) were generously provided by Dr. I. Stroynowski (University of Texas Southwestern Medical Center, Dallas, TX). DC were isolated from collagenase (Roche Diagnostics, Laval, Que.) treated splenocytes using anti-CD11c-conjugated microbeads (Miltenyi Biotec, Auburn, CA). Peritoneal exudate cells were isolated by PBS lavage.

2.3. Antibodies, MHC-reagents, and flow cytometry

Anti-Ly49Q mAb 2E6 was purchased from MBL (Japan) and biotinylated using a kit (Roche). Anti-Ly49Q mAb NS-34 was previously described (Toyama-Sorimachi et al., 2004). The H-2K^b:Ig and H-2D^b:Ig fusion proteins were purchased from BD Biosciences (Toronto, Ont.). H-2K^b:Ig was incubated overnight in the presence of four molar excess of ovalbumin SIINFEKL peptide (a kind gift Dr. A. Veillette, IRCM) in PBS at 37 °C. H-2D^b:Ig was similarly prepared with LCMV gp33 peptide KAVYNFATM (Sigma, Oakville, Ont.). Antibody or fusion protein coating of flat-bottom 96-well plates was performed in PBS at the indicated concentrations for 90 min followed by several PBS washes. FITC-conjugated affinity purified donkey anti-human IgG (H+L) (Jackson Lab., West Grove, PA) was used as a secondary antibody to reveal Ly49Q-Ig. All staining reactions included 20% normal rat serum and 10 μg/ml of 2.4G2 (anti-CD16/32) mAb for blocking non-specific binding. PE-conjugated H-2K^b/OVA tetramer contains human β2m and was purchased from Beckman Coulter (Fullerton, CA). Flow cytometry and analysis was performed using a FACsCalibur (BD Biosciences, Mountain View, CA).

2.4. Construction of the CD3ζ/NKR-P1B/Ly49Q chimeric receptor and BWZ assay

A pMSCV2.2-CMV-IRES-GFP (pMCIG) retroviral vector was modified to include the intracellular region of CD3ζ and the membrane proximal and transmembrane regions of NKR-P1B, as previously described (Carlyle et al., 2006). The extracellular domains of Ly49Q^{BALB} and Ly49Q^{B6} were amplified from splenocyte cDNA using primers Q-EC-Xho-for, CTC GAG AAC ATT TTG CAG TAT AAG CAA G and Q-EC-Not-rev, GCG GCC GC TTA ACT GTT GTT GGG GAG CG (restriction sites are underlined). PCR products were TOPO TA-cloned into pCR2.1 (Invitrogen Life Technologies), cut with *Xho*I and *Not*I, and then ligated in-frame adjacent to the CD3ζ/NKRP1B cassette. BWZ.36 cells were transduced with retroviral supernatants (24–48 h) from transient triple-transfected 293T cells, as described previously (Carlyle et al., 2004), then sorted at days 3–4 following transduction. Stable BWZ transductants were analyzed using plate-bound mAb or cell mixtures, as previously described (Carlyle et al., 2004).

2.5. Preparation of Ly49Q-human IgG$_1$ fusion protein

The CD5 leader-IgG$_1$ vector, kindly provided by Dr. B. Seed (Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MA), was employed for the production of a Ly49Q^{B6} protein fused to the human IgG$_1$ constant region. A stop codon at the 3′ end of the CD5 leader-IgG1 cassette was replaced with an *Eco*RI-cloning site by PCR-based mutagenesis. The extracellular region of Ly49Q^{B6} was amplified by PCR using *Eco*RI-containing primers, digested with *Eco*RI, and ligated into the CD5 leader-IgG$_1$ vector at the 3′ end of the IgG$_1$ sequence. Ly49Q-Ig chimeras were collected as serum-free supernatants of CHO transfectants, and used after concentrating using ultrafilter membranes.

3. Results

3.1. Construction of a Ly49Q-ligand reporter cell assay

To elucidate the expression of a cognate ligand for Ly49Q, we employed a modification of the BWZ colorimetric reporter cell assay (Carlyle et al., 2004, 2006; Sanderson and Shastri, 1994). BWZ.36 cells containing a *lacZ* transgene under the control of an NFAT-inducible promoter (Sanderson and Shastri, 1994) were stably transduced to express a chimeric protein consisting of the CD3ζ cytoplasmic tail and NKR-P1B CxCP-motif/transmembrane region (Carlyle et al., 2004; Carlyle et al., 2006), and the extracellular domain of Ly49Q^{BALB} or Ly49Q^{B6}. Ly49Q^{B6} and Ly49Q^{BALB} are allelic variants that differ at four and two amino acid substitutions in the stalk and lectin-like domains, respectively (Proteau et al., 2004). Expression of the CD3ζ/NKR-P1B/Ly49Q fusion receptor was monitored using GFP fluorescence (driven by an IRES-GFP element in the retroviral cassette) (Carlyle et al., 2006). To ensure that the fusion receptor was expressed on the surface of BWZ.36 transductants and that proper folding had taken place, CD3ζ/NKR-P1B/Ly49Q-transduced BWZ.36 cells (hereafter referred to as BWZ.Ly49Q) were stained with the anti-Ly49Q mAb, NS-34. Flow cytometric analysis of sorted BWZ.Ly49Q^{BALB} and BWZ.Ly49Q^{B6} transductants revealed that both cell lines expressed the fusion protein at the cell surface (Fig. 1A). While high surface levels of the fusion receptors were difficult to attain, both cell lines expressed similar but low levels of GFP (Fig. 1A). Notably, this low level of fusion-receptor and IRES-GFP expression has been observed before, and may be related to co-expression of a low-level or weak Ly49Q ligand on the parental BWZ cells themselves (JRC, unpublished observations).

To test if the reporter cell assay was functioning properly, parental BWZ.36, BWZ.Ly49Q^{BALB}, or BWZ.Ly49Q^{B6} cells were seeded in wells that had been pre-coated at various concentrations with anti-Ly49Q mAb or an isotype control mAb. BWZ.36 parental cells showed only background β-galactosidase enzyme activity after incubation with anti-Ly49Q mAb (Fig. 1B). In contrast, strong β-galactosidase activity was observed using BWZ.Ly49Q^{BALB} or BWZ.Ly49Q^{B6} reporter cells in response to plate-bound anti-Ly49Q mAb. Importantly, only background activity was seen for all cell lines in response to plate-bound isotype control mAb. These results demonstrate that the Ly49Q fusion receptor is folded correctly and that the BWZ.Ly49Q reporter cells are specifically stimulated by mAb-mediated receptor ligation.

3.2. Identification of Ly49Q-ligand bearing tumor cells

An unbiased screening of a panel of tumor and transformed cell lines for BWZ.Ly49Q cell stimulation was undertaken. Notably, BWZ.Ly49Q^{BALB} but not control BWZ.36 cells were stimulated with RAW, EL-4, DC2.4, C1498, and MC57G cells

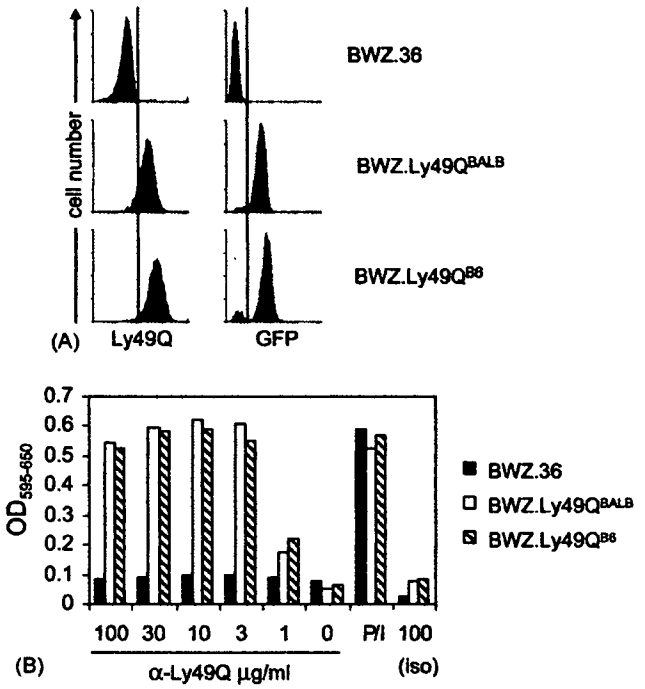

Fig. 1. Ly49Q-CD3ζ fusion receptor-bearing BWZ cells are stimulated by Ly49Q cross-linking. (A) BWZ.36 parental cells or transductants expressing a chimeric protein (introduced by a retrovirus containing IRES-GFP) with the extracellular domain of either B6 or BALB/c Ly49Q proteins were stained with anti-Ly49Q mAb and analyzed by flow cytometry. Both anti-Ly49Q (NS-34) staining (left column) and GFP expression (right column) are shown for parental and transduced cells. (B) BWZ.36 parental cells and Ly49Q-CD3ζ transductants were seeded in wells pre-coated with various concentrations of anti-Ly49Q (2E6) mAb or isotype (iso) control antibodies. After overnight incubation, lacZ production was revealed by addition of CPRG substrate and optical density measurements at the indicated wavelengths. LacZ production capability by BWZ cells is shown by PMA/ionomycin (P/I) treatment.

(Fig. 2A). The prevalence among the stimulating tumor cells lines of the H-2^b haplotype suggested that this haplotype contains a ligand for Ly49Q. Similar results were observed for the BWZ.Ly49Q^{B6} reporter cells, although the responses were significantly weaker (data not shown). This may be due to weaker receptor–ligand interactions for BWZ.Ly49Q^{B6} relative to BWZ.Ly49Q^{BALB} cells (Fig. 1A).

An early hypothesis was that the ligand for the relatively divergent and pDC-specific Ly49Q receptor might be a class Ib MHC, as is the case with the rat Ly49i5 and Ly49s5 receptors (Naper et al., 2005). To test this hypothesis, a panel of cell lines expressing some of the well-characterized class Ib MHC molecules was tested for BWZ.Ly49Q^{BALB} reporter cell stimulation (Fig. 2B). The class Ib MHC panel tested included CD1d1, Qa1^b, H2-Q9, and H2-Bl (also known as blastocyst MHC). Although the known class Ib repertoire is much larger, none of these class Ib MHC molecules was able to stimulate the BWZ.Ly49Q^{BALB} reporter cells when expressed on the surface of various tumor cell lines (Fig. 2B). In contrast, BWZ.Ly49Q^{BALB} reporter cells were stimulated by RMA (H-2^b), but not RMA-S or B16 tumor cells (Fig. 2B). B16 has very low levels of surface MHC. Similarly, RMA-S is a *Tap2*-deficient subline of the RMA parental cell line that lacks surface expression of MHC Ia molecules due to an inability to transport antigenic peptides into the ER during MHC I biogenesis (Powis et al., 1991). Together, these findings indicated that Ly49Q may recognize a H-2^b MHC Ia-like ligand.

3.3. The Ly49Q ligand is class Ia MHC-dependent

To test whether the ligand engaging Ly49Q was also expressed on normal H-2^b-derived cells *ex vivo*, the reporter cell assay was used to analyze various B6-derived leukocyte populations directly, including peritoneal cells, splenocytes, bone marrow cells, thymocytes, mesenteric and popliteal lymph node cells. Strong BWZ.Ly49Q^{BALB} stimulation was observed with

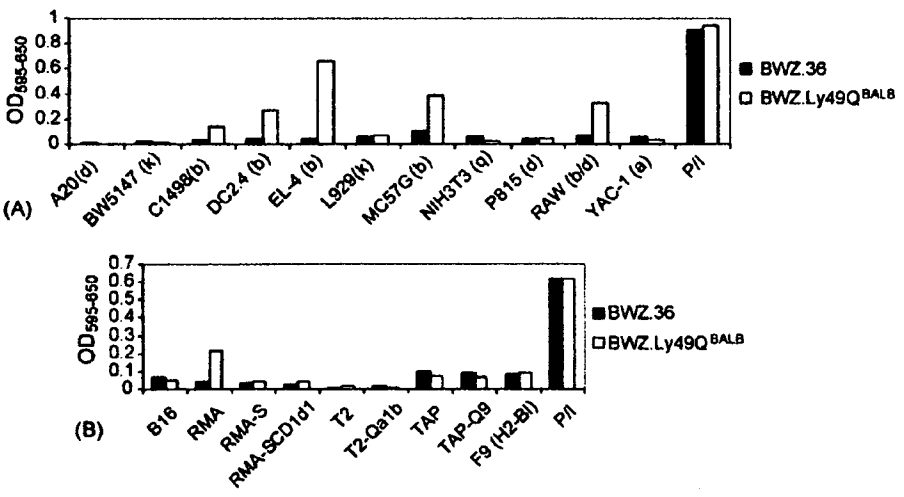

Fig. 2. H-2^b tumor cells stimulate BWZ.Ly49Q reporter cells. BWZ.36 parental or BWZ.Ly49Q^{BALB} cells were co-incubated with various indicated (A) tumor lines and (B) class Ib MHC-transfected sublines at a 1:1 ratio overnight followed by addition of CPRG substrate. The H-2 haplotype is indicated in parentheses in (A). Data representative of three independent experiments is shown.

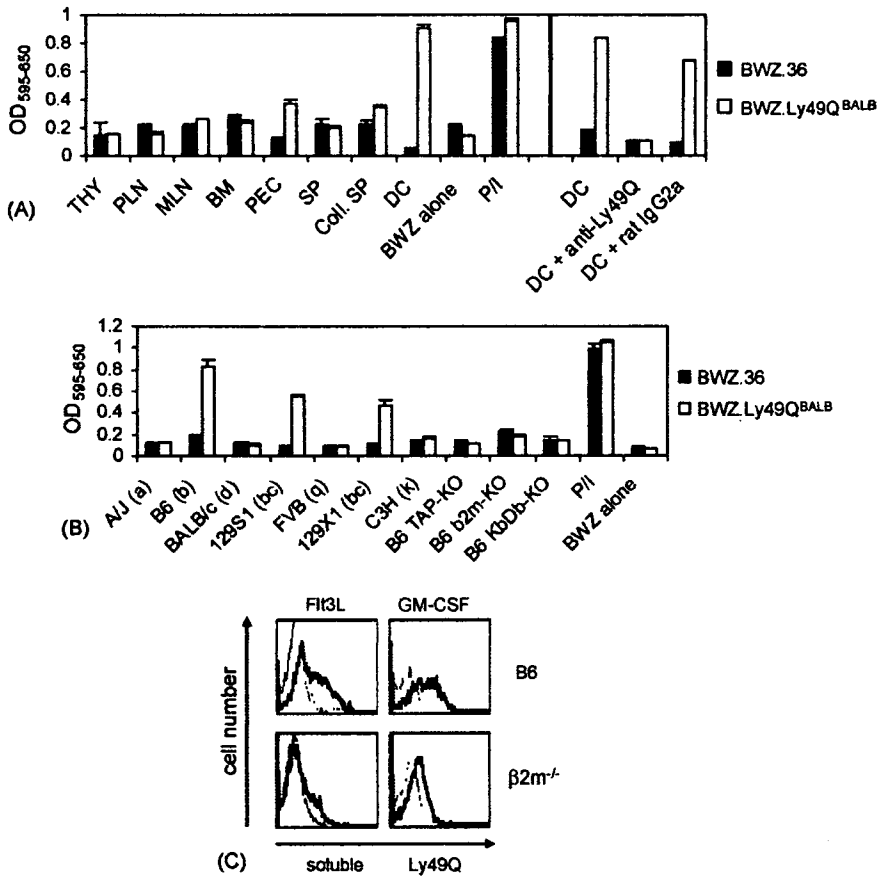

Fig. 3. H-2^b-derived DC stimulate BWZ.Ly49Q in a class Ia MHC-dependent manner. (A) Single cell suspensions were prepared from the indicated tissues of B6 mice and incubated with BWZ.Ly49Q^{BALB} reporter cells followed by addition of CPRG substrate (left panel). DC were isolated using anti-CD11c microbeads and incubated with BWZ cells in the presence of 2E6 (anti-Ly49Q) or isotype-matched control mAb (right panel). THY: thymus; BM: bone marrow; SP: spleen; PEC: peritoneal exudate cells; MLN: mesenteric lymph nodes; PLN: popliteal lymph nodes; Coll. SP: collagenase-treated spleen. (B) CD11c$^+$ DC isolated from the indicated inbred and gene-knockout mouse strains were co-incubated with BWZ reporter cells overnight followed by addition of CPRG substrate. The H-2 haplotype of the inbred strains is indicated in parentheses. (C) Flt3L-cultured (left column) or GM-CSF-cultured (right column) bone-marrow DC from B6 or $\beta2m^{-/-}$ mice were treated with IFN-α and then stained with a soluble Ly49Q-Ig fusion protein. Surface binding of CD11b$^+$CD11c$^+$ gated cells was analyzed by flow cytometry and are shown as histograms. Thick black line: soluble Ly49Q; thick gray line: human IgG (control); thin black line, secondary antibody alone. Data representative of three independent experiments is shown.

fresh peritoneal exudate cells (PEC), all of which were CD11b^{hi} by flow cytomctry (Fig. 3A, left panel and data not shown). Interestingly, collagenase-treated splenocytes also weakly stimulated BWZ.Ly49Q^{BALB} cells compared to splenocytes isolated without the use of collagenase, suggesting the ligand may be expressed at higher levels on a stromal cell type.

As DC are readily liberated from tissues by collagenase treatment, these cells were tested directly. B6-derived CD11c$^+$ splenocytes were isolated by positive selection and incubated with BWZ.Ly49Q^{BALB} cells. Strikingly, a rapid and strong stimulation was observed (Fig. 3A, left panel). BWZ.Ly49Q^{B6} were also specifically stimulated by DC, albeit more weakly (data not shown). Furthermore, stimulation of reporter cells by DC could be blocked by addition of anti-Ly49Q mAb, but not isotype-matched control mAb, demonstrating the receptor specificity of the stimulation (Fig. 3A, right panel). Thus, in addition to several H-2^b tumor cell lines, a ligand for Ly49Q is expressed on B6-derived DC and PEC *ex vivo*. Notably, these populations are known to express high levels of both MHC and adhesion molecules, further highlighting their utility in the reporter cell assay. Therefore, DC were used in subsequent experiments as a source of ligand-bearing cells, due to their high stimulation index and ease of isolation.

The haplotype specificity of Ly49Q recognition was next tested by isolating DC from different inbred mouse strains and testing their ability to stimulate BWZ.Ly49Q cells. DC from B6 (H-2^b), 129X1 (H-2^{bc}), and 129S1 (H-2^{bc}), but not FVB (H-2^q), BALB/c (H-2^d), A/J (H-2^a), or C3H (H-2^k) mice were able to stimulate BWZ.Ly49Q^{BALB} reporter cells (Fig. 3B). The class Ia MHC (H-2K and H-2D) of B6 and the 129-group strains are identical, while the class Ib MHC regions have significant differences in gene content (Kumanovics et al., 2002). To directly test whether an MHC I molecule is a ligand for Ly49Q, DC were isolated from both β_2m-deficient and TAP-1-deficient mice of the H-2^b haplotype and used in the reporter cell assay. Neither of these MHC I-deficient DC were able to stimulate BWZ.Ly49Q^{BALB} (Fig. 3B). Furthermore, a low but detectable binding of a soluble Ly49Q-Ig fusion protein to

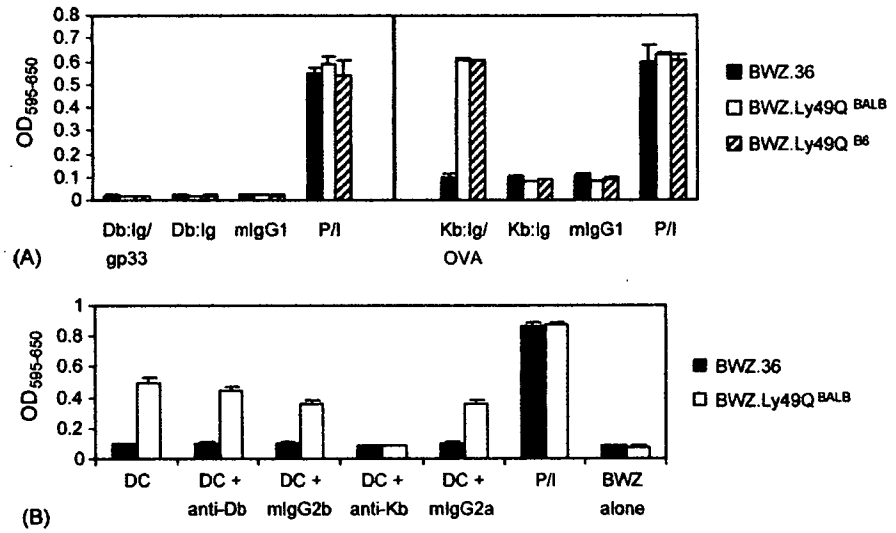

Fig. 4. H-2K^b, but not H-2D^b, is a ligand for Ly49Q. (A) BWZ.36 parental, BWZ.Ly49Q^{B6}, and BWZ.Ly49Q^{BALB} reporter cells were seeded onto wells that had been pre-coated with peptide-bearing or peptide-receptive H-2K^b:Ig or H-2D^b:Ig fusion proteins, or wells pre-coated with control mAb matched to the isotype of the MHC fusion protein (mouse IgG$_1$). (B) BWZ reporter cells were incubated with B6-derived DC in the presence of blocking anti-H-2K^b, anti-H-2D^b, or control mAbs. Analysis was performed as described in Fig. 1. Data representative of three independent experiments is shown.

B6 bone-marrow-derived DC was observed by flow cytometry (Fig. 3C). Importantly, the binding of the soluble Ly49Q protein was not observed when β_2m-deficient DC were analyzed (Fig. 3C). While not all MHC I-related proteins require TAP-1/2 or β_2m for cell surface expression, these data strongly suggest that the ligand for Ly49Q is MHC I-like.

To directly test class Ia MHC molecules as candidate ligands, BWZ.Ly49Q^{BALB} cells were incubated with DC from H-2K^{b}D^b double-deficient mice. H-2K^{b}D^b-deficient DC failed to stimulate BWZ.Ly49Q^{BALB} cells, despite having mostly normal expression of class Ib MHC (Fig. 3B). Thus, the candidate ligand for Ly49Q is likely a class Ia MHC, and the DC-expressed ligand must either be H-2K^b, H-2D^b, or an MHC Ib molecule dependent on MHC Ia expression. Notably, Qa1^b molecules present the Qdm leader peptide derived from the H-2D^b class Ia MHC; however, Qa1^b was formally ruled out using stably transfected cells (Fig. 2B).

3.4. H-2K^b is a ligand for Ly49Q

To directly test H-2K^b and H-2D^b for Ly49Q binding, soluble mouse IgG$_1$ fusions of both MHC proteins (bearing specific peptides) were used to coat wells onto which BWZ.Ly49Q cells were seeded. Following incubation, strong stimulation of both BWZ.Ly49Q^{BALB} and BWZ.Ly49Q^{B6} was detected in H-2K^b:Ig-coated wells (Fig. 4A). The stimulation was similar to that seen with anti-Ly49Q mAb-coated wells and only slightly less robust than that induced by PMA/ionomycin treatment. Importantly, no stimulation was seen in wells coated with H-2D^b:Ig or control isotype Ig (mAb). Thus, H-2K^b binds directly to Ly49Q.

Other Ly49 receptors have been shown to bind H-2K^b, including Ly49C^{B6}, C^{BALB}, I^{B6}, I^{129}, and V^{129} (Hanke et al., 1999; Makrigiannis et al., 2001). Interestingly, evidence has been previously presented that Ly49C is in fact a receptor for empty or "peptide-receptive" H-2K^b (Su et al., 1999). To test the peptide-dependence of Ly49Q recognition, the H-2K^b:Ig fusion protein was used to coat wells in the absence of added peptide. Neither BWZ.Ly49Q^{BALB} nor BWZ.Ly49Q^{B6} cells were stimulated with H-2K^b lacking peptide (Fig. 4A). This suggests that recognition of H-2K^b by Ly49Q is dependent on the presence of bound peptide.

As independent confirmation of Ly49Q specificity, antibody-mediated blocking of reporter cell stimulation was also performed. As expected, addition of anti-H-2K^b but not anti-H-2D^b or isotype control mAb to DC co-cultures inhibited BWZ.Ly49Q stimulation (Fig. 4B). Ly49Q specificity was subjected to further testing using YB2/0-derived transfectants expressing various alternate alleles. No specific stimulation was observed for YB-D^b, YB-D^d, YB-K^k, or YB-L^d (data not shown). Thus, Ly49Q appears to be H-2K^b-specific, unlike Ly49C and V, which show affinity for multiple MHC alleles in addition to H-2K^b (Hanke et al., 1999; Makrigiannis et al., 2001). Thus, H-2K^b, but not H-2D^b, is a ligand for the BALB/c and B6 alleles of Ly49Q.

In order to independently confirm the results of the BWZ assay, binding of H-2K^b to real pDC was evaluated. Splenic pDC were isolated using anti-mPDCA1 microbeads and then stained with soluble PE-labeled H-2K^b tetramer. Significant binding of the MHC tetramer was observed by pDC (Fig. 5A). Contaminating lymphocytes served as an internal control and were not stained by H-2K^b tetramer. Interestingly, pDC from K^{b}D^b-deficient mice bound higher levels of H-2K^b tetramer suggesting that, similar to other Ly49 (Doucey et al., 2004), Ly49Q interacts with its ligand in *cis*. H-2K^b tetramer binds to Ly49Q on pDC, and this can be blocked by pre-incubating pDC with anti-Ly49Q mAb but not control mAb (Fig. 5B). Thus, H-2K^b binding by Ly49Q is confirmed independently of the BWZ assay.

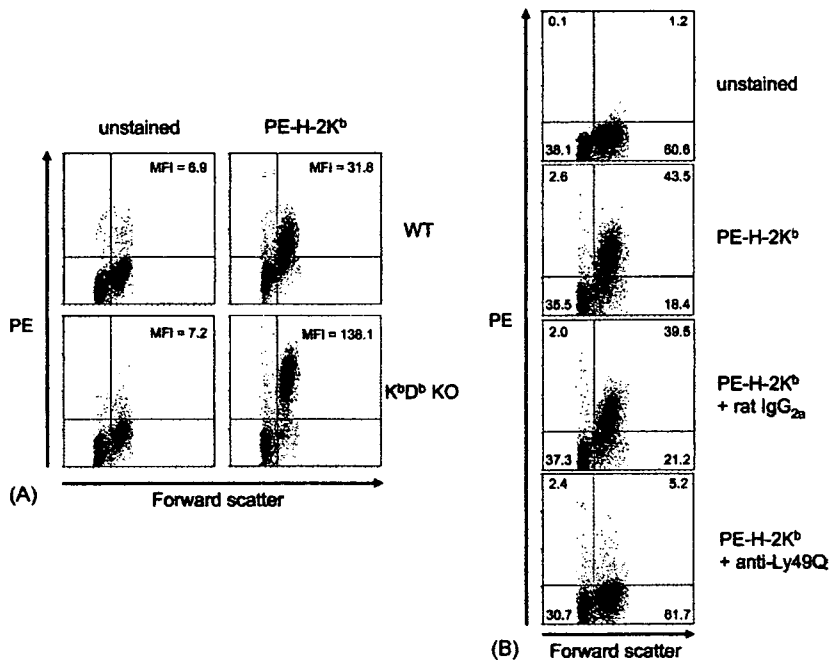

Fig. 5. Soluble H-2K^b binding to pDC is Ly49Q-dependent. (A) Splenic pDC were isolated using anti-mPDCA1 microbeads from wildtype or K^{b}D^b-deficient mice and stained with soluble PE-H-2K^b tetramer bearing OVA peptide. Cells were gated to include pDC and lymphocytes. The MFI of pDC only is indicated. (B) Splenic pDC from K^{b}D^b-deficient mice were pre-incubated with anti-Ly49Q mAb or isotype control and then stained with PE-H-2K^b/OVA tetramer. Cells were gated to include pDC and lymphocytes. The percentage of cells in each quadrant is indicated.

4. Discussion

Class Ia MHC ligands have been identified for most inhibitory or ITIM-containing members of the Ly49 family of NK cell receptors. This study presents evidence that the ligand of the divergent pDC-expressed Ly49Q protein is also class Ia MHC, specifically H-2K^b. The expression of Ly49Q on pDC, but not NK cells, sets Ly49Q apart from the other inhibitory Ly49, but pDC and NK cells have related functional characteristics. Plasmacytoid DC and NK cells express receptors such as TLR9 and Ly49H, respectively, that are directly capable of detecting viral infection. NK cells are also potently activated by IFN-α, a cytokine that pDC make in abundance. Thus, the roles of pDC and NK cells are strongly intertwined and these two cell types likely work in concert during anti-viral and perhaps other innate immune responses. The finding that both cell types are sensitive to the presence or absence of MHC supports this hypothesis.

The finding that H-2K^b is a ligand for Ly49Q was somewhat unexpected, given that fresh *ex vivo* T and B cells contained in the primary and secondary lymphoid tissue suspensions express H-2K^b, but did not significantly stimulate the BWZ.Ly49Q cells (Fig. 3A). One possibility for this apparent paradox is variation in the level of H-2K^b expression, as well as adhesion molecule levels, on stimulating versus non-stimulating cell types. Flow cytometric analysis reveals that H-2K^b, but not H-2D^b, surface expression is indeed higher on B6-derived DC (MFI = 487) compared to whole splenocytes (MFI = 381; unpublished observations). In addition, DC (and PEC) possess greater levels of adhesion and costimulatory molecules than resting lymphocytes, which combined with a high surface level of H-2K^b may overcome a threshold requirement for BWZ cell stimulation. Nonetheless additional cellular signals are not required to induce BWZ stimulation via the CD3ζ fusion receptor, as supported by the cell-free stimulation using plate-bound anti-Ly49Q mAb or K^b:Ig fusion protein. Furthermore, the stimulation of BWZ.Ly49Q by EL-4 and RMA (T cell), C1498 (leukemia), and MC57G (fibrosarcoma) tumor lines may again be facilitated by increased levels of adhesion molecules as well as greatly increased cell surface area. Thus, freshly isolated lymphocytes are smaller than DC and PEC cells, and much smaller than BWZ reporter cells and the stimulating tumor cells tested. Finally, as noted previously, the inability to achieve high level expression of the fusion-receptor and IRES-GFP cassette in BWZ.Ly49Q transductants (Fig. 1A) has been observed before, and may be due to co-expression on the parental BWZ cells themselves of a low-level or weak Ly49Q ligand. Thus, chronic basal stimulation (in *cis* or in *trans*) of the BWZ reporter cells in isolation can result in reporter cell death (loss of Ly49Q^{hi}/GFP^{hi} transductants), as well as an increased threshold requirement for stimulation (JRC, unpublished observations). The higher binding of H-2K^b tetramer by pDC of K^{b}D^b-deficient mice relative to wildtype pDC supports *cis* interactions of Ly49Q and H-2K^b (Fig. 5A) (Doucey et al., 2004).

Related to the above, our inability to detect significant BWZ.Ly49Q stimulation with other H-2K alleles and indeed other H-2 haplotypes by no means indicates that alternative, weaker ligands do not exist. In contrast, as BWZ cells are of the non-stimulating H-2^k haplotype, low endogenous levels of H-2^k or weak Ly49Q:H-2^k interactions may actually set the threshold

for detection of receptor–ligand interactions. In other words, it is possible that there is a ligand for Ly49Q^{BALB} in BALB/c mice, but that the affinity of the interaction is below the threshold of detection in this assay. This is supported by the apparent weaker stimulation of BWZ-Ly49Q^{B6} compared to BWZ-Ly49Q^{BALB} by DC and tumour cells, while both were equally stimulated by plate-bound H-2K^b:Ig (Fig. 4A). Alternatively, an allele of Ly49Q that recognizes BALB/c MHC may exist, but in non-BALB/c mice. Thus, the apparent absence of an MHC ligand for Ly49Q in BALB/c mice (H-2^d haplotype) may represent an artificial circumstance related to the derivation of man-made inbred mouse strains. At present, we cannot exclude these possibilities; however, with knowledge of H-2K^b as a high-affinity ligand for Ly49Q, future analysis of potential weak interactions may be facilitated using high-avidity reagents such as MHC tetramers or multimers.

Importantly, both the BALB/c and B6 alleles of Ly49Q directly recognized H-2K^b (Figs. 4A and 5B). However, BALB/c-derived DC did not stimulate Ly49Q^{BALB}-expressing BWZ cells. Therefore, notwithstanding the above, it would appear that the Ly49Q allele in BALB/c mice may either recognize a weak self ligand or have no self ligand at all. This would not be unique to Ly49Q, as this is also the case for other inhibitory Ly49 receptors. For example, Ly49A^{B6}, Ly49O^{129}, and Ly49G^{B6} can bind H-2D^d, but have no known ligands in B6 or 129 mice (Hanke et al., 1999; Makrigiannis et al., 2001). Nonetheless, this may not be a detrimental situation with respect to NK cell function, since there are usually other inhibitory receptors that recognize self MHC; however, pDC appear to have only a single MHC-specific receptor, Ly49Q.

What is the role for an inhibitory MHC-specific receptor on pDC? Such receptors on NK cells are hypothesized to mediate "missing-self" recognition of pathological MHC-deficient host cells during viral infections or cellular transformation. Inhibitory receptors for classical MHC have recently been suggested to also mediate the 'licensing' or 'disarming' of developing NK cells in their acquisition of self-tolerance maturation signals (Fernandez et al., 2005; Kim et al., 2005). Essentially, the expression of self-MHC-specific inhibitory Ly49 receptors permits the functional maturation of NK cells that would otherwise be rendered hyporesponsive or 'anergic' due to low-level chronic engagement of stimulatory receptors such as NKG2D, NKR-P1A/C/F, or Ly49D/H. These self-tolerant mature NK cells are better able to mediate cytotoxicity and cytokine production in response to imbalances in self–non-self discrimination stimuli. By analogy, therefore, it is conceivable that Ly49Q may be responsible for similar functions in the functional maturation of pDC. If this is the case, then why would pDC express only a single tolerizing receptor instead of multiple inhibitory receptors like NK cells? One might hypothesize that Ly49Q might function as a pan-specific MHC receptor, like Ly49C or Ly49V, thereby obviating the need for multiple allele-specific receptors. While the data presented herein do not support this hypothesis, more sensitive methods for the detection of low affinity interactions need to be applied before making any final conclusions.

Recent reports suggesting that ITAM signaling is actually inhibitory in nature in myeloid lineage cells, including pDC, also need to be taken into consideration when assigning a role for the ITIM-containing Ly49Q receptor. Like the coexpression of Ly49Q and H-2K^b on pDC, the DAP12-coupled TREM2 receptor is expressed on macrophages along with an unknown ligand. The loss of either TREM2 or DAP12 results in macrophages that are hyperresponsive to TLR stimulation and secrete higher than normal amounts of TNF-α (Hamerman et al., 2006). Analogous findings have been reported for pDC. Specifically, the DAP12-coupled Siglec-H and Nkp44 receptors have inhibitory effects on pDC, resulting in suppression of type I interferon secretion (Blasius et al., 2006a; Fuchs et al., 2005). Normally, ITIM-mediated signals are dominant over ITAM-dependent pathways, yet whether the Ly49Q interaction with MHC results in suppression of stimulatory or inhibitory signals in pDC needs to be explored. Thus, the role of Ly49Q on pDC might be to facilitate the activation, rather than inhibition, of the innate immune system.

Acknowledgements

We would like to thank Jason Fine, Peter Chen, and Wayne Chou for technical assistance with reporter cell assays and Stephen K. Anderson for critical reading of the manuscript.

This work was supported by Operating Grants from the Canadian Institutes of Health Research (CIHR MOP 74754, to JRC, and MOP 62841, to APM). AM was supported by a Life Sciences Award from the University of Toronto. L-HT is supported by an IRCM-Invitrogen Award, and M-LG was supported by an IRCM-Master's Award. JRC is supported by a Career Development Award from the International Human Frontier Science Program Organization (CDA0037-2005). APM is supported by a New Investigator Award from the CIHR.

References

Anderson, S.K., Dewar, K., Goulet, M.L., Leveque, G., Makrigiannis, A.P., 2005. Complete elucidation of a minimal class I MHC natural killer cell receptor haplotype. Genes Immun. 6, 481–492.

Anderson, S.K., Ortaldo, J.R., McVicar, D.W., 2001. The ever-expanding Ly49 gene family: repertoire and signaling. Immunol. Rev. 181, 79–89.

Blasius, A.L., Cella, M., Maldonado, J., Takai, T., Colonna, M., 2006a. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. Blood 107, 2474–2476.

Blasius, A.L., Giurisato, E., Cella, M., Schreiber, R.D., Shaw, A.S., Colonna, M., 2006b. Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. J. Immunol. 177, 3260–3265.

Carlyle, J.R., Jamieson, A.M., Gasser, S., Clingan, C.S., Arase, H., Raulet, D.H., 2004. Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. Proc. Natl. Acad. Sci. U.S.A. 101, 3527–3532.

Carlyle, J.R., Mesci, A., Ljutic, B., Belanger, S., Tai, L.H., Rousselle, E., Troke, A.D., Proteau, M.F., Makrigiannis, A.P., 2006. Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. J. Immunol. 176, 7511–7524.

Doucey, M.A., Scarpellino, L., Zimmer, J., Guillaume, P., Luescher, I.F., Bron, C., Held, W., 2004. Cis interactions of Ly49A with MHC class I restricts natural killer cell inhibition. Nature Immunol. 5, 328–336.

Fernandez, N.C., Treiner, E., Vance, R.E., Jamieson, A.M., Lemieux, S., Raulet, D.H., 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. Blood 105, 4416–4423.