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厚生労働科学研究委託費
創薬基盤推進研究事業

（委託業務題目）
血中PD-1リガンド検出エライザー法によるPD-1抗体がん治療法の
有効性診断薬開発

平成２６年度　委託業務成果報告書

業務主任者　本庶　佑

平成２７（２０１５）年　３月

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厚生労働科学研究委託費（創薬基盤推進研究事業）
委託業務成果報告（総括）

血中PD-1リガンド検出エライザー法によるPD-1抗体がん治療法の有効性診断薬開発に関する研究

総括　本庶　佑　京都大学医学研究科客員教授

研究要旨

PD-1 抗体が 2014 年に、悪性黒色腫の治療薬として初めて承認され、現在、このがんにおいて、最も有効性の高いがん治療法として注目されている。将来的には、PD-1 抗体がより多くのがんに対する治療薬として承認されることが予想される。本研究では、血中 PD-1 リガンド（PD-L）を高感度で検出するエライザー法を開発し、健常人、および術前、がん原発巣切除後の患者由来血液検体を測定、また、組織学的に PD-L の発現を検討することにより、PD-1 抗体によるがん治療における有効性事前診断を可能とする事を目標とする。また、多くの難治がん症例が集約する、京都大医学部付属病院の診療科より協力を得る。ここから得られる検体よりデーターを得て、将来的に、より多種のがんにおいて、当治療法の奏功率を画期的に上昇させられる様、研究を行う。

業務項目の担当責任者
本庶　佑　京都大学医学研究科客員教授
湊　長博・京都大学医学研究科教授
竹馬俊介・京都大学医学研究科助教

A．研究の目的

２０１４年に、悪性黒色腫の治療薬として初めて承認された PD-1 抗体は、他のがん腫における前臨床試験においても、術後再発例や、化学療法抵抗性の末期患者において奏功率２０～３０％という画期的な成果をもたらしている。

私たちは、これまでの研究成果より、マウスにおけるがん細胞の PD-1 リガンド(PD-L)発現の有無と PD-1 阻害によるがんの治療効果相関実験（Iwai et al. PNAS)、および卵巣がん手術後の患者の予後不良とがん細胞の PD-L 発現との相関を明らかにしており（Hamanishi et al. PNAS)、PD-L の発現が PD-1 抗体の治療有効性の有望なマーカーとして期待される。

このため腫瘍から放出される PD-L を患者の血漿で検出可能な超高感度エライザー法を開発し、治療前の有効性診断薬を開発する。

初年度には PD-L エライザー試薬を開発する。第２年度に腎がん、肺がん、卵巣がん患者の血清中 PD-L の検出可能性を検索する。第３年度に、PD-1 抗体治療患者の有効例と無効例との間で血中 PD-L 検出の有無との相関を検定する臨床研究を行い、コンパニオン診断薬開発を進める。

B．研究の方法

①ヒト PD-L 抗体に対する抗体の選定を行い、サンドイッチエライザー法によって極微

量の PD-L 検出を可能とするエライザーセットを確立する。臨床検体の測定に十分なよう、感度を向上させる。②次いでこれらのエライザーキットを自動計測装置に組み込む。③また、これらの検出リガンドが正しいかどうかを抗体による免疫沈降物の質量分析等による化学的検定を行う。また組織染色における抗体の特異性を検討する。④培養細胞における細胞表面リガンド発現と培養液中への放出の量の相関を検討すると共に非発現細胞と比較してバックグランドレベルの確定を行う。

（倫理面への配慮）

本研究で行われる試験に携わる全ての研究者はヘルシンキ宣言および臨床研究に関する倫理指針（平成 20 年厚生労働省告示第 415 号）に従って本試験を実施し、患者の人権保護に努める。インフォームドコンセントは、倫理審査委員会の承認を得た本試験の同意説明文書を患者本人に渡し、試験の内容を口頭で詳しく説明した上で、患者本人より同意への署名を取得する。その際には、患者の人権および個人情報の保護、試験への参加を拒否した場合でも不利益を被らないこと、代替治療の説明等を行い、倫理面への配慮を十分に行う。

C．研究結果

1. エライザー系の確立

湊研究室提供および市販の10種類以上抗体を用い、サンドイッチエライザーに用いる抗 PD-L1 抗体の組み合わせに関して検討した。結果、検出が最適となる固相化抗体、検出用抗体のペアを最適化できた。上記、抗体のペアで、可溶性PD－L1 の検出感度を測定した。この検討では、10～100000pg/mlまで良好な検出を行うことができ、臨床サンプルの測定に十分高感度であると判断した。

2.特異性の検定

上記抗体のペアで、可溶性PD－L1 および、PD－L1が属するその他のB7ファミリータンパク(6種)に対する特異性検討を行ったところ、高い特異性を持ってPD－L1 のみが検出された。

3. 検討会の実施

呼吸器外科学、泌尿器科学、産科婦人科学の臨床 3 科と定期的に会合を行った。その結果、手術由来組織検体、および血液検体の収集体制の整備が出来た。また、検体収集に関し、あらたに京大病院病理部（手術組織）、および京大癌バイオバンク(血液サンプル)から、収集の助けを得ることができる事となった。これにより、臨床3科から集まる検体の効率的、かつ、手技などに左右されない均一なサンプリングが可能となった。

D．考察

PD-1 抗体が、一部の患者には効かない原因として、がん腫が免疫回避のため発現する PD-L の多寡が影響すると考えている。そのため、PD-L を測定することは重要であるが、がん種の組織学的な解析のみでは、PD-L 発現の総量を測定することは困難である。

その点で、検査血清より PD-L 値を得る、この測定を整備出来た本年度の成果は重要である。

PD-L には、PD-L1 および PD-L2 の 2 種のリガンドが存在するが、PD-L2 に関しては予備検討や文献から、がん免疫に直接関係しない

という見解が得られている。今後はPD-L1を主に測定することに重点を置く方針である。

E．結論

1．患者由来血液サンプルより、高感度でPD-Lを測定する系を開発した。
2．次年度より、多数のがん患者、および健常ボランティアからサンプリングする体制を確立した。

F．健康危険情報

なし

G．研究発表

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Immunosenescence and Autoimmunity（口頭） 湊 長博 International Symposium of Japanese Immunilogy Society（京都） 2014.12.11 国内

厚生労働科学研究委託費（創薬基盤推進研究事業）
委託業務成果報告（業務）

臨床検体の調整とエライザーの有効性検定

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小川　修・京都大学医学研究科教授
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研究要旨

PD-1 抗体によるがん治療法は、今後、より多くのがん種で、幅広く承認されることが予想される。本業務では、卵巣がん、肺がん、および腎がんの手術対象症例より臨床サンプルを幅広く収集し、手術前、および術後一定期間における、血液サンプルを収集する。血中 PD-1 リガンド（PD-L）エライザーを用い、健常人ボランティア等との比較を行う。また、組織学的に PD-L の発現を検討することにより、PD-1 抗体によるがん治療における有効性事前診断を可能とする事を目標とする。

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伊達洋至・京都大学医学研究科教授

A．研究の目的

PD-1 抗体は、すでに承認されたメラノーマ以外に、腎がん、肺がん、卵巣がんにおいても臨床治験が進められ、いずれも２～３割の奏効率を示している。しかしながら、７割の患者に奏効しない理由は不明である。これまでの検討から、PD-L を強く発現している腫瘍は、より PD-1 依存性に免疫回避をしていることが予想される。そのため、本研究では、患者の血中、および摘出腫瘍サンプルより、PD-L を鋭敏に検出する可能性を検索する。PD-1 抗体治療患者の有効例と無効例との間で血中 PD-L 検出の有無との相関を検定する。

各々のがん種につき、数十検体を収集し、これを用いて臨床的検討を行う。

B．研究の方法

1．PD-L エライサーの有効性判定
市販血清、血漿を用いて、開発したエライザーの確認を行う。

2．組織学的検討

3．臨床検体の測定の整備
協議によって３科における検体収集方法の一本化を行い、安定なサンプリングを行えるよう体制を確立する。京都大学医の倫理委員会の承認を得る

（倫理面への配慮）

本研究で行われる試験に携わる全ての研究者はヘルシンキ宣言および臨床研究に関する倫理指針（平成 20 年厚生労働省告示第 415 号）に従って本試験を実施し、患者の人権保護に努める。インフォームドコンセントは、倫理審査委員会の承認を得た本試験の同意説明文書を患者本人に渡し、試験の内容を口頭で詳しく説明した上で、患者本人より同意への署名を取得する。その際には、患者の人権および個人情報の保護、試験への参加を拒否した場合でも不利益を被らないこと、代替治療の説明等を行い、倫理面への配慮を十分に行う。

C．研究結果

1．PD-L エライサーの有効性判定

市販の健常人血漿、および血清サンプルより、PD-L1 を測定した。測定値は 250-350pg/ml と、一定の範囲に収まった。また、後述の癌バイオバンクでも血漿を用いることから、血清と血漿の比較を行い、血漿で問題なく PD-L1 の測定を行えるようになった。

2．組織学的検討

エライザーに用いた抗体のうち、1種を用いた組織染色では、市販の肺がん組織を良く染色し、この染色は、組み換えPD－L1を加え、競合させることにより阻害されたことから、高い特異性を持つことが示された。

3．倫理委員会の承認、および検討会の実施

当該研究の実施に関し、京都大学「医の倫理委員会」の承認を得た。また、臨床 3 科、および本庶研究室と定期的に会合を行った。その結果、手術由来組織検体、および血液検体の収集体制の整備が出来た。また、検体収集に関し、あらたに京大病院病理部（手術組織）、および京大癌バイオバンク(血液サンプル)から、収集の助けを得ることができる事となった。これにより、臨床 3 科から集まる検体の効率的、かつ、手技などに左右されないサンプリングが可能となった。

D．考察

PD-1 抗体が、一部の患者には効かない原因として、がん腫が免疫回避のため発現する PD-L の多寡が影響すると考えている。そのため、PD-L を組織学的、血液サンプルより検出できることは有用であり、この測定を整備出来た本年度の成果は重要である。

今回、エライザー検定に用いた健常人サンプルは、国籍、人種などが多様な市販検体であり、本試験では年齢、国籍（日本人）のマッチしたサンプルを用いることにより、患者サンプルとのより正確な比較ができると考えられる。

E．結論

1. 開発した PD-L 測定エライザーは、臨床検体の測定に十分な感度と特異性を持つことがわかった。
2. PD-L を、がん組織で検出し、スコア化する方法を確立した。
3. 次年度より、がん患者、および健常ボランティアからサンプリングする体制を確立した。

F．健康危険情報

なし

G．研究発表

1．論文発表

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2．学会発表

なし

学 会 等 発 表 実 績

1．学会等における口頭・ポスター発表

発表した成果(発表題目、口頭・ポスター発表の別)	発表者氏名	発表した場所(学会等名)	発表した時期	国内・外の別
Academic innovation of cancer immunotherapy by PD-1 antibody(口頭)	本庶 佑	Bordeaux, France(京都大学ボルドー大学シンポジウム)	2014.5.5	国外
PD−1:発見から臨床応用まで(口頭)	本庶 佑	東京(医薬ライセンシング協会(JPLA)月例会講演)	2014.5.21	国内
Academic Innovation of cancer immunotherapy by PD-1 antibody.(口頭)	本庶 佑	London, UK(Seminar at King's College London School of Medicine)	2014.6.17	国外
Academic Innovation of cancer immunotherapy by PD-1 antibody.(口頭)	本庶 佑	香川県, 小豆島(第16回免疫サマースクール in 小豆島)	2014.7.30	国内
免疫の可変抵抗器 PD-1 の特性とその臨床応用における利点(口頭)	本庶 佑	神奈川県, 横浜(第 52 回日本癌治療学会学術集会)	2014.8.3	国内
Biology of PD-1 and Cancer Immunolotherapy by PD-1 Antibody(口頭)	本庶 佑	La Jolla, USA(The Usha Mahajani Symposium)	2014.9.8	国外
Academic innovation of cancer immunotherapy by PD-1 antibody(口頭)	本庶 佑	台北,台湾(Tang Prize: Laureate Lecture)	2014.9.19	国外
A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application(口頭)	本庶 佑	台北,台湾(2014 NHRI/IBMS Joint International Conference on Inflammation & Disease)	2014.10.16	国外
Durable cancer immunotherapy by PD-1 antibody(口頭)	本庶 佑	台北,台湾(Lecture at Taipei Medical University)	2014.10.17	国外
PD-1 抗体によるがん治療(口頭)	本庶 佑	京都(JCA-CHAAO 賞受賞記念講演会)	2014.10.20	国内
PD-1: Biology and therapy(口頭)	本庶 佑	京都(DSK Symposium 2014: Perspectives in Anticancer Drug Discovery and Development)	2014.11.11	国内
PD-1 抗体によるがん治療(口頭)	本庶 佑	京都(第 55 回日本肺癌学会学術集会招聘講演)	2014.11.16	国内
新しいがん治療薬 PD-1 抗体(口頭)	本庶 佑	山口,宇部(渡辺裕策翁生誕 150 年記念講演会)	2014.11.22	国内

PD-1 抗体によるがん治療(口頭)	本庶 佑	京都(新世代のがん分子標的療法戦略シンポジウム)	2014.12.6	国内
PD-1 抗体によるがん治療(口頭)	本庶 佑	京都(第 43 回日本免疫学会学術集会)	2014.12.11	国内
Cancer Immunotherapy by Blockade of a Negative Immuno Regulator PD-1(口頭)	本庶 佑	京都(Satellite Symposium of Ist Kyoto University-UC SanDiego Joint Symposium)	2015.3.13	国内
Immunosenescence and Autoimmunity(口頭)	湊 長博	京都(International Symposium of Japanese Immunilogy Society)	2014.12.11	国内
Immunosenescence, Malignancy and Autoimmunity(口頭)	湊 長博	京都(International Symposium of Japanese Society, of Allergy)	2014.5.10	国内
T cell senescence and Autoimmunity(口頭)	湊 長博	東京(The Uehara Memorial Foundation Symposium)	2014.6.15	国内

2. 学会誌・雑誌等における論文掲載

掲載した論文(発表題目)	発表者氏名	発表した場所(学会誌・雑誌等名)	発表した時期	国内・外の別
γ δ T Cells and Their Potential for Immunotherapy.	Wu, Y-L, Ding, Y-P, Tanaka, Y, Shen, L-W, Wei, C-H, Minato, N, and Zhang, W.	Int. J. Biol. Sci.	10, 119-135, 2014	国外
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Review

γδ T Cells and Their Potential for Immunotherapy

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Abstract

Vγ9Vδ2 (also termed Vγ2Vδ2) T cells, a major human peripheral blood γδ T cell subset, recognize microbial (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate and endogenous isopentenyl diphosphate in a TCR-dependent manner. The recognition does not require specific accessory cells, antigen uptake, antigen processing, or MHC class I, class II, or class Ib expression. This subset of T cells plays important roles in mediating innate immunity against a wide variety of infections and displays potent and broad cytotoxic activity against human tumor cells. Because γδT cells express both natural killer receptors such as NKG2D and γδ T cell receptors, they are considered to represent a link between innate and adaptive immunity. In addition, activated γδ T cells express a high level of antigen-presenting cell-related molecules and can present peptide antigens derived from destructed cells to αβ T cells. Utilizing these antimicrobial and anti-tumor properties of γδ T cells, preclinical and clinical trials have been conducted to develop novel immunotherapies for infections and malignancies. Here, we review the immunological properties of γδ T cells including the underlying recognition mechanism of nonpeptitde antigens and summarize the results of γδ T cell–based therapies so far performed. Based on the results of the reported trials, γδ T cells appear to be a promising tool for novel immunotherapies against certain types of diseases.

Key words: γδ T cells, nonpeptide antigen, tumor, infection, autoimmune and allergic diseases, immunotherapy

Introduction

T cells are subdivided into two major populations distinguished by their surface expression of αβ and γδ T cell receptors (TCR). Both αβ and γδ T cells arise from common multipotent double negative (DN) precursors in the thymus, which can be further separated into four DN subsets based on CD44 and CD25 expression [1, 2]. The T cells undergo extensive DNA rearrangements at the β, γ and δ TCR loci aiming to express functional TCR chains and make a selection between two developmental programs during the DN3 stage, thus generating two distinct characteristics and functions of T cell subsets [3, 4]. Cells with the αβ TCR generally express CD4 or CD8 lineage markers and mostly fall into helper or cytotoxic/effector subsets, whereas, cells with the γδ TCR in humans usually do not express the lineage markers. They do not require conventional antigen presentation in the context of MHC [5]. In fact, the αβ and γδ T cell populations recognize different types of antigens. αβ T cells recognize non-self peptide fragments restricted

by MHC molecules. γδ T cells, on the other hand, recognize unconventional antigens including stress molecules like MICA and MICB and non-peptidic metabolites of isoprenoid biosynthesis [6-9] and so on. Ligand recognition by γδ T cells appears to be dispensable for the selection of γδ T cells in the adult thymus [10].

Human γδ T cells can be divided into three main populations based on δ chain expression. γδ T cells expressing Vδ1 chains are prominent in the intraepithelial layer of mucosal surfaces, where they are involved in maintaining epithelial tissue integrity when facing damage, infection, or transformation [11], responding to stress antigens on epithelial cells [12] and producing IL-10 but little or no IL-2, IL-4 or IFN-γ [13]. They also appear in the peripheral blood, but the intraepithelial and blood populations seem to mix little or not at all [14, 15]. The second population of γδ T cells expresses Vδ2 chain products and represents the majority of circulating γδ T lymphocytes in healthy human adults, comprising up to 50%–90% of the peripheral γδ T-cell population. The Vδ2 chain pairs almost exclusively with Vγ9 (also termed Vγ2). The Vγ9Vδ2 pairing is present only in humans and nonhuman primates [16]. One attractive feature of Vδ2 T cells is that they can serve as professional antigen-presenting cells (APCs) [17]. Activated Vδ2 T cells acquire characteristics of professional APC, such as the expression of antigen presenting, costimulatory, and adhesion molecules, including MHC-II, CD80, and CD86 [18]. It is worthy of note that rapamycin or IL-18 treatment is reported to increase the expression of MHC-II, CD80, and CD86 on Vδ2 T cell lines [18]. The third population is Vδ3 T cells, which take up about 0.2% of circulating T cells including $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ subsets. They are expressed CD56, CD161, HLA-DR, and NKG2D but without NKG2A and NKG2C [19]. The Vδ3 T cells are found only a little in blood but are rich in liver and in patients with leukemias and some chronic viral infections. Through mitogen stimulation with IL-2, Vδ3 T cells were expanded to recognize CD1d. Once activation, they can kill $CD1d^+$ target cells, release cytokines such as Th1, Th2, and Th17, and induce maturation of dendritic cells (DCs) into APCs [19].

γδ T cell receptor

Since the discovery of γδ T cells more than twenty five years ago, many articles have been published to address the physiological functions of these lymphocytes in animals and humans. These studies have emphasized a marked molecular, phenotypic, and functional heterogeneity of γδ T cells. γδ T cells account for 1-10% of $CD3^+$ peripheral blood T lymphocytes of healthy adults [20] and the ratio of Vδ2/Vδ1 cells in blood is approximately 3–10. It is thus most likely that environmental or genetic factors control the balance of the subsets [21, 22]. Compensatory changes for Vδ2 and Vδ1 populations in blood demonstrate a homeostatic balancing mechanism that controls the overall blood count [23]. Caccamo and colleagues showed the influence of gender and age on blood Vδ2 levels, with women having lower levels, compared to age-matched men [24]. These studies suggest that Vδ2 T cell levels be controlled by multiple mechanisms, resulting in the difference in peripheral blood γδ T cell populations among people.

The crystal structure of a human Vγ9Vδ2 γδ TCR [25] illustrates that the receptor shares similarities with the heavy and light chains of immunoglobulins, including the formation of a cleft between the CDR3 loops that is similar to the combining site of immunoglobulin. The amino acid residues consisting of the pocket are mostly germ-line-encoded. This strongly suggests that γδ T cells directly recognize nonpeptide antigens, in a manner similar to antibodies. The recognition of phosphoantigens, however, requires cell–cell contact [26], which indicates that an as yet unidentified molecule(s) is also required to present phosphoantigens to γδ TCR or to transduce the γδ TCR mediated-signaling. Recent reports suggest that ATP synthase-F1/apolipoprotein A-1 complex and butyrophilin 3 are involved in the recognition of nonpeptide antigens by γδ T cells [27, 28].

It is difficult to identify antigens for γδ T cells other than phosphoantigens, because immunization, vaccination, or infection does not generally elicit antigen-specific γδ T cells. It is suggested that the γδ TCR repertoire is directed against "self," most likely represented by inducible cell surface molecules that reflect the status of a cell or tissue [29]. Apart from γδ TCR, γδ cells express most T-lineage-specific genes, including cell surface receptors, signaling effectors, cytokines, and transcription factors, and they phenotypically and functionally resemble αβ cells in both activated and resting states [30].

Classification of γδ T cells

γδ T cells are distinguished from their αβ T cell counterparts by utilizing a distinct set of somatically rearranged variable (V), diversity (D), joining (J), and constant (C) genes. Compared to αβ T cells, available germ line repertoires of Vγ and Vδ genes are limited. γδ T cells contain fewer V, D, and J segments than αβ T cells. Regarding evolutionary forces that shape the V, D, and J gene segments, distinct forces were involved in the formation of the γ and δ loci. In fact, the primate Vγ and Jδ genes are highly conserved, whereas, the γ-locus is split: the Vγ9, Vγ10, and Vγ11 genes represent the conserved region of the Vγ gene

locus, but the remaining Vγ genes have been evolving rapidly [31].

According to different TCR γδ chain expression, human γδ T cells can be classified into two subsets: Vδ1 and Vδ2 γδ T cells. Vδ1 T cells with different Vγ predominate in the intraepithelial subset of mucosal γδ T cells where the TCRs appear to recognize stress molecules on epithelial cells [32]. Vδ2 T cells that generally coexpress Vγ9 are abundant in the peripheral blood and lymphatic system. Besides, they can also be classified into four subpopulations based on their expression of CD27 and CD45RA: naive ($CD27^+CD45RA^+$), effector memory ($CD27^-CD45RA^-$), central memory ($CD27^+CD45RA^-$) and terminally differentiated ($CD27^-CD45RA^+$) [33]. These subpopulations can exhibit unique functions during mycobacterial infection that correspond to the functions of their αβ T cell analogues [33].

Although the number of available Vδ genes is quite small, the combinatorial diversity of the γδ TCR repertoire is at least as large as the αβ TCR repertoire, owing to extensive non-genetically determined mechanisms: for example, N region insertions, imprecise joining, usage of three reading frames, and so on.

Mechanism of ligand recognition

γδ T cells can recognize a variety of structurally different ligands that vary much in size, composition and molecular structure, including non-peptidic antigens, MHC and non-MHC cell surface molecules, soluble proteins, sulfatide and so on.

Non-peptidic antigens

Pfeffer et al. [34, 35] suggested that non-peptide antigens may be important targets for T-cell recognition, which may occur for infections with fungus, bacteria, or protozoa. Porcelli et al. [36] demonstrated that monoalkyl phosphates (MEP) might stimulate γδ T cells in a TCR-dependent manner which was similar to naturally occurring non-peptide mycobacterial antigens. The natural non-peptide antigens are usually fixed in their structure with less antigenic modulation and are much smaller, so the recognition of these antigens is more quickly and effectively with as fast as 2-3 minutes after exposure, indicating that it is not need for antigen uptake and processing. Studies also have confirmed that γδ T cells are specific to recognize phosphate-containing compounds with short, non-bulky carbon chains [37, 38]. Recognition of the prenyl pyrophosphates by the Vγ9Vδ2 γδ TCRs depends on all CDRs [39], as these small molecules are usually presented on the surface of target cells. These findings provide convincing evidence that human γδ T cells can recognize a variety of small phosphorylated non-peptide antigens.

Butyrophilin BTN3A1 has been identified as phosphorylated antigen-presenting molecule (APM) to Vγ9Vδ2 T cells [40], which belongs to a family of immunoglobulin-like molecules with immunomodulatory functions [28, 40, 41]. It plays an important role in Vγ9Vδ2 TCR recognition of prenyl pyrophosphates. Researches have found that it seems function as a sensor for the intracellular levels of phosphorylated antigens, not the same as a costimulatory ligand or a classical presenting molecule binding prenyl pyrophosphates with its extracellular domains [42]. BTN3A1 molecules are modified via specifically binding with these antigens, thus leaving more free space to facilitate the binding of antigens [40]. The identification of BTN3A1 as an additional APM enriches the field of human immunological recognition, with potentially far-reaching implications for clinical immunotherapy.

MHC molecules

γδ T cells can recognize MHC molecules such as group 1(CD1a, b, c) and group 2 (CD1d) CD1 molecules [43, 44]. The stress-induced MHC class I-related molecules MICA and MICB including α1 and α2 domains are also involved in the recognition by human γδ T cells, and do not need Ag processing [45]. Willcox et al. demonstrated a new kind of EPCR-dependent recognition of cytomegalovirus (CMV)-infected cells by γδ T cells [46]. They found that human γδ TCR could directly bound endothelial protein C receptor (EPCR), which is a MHC-like molecule that binds lipids similar to the antigen-presenting molecule CD1d and can make γδ T cells recognize endothelial cells which are targets for cytomegalovirus infection and epithelial tumors. CMV infection can activate γδ T cells specific for self antigens inducing by a multimolecular stress signature on the surface of infected cells, containing EPCR and costimulatory ligand (s). The γδ TCR bound EPCR in an antibody-like way, independently of lipids. Stress surveillance by γδ T cells may consist of innate-like (NKG2D-mediated) components and adaptive (TCR-specific) components, with the γδ TCR engaging a stress-regulated self antigen.

Soluble proteins

Some soluble proteins are also involved in the recognition by γδ T cells, for example, bacterial proteins including the unrelated staphylococcal enterotoxinA (SEA) [47] and the toxin listeriolysin O (LLO) [48]. γδ T cells can also recognize heat shock proteins (HSPs), which does not need antigen processing and can occur in the absence of any APCs [49]. There is no substantial evidence demonstrating that HSP recog-

nition involves the TCR. HSPs may signal through TLRs, directly or indirectly through binding peptides or hydrophobic ligands, such as LPS [50, 51].

In addition, researches show that the myelin-derived glycosphingolipid sulfatide can also be recognized by human γδ T cells [52], indicating that there may also exist other kinds of non-peptidic Ags for γδ T cells which have not yet been identified.

NKG2D, which is expressed on Vγ9Vδ2 T cells, plays an important role in the ligand recognition by γδ T cells, and also is key molecular determinants for the Vγ9Vδ2 T-cell recognition of various carcinomas [53, 54]. NKG2D is a C-type lectin receptor, which provides activation signals by binding to its ligands such as MIC and ULBP families. NKG2D ligands are not expressed by most normal tissues but are upregulated by many tumor-cell types, which are required for tumor cell-recognition by Vγ9Vδ2 T cells.

The precise molecular mechanisms of ligand recognition by γδ T cells remain to be elusive: one supposing that the recognition of nonpeptide antigens by γδ T cells is essentially the same as that by immunoglobulins (Igs) [55], just binding to the specific ligand; the other one is the mechanism similar to that of αβ TCRs, involving a specific ligand and a self-identifying presenting molecule [56]. Binding studies exihibit the existence of Ig-like recognition and a crystallographic analysis supports the direct receptor–ligand interaction for proteinaceous antigens [57]. But further investigation has to be conducted to verify the underlying mechanism of the γδ T cell recognition of nonpeptide antigens, because third molecule may possibly be involved in the efficient recognition.

Activation of γδ T cells

Early studies demonstrated that most Vγ9Vδ2 T cells are strongly activated by a variety of killed microorganisms including bacteria such as *Mycobacterium tuberculosis* [58] and parasites such as *Plasmodium falciparum* [59]. Later, antigens have been identified as (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMB-PP), an immediate upstream metabolite of isopentenyl diphosphate (IPP), so called phosphoantigens [60]. The phosphoantigens can specifically activate Vγ9Vδ2 T cell subset at pico- to nanomolar concentrations. The bacterial HMB-PP levels determine the magnitude of Vγ9Vδ2 T cell expansion *in vitro* or upon transfer of human PBMC and subsequent infection with bacteria. Analogues with potent and selective stimulatory activity for Vγ9Vδ2 T cells can be synthesized. For instance, bromohydrin diphosphate (BrHPP) is active at nanomolar concentrations [61].

In contrast to many bacteria and protozoas, human cells utilize the mevalonate pathway for isoprenoid and IPP biosynthesis [62, 63], which also activates Vγ9Vδ2 T cells *in vitro* following presentation by professional antigen-presenting cells or tumor cells [64, 65], but only at concentrations not achieved physiologically in non-transformed cells. However, certain tumors yield higher concentrations of IPP, which can be sensed by Vγ9Vδ2 T cells [66, 67].

The intracellular levels of IPP can be manipulated by the therapeutically administered drugs. Nitrogen-containing bisphosphonates (N-BPs) such as pamidronate (Pam) and zoledronic acid (Zol), which are in clinical use for osteoporosis and hypercalcemia of malignancies, can enhance intracellular levels of IPP by the inhibition of farnesyl diphosphate synthase (FPPS) [68-70], contributing to the activation and expansion of human Vγ9Vδ2 T cells (Fig. 1).

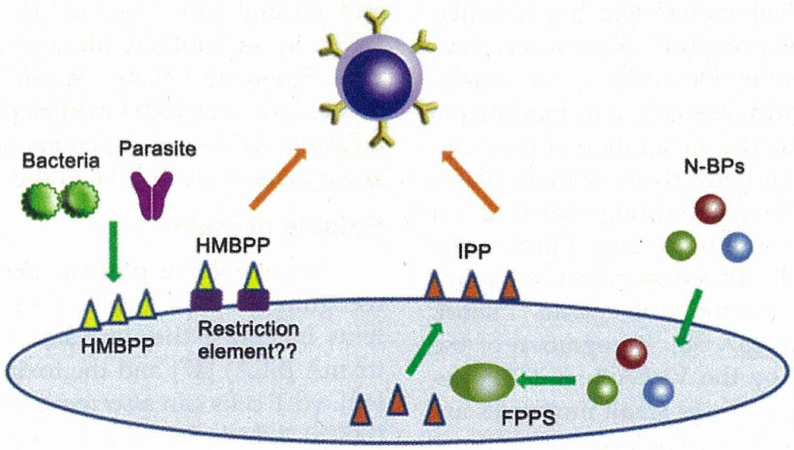

Figure 1. Nonpeptide antigens for γδ T cell stimulation.

Because the activation of γδ T cells does not require antigen processing or MHC molecules, but relies on cell-cell contact with APCs [71], stimulated γδ T cells themselves seem to serve as APCs, but the self-activation or presentation is not effective, compared to the optimal stimulation by monocytes or tumor cells [72]. This indicates that TCR recognition of phosphoantigens requires antigen presentation molecules on APCs. In fact, tetramers of human γδ TCRs bind to APCs in an antigen-dependent manner [73-75].

Recently, Harly and coworkers [27] made a significant advancement on the mechanism underlying the activation of human Vγ9Vδ2 T cells. They found that CD277, a member of butyrophilin molecules, played a central role during the γδ T cell activation. It is, however, still unclear how the Vγ9Vδ2 T cells recognize the phosphoantigen (or anti-CD277 mAb)-induced perturbation of the CD277 surface molecule [76]. The requirement of CD277 for the recognition may explain why human γδ T cells recognize phosphoantigens in a species-specifc manner, because there is no CD277 ortholog in rodents.

In addition, the activation of γδ T cells also requires a variety of costimulatory molecules, including immunoglobulin (Ig) superfamily coreceptors (like CD28 or JAML), tumor necrosis factor receptor (like CD27) and atypical costimulatory molecules such as NKG2D or CD46.

Ig superfamily coreceptors

Several functional assays have suggested CD28 plays an active role in γδ T cell activation [77, 78], which may produce both qualitative and quantitative changes resulting in lower activation threshold and enhanced T cell activity. Anti-CD28 agonist antibodies can enhance human γδ T cell proliferation [79], while blocking antibodies inhibit it obviously [80].

Junctional adhesion molecule-like protein (JAML) has been considered as a key co-receptor in mouse DETC (express an oligoclonal Vγ5Vδ1 TCR) activation [81], whose costimulation can induce DETC proliferation and the secretion of TNF-α, IFN-γ and IL-2. However, it remains unknown whether JAML plays any role in the costimulation of other (including human) γδ T cell subsets.

Tumor necrosis factor receptor (TNFR)

CD27, one of TNFR superfamily co-receptors, has also been shown important contributions to T cell activation. About 80% of Vγ9Vδ2 T cells express CD27 (TNFRSF7) [82]. Upon activation with PMA and ionomycin, most of $CD27^+$ Vγ9Vδ2 T cells produce IFN-γ with less than 1% is IL-17 [82]. The proliferation of $CD27^+$ Vγ9Vδ2 T cells is sensitive to CD70-CD27 modulation, which provides survival and proliferative signals to control γδ T-cell activation. CD27 signals can activate the non-canonical NF-kB pathway and enhance the expression of anti-apoptotic and cell cycle-related genes [83]. Besides, CD27 costimulation plays important roles in the protection from activation induced cell death (AICD) following phosphoantigen stimulation [82] and the expansion of tumour-specific cytotoxic T lymphocytes (CTLs) [84, 85].

Atypical costimulatory molecules

The C-type lectin-like NKG2D receptor plays critical roles in the activation of T cells. NKG2D shows costimulatory function in γδ T cells, which can enhance the response of Vγ9Vδ2 T cells upon TCR activation. NKG2D ligation in Vγ9Vδ2 T cells can upregulate the activation marker CD69 independently of TCR stimulation [48]. NKG2D can either directly activate T cells, as happens for NK cells, or act as a co-receptor to the TCR just as $CD8^+$ T cells do [82]. Upon activation, these NKG2D-expressing T cells can kill tumor [86] or pathogen-infected cells [87].

Furthermore, Vγ9Vδ2 T cells express the BC2 isoform of CD46, which can reduce TNF-α and IFN-γ secretion in HMBPP-stimulated Vγ9Vδ2 T cell cultures [88] when signaling through this isoform, indicating a novel role for CD46 in regulating the production of (Th1-like) pro-inflammatory cytokines in human γδ T cells.

Functions of γδ T cells

γδ T cells display a variety of functions. They can produce different kinds of cytokines and chemokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and growth factors such as IGF-1. Besides, they can regulate immune responses via interactions with other cells, for example, they can provide help for B cells and present antigens for αβ T cells. Other important roles are involved in the macrophage recruitment, cytolytic activity and so on. Such main functions are detailed below.

Firstly, γδ T cells can produce a vast variety of cytokines and chemokines to regulate other immune (e.g., Tregs) and non-immune cells. The types and physiological roles of these cytokines, chemokines and others are in detail discussed in the part of the subtitle "Cytokine secretion".

Secondly, they can provide help for B cells [89]. Caccamo group found that a subset of $V\gamma9V\delta2^+$ T cells that expresses CXC-chemokine receptor 5 (CXCR5) which defines follicular B Th (Th_f) cells were able to induce significant production of Igs even in the absence of Ag, and provided help for naive B cells to produce IgM, IgG, and IgA Abs, suggesting that they are highly efficient at providing B cell help and play a

significant regulatory role in humoral immunity [90].

Thirdly, γδ T cells can trigger dendritic cell (DC) maturation. Vγ9Vδ2 lymphocytes can induce full activation of immature dendritic cells (iDCs) infected with pathogens, such as *Mycobacterium bovis* bacillus Calmette-Gue´rin (BCG) [91]. Human γδ T cells induced DC maturation [92] via TCR-CD1 [93] and Fas–FasL interactions [94]. Because DCs are uniquely resistant to Fas-induced cell death, Fas-FasL interaction can transduce maturation signaling. When human Vγ9Vδ2 T cells were co-cultured with iDCs, they induced increased expression of CD86 and MHC class I on iDCs [95], mainly via γδ T cell-derived TNF-α and IFN-γ. DC maturation induced by LPS was further enhanced by γδ T cells [96].

Fourthly, γδ T cells are involved in the macrophage recruitment. In listeriosis, γδ T cells seem to control the production of macrophage chemoattractant protein I, because it was absent in mice genetically deficient in γδ T cells [97]. A recent investigation of wound healing demonstrated that epidermal γδ T cells could regulate keratinocyte proliferation after wounding [98], and induce hyaluronan production by the epithelial cells [99]. During the subsequent tissue repairing processes, hyaluronan is deposited in the extracellular matrix where it becomes involved in the recruitment of macrophages to the wound lesions. In addition, γδ T cells facilitate cellular differentiation of monocytes and macrophage lineage cells [100], as monocytes differentiate into inflammatory macrophages during bacterial infections, but fail to undergo maturation when γδ T cells are absent.

Fifthly, γδ T cells exhibit varying degrees of cytolytic activity to various kinds of malignancies, such as neuroblastomas [101-103]. Chargui et al. [104] suggested that the expanded γδ T cells which were derived from neuroblastoma patients effectively lysed autologous and allogeneic neuroblastoma cells, and zoledronate or BrHPP could increase the susceptibility of neuroblastoma cells to γδ T cells. The cytolytic activity of γδ T cells could be induced through appropriate cellular contact [105, 106] and augmented by exposure to cytokines during ex vivo expansion, for example, IL-15 and/ or IL-18 [68, 107]. So ex vivo expanded γδ T cells can function as a promising tool for anti-malignancy immunotherapy.

Sixthly, γδ T cells produce growth factors that maintain epidermal integrity [98]. γδ T cells in Psoriasis, an immune mediated disease associated with hyperproliferation of keratinocytes and angiogenesis, have been showed to produce growth factors such as IGF-1, VEGF and FGF-2[108]. They can up-regulate IGF-1 production when stimulated with HMB-PP or PMA/Ionomycin, and produce low amounts of VEGF (<100pg/ml) and FGF-2 (<15 pg/mL) upon activation.

Seventhly, they can present antigens for αβ T cells. It was reported by Brandes et al. that Vδ2$^+$ T cells showed principal characteristics of professional antigen-presenting cells such as dendritic cells. After activation, these cells can rapidly express CC-chemokine receptor 7 (CCR7), which drives their migration to lymph nodes, and upregulate their expression levels of MHC class I and class II molecules and the co-stimulators CD80 and CD86 [109]. They can efficiently process antigens and provide co-stimulatory signals which can strongly induce naive αβ T cell proliferation and differentiation. Human Vγ9Vδ2$^+$ T cells can also present antigens to CD4$^+$ T cells and cross-present antigens to CD8$^+$ T cells [109].

Cytokine secretion

Activated γδ T cells exhibit broad cytotoxic activity against a wide variety of tumor cells, in which they utilize death receptor/ligand (e.g. Fas/Fas-ligand)-dependent and perforin/granzyme- or granulysin-dependent pathways. γδ T cells secrete various cytokines [110] and chemokines including proinflammatory Th1-like cytokines such as IFN-γ and TNF-α, and contribute to reduced survival of autologous tumor cells [111]. Besides, they also produce Th2 cytokines such as IL-4 in the bronchoalveolar lavage fluid of patients with allergic asthma [112, 113].

Furthermore, some γδ T cells give rise to particular cytokines such as keratinocyte growth factor and connective tissue growth factor (CTGF), showing important functions in the control of epithelial integrity, fibrinogenesis and wound repair. Other γδ T cells also produce antimicrobial peptides such as cathelicidin/LL37, indicating the capacity in the local epithelial defense.

In addition, γδ T cells have been reported to secrete interleukin-10 (IL-10) [114], controlling CD8$^+$ T cell expansion and regulating TNF-α secretion by activated CD8$^+$ T cells, and IL-17 [115], regulating the expansion and recruitment of neutrophils and monocytes to initiate inflammatory responses. The role of IL-17-producing γδ T cells has been evaluated in various models of infection [116] and autoimmunity. Secretion of multiple cytokines and chemokines by activated γδ cells and their physiological roles are shown in Fig. 2