厚生労働科学研究費補助金

第３次対がん総合戦略研究事業

ヒト化抗 CD20 抗体を細胞外ドメインとした新規キメラ抗原レセプター（CAR）遺伝子導入 T 細胞の作成と評価

平成24-25年度　総合研究報告書

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総 合 研 究 報 告 書

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ヒト化抗CD20抗体を細胞外ドメインとした新規キメラ抗原レセプター（CAR）遺伝子導入T細胞の作成と評価

研究代表者：寺倉　精太郎　名古屋大学医学部附属病院　血液内科医員

研究要旨：

ヒト化抗 CD20 抗体を細胞外ドメインとした新規キメラ抗原レセプター(CAR) を開発し、これを遺伝子導入したT細胞を用いてその評価を行った。CD20 を特異的に認識する CAR 遺伝子を開発し得た。現在臨床で用いられる抗 CD20 抗体は、連用することで腫瘍細胞表面上の CD20 発現が低下し、抗CD20 抗体療法が不応になることが知られているが、CD20-CAR+ T 細胞はこうした CD20 低発現細胞株も有効に認識・傷害した。また、腫瘍細胞表面上の CD20 が低発現となった患者からの臨床分離株においても有効な認識・傷害を示した。

これまで用いてきたCD28細胞内ドメインに加え、4-1BBおよびCD27細胞内ドメインをもつCARを作成した。さらに抗体部分のaffinityの異なるCARを複数種類作成し、affinity と細胞内ドメインの最適な組み合わせについて検討した。

CD20CAR遺伝子導入T細胞の調製にあたり、実験で行うよりも大きな規模の培養が必要になる。そのため、病院の細胞調製施設を使って試験培養を行う予定としていた。病院の細胞調製施設の規則によって、試験培養は行うことが出来なかったが、その代替として実験室での拡大培養を行った。これによって培養が可能であることが分かった。

研究分担者氏名・所属研究機関名及び所属研究機関における所属

直江知樹・名古屋大学大学院医学系研究科　教授（当時）

村田誠・名古屋大学医学部附属病院血液内科　講師

A.研究目的

本研究ではヒト化CD20抗体を細胞外ドメインとして用いたCARを作成･評価し前臨床試験までを行うことを目的とした。既に抗体のAffinityが報告されているヒト化CD20抗体の遺伝子情報を用いて開発に着手できる。ヒト化抗体を用いた CAR の開発はまだ報告が少ないが、導入した遺伝子産物に対する免疫反応が起こりにくいと考えられるヒト化抗体を用いて免疫反応を避ける必要性は高い。本研究で用いるCD20抗体はAffinityが既知であるために、標的細胞側の抗原発現量がリガンド結合後のT細胞機能に及ぼす影響を、異なる Affinity の CAR を用いて検討可能である。CD20 低発現細胞株や臨床分離細胞を用いてCD20 低発現の標的に対する CD20-CAR の作

用について検討する。さらに Affinity/avidity を変化させたときに CD28/CD27/4-1BB の細胞内ドメインの違いが及ぼす影響について、とくに抗原刺激後の T 細胞の増殖・メモリー化に及ぼす影響について in vitro で検討する。

CD20 刺激後のサイトカイン分泌や細胞分裂能から最適な CAR の構造を決定し、これを用いた臨床試験の準備を行う。分担研究者において既に稼働している Cell processing center を用いて実際の患者から分離した T 細胞を用いた細胞調整の試験を数例程度行い、GMP 基準に則った細胞調整が可能であることを確認する。

開発した CD20-CAR を用いて臨床試験を行い、実際に臨床的有用性が示されれば、現在臨床で使用されている維持抗体療法・化学療法の代替としてより副作用が少なく、維持療法よりもむしろ安価な治療として認知されることが期待される。

B. 研究方法

新規ヒト化抗CD20抗体の可変領域を細胞外ドメインとするCARを分子生物学的手法を用いて作成した。作成したCD20-CARをレトロウイルス作成用のプラスミド・ベクターに組み込み、レトロウイルス・ベクターを作成した。作成したレトロウイルス・ベクターを用いてドナー末梢血T細胞にCD20-CARを遺伝子導入し、CD20-CAR+ T細胞を作成した。さらにCD8陽性細胞あるいはCD4陽性細胞に純化して実験に用いた。種々のレベルのCD20を発現するCD20-CEM細胞を標的としてクロム放出試験を行った。また、本学にて冨田らが樹立・報告しているCD20低発現細胞株およびCD20低発現臨床分離細胞を標的としてクロム放出試験や細胞内インターフェロンγ染色にて、CD20-CAR+ T細胞が標的をCD20特異的に認識・傷害するかどうかについて検討した。細胞上清に含まれるインターフェロンγやIL-2はELISAを用いて測定した。

これまで用いてきたCD28細胞内ドメインに替えて4-1BB/CD27の細胞内ドメインを組み込んだプラスミド・ベクターを作成した。上記同様にレトロウイルス・ベクターを作成した。CARがCD20に結合した後、伝達されるシグナルを比較検討するため、JurkatおよびSUPT1細胞株にreporter vectorを組み込んだものを作成している。これにより、より定量的にシグナル伝達を評価することにした。

（倫理面への配慮）

患者あるいはドナーから細胞その他の材料を採取する場合には、当院 IRB で審査を受け、適切なインフォームド・コンセントのもと行う。研究遂行にあたって必要な倫理指針などを遵守して行う。

C. 研究結果

新規にヒト化抗 CD20 抗体を細胞外ドメインとして用いた CAR を作成し、細胞表面上に発現する CD20 の抗原量と CD20-CAR+ T 細胞の反応しうる限界について検討した。新たに作成した CD20-CAR を遺伝子導入した CD20-CAR+ T 細胞は CD20 特異的に標的細胞を認識・傷害した。この細胞を用いて様々な程度の CD20 を発現する CEM 細胞株群に対する細胞傷害活性を検討した。CD20-CAR+ T 細胞は極めて低い CD20 発現を示す CD20+ CEM 細胞に対しても高い細胞傷害活性を示した。さらに、CD20 低発現となり臨床的に抗 CD20 抗体療法に対して不応となった患者から樹立された細胞株・臨床分離検体に対しても比較的高い認識・細胞傷害活性を示した。

CD27 細胞内ドメインを用いた CAR を CD28 あるいは 4-1BB の細胞内ドメインを用いた CAR と比較して有用性を検討することを目的として、これまで用いてきた CD28 細胞内ドメインに替えて、4-1BB/CD27 細胞内ドメインに入れ替えたものを作成した。これらのプラスミド・ベクターを用いてレトロウイルス・ベクターを作成した。Reporter vector を遺伝子導入した Jurkat および SUPT1 細胞にこれらの CD20-CAR を遺伝子導入し、CD20

刺激後に蛍光を評価する系を樹立すべく、実験を進めた。Reporter vector を遺伝子導入した細胞株は樹立できたが、刺激後の活性化が見られなかった。作成した Affinity の異なる 5 種類の CAR を比較するため、ドナー由来 T 細胞に遺伝子導入し、比較検討した。Affinity が高まるにつれて、一定以上の Affinity があればCD20 特異的にCAR を通した細胞の活性化は見られた。しかしながら、一定以上に Affinity を高めても、同程度のシグナルが伝わっていることが分かった。CAR として有効性が出るための抗体の Afftinity の閾値が存在するものと考えられた。

最適と考えられるCD20-CAR の構造が決定した後、これを用いた臨床試験の準備として GMP 基準に則った細胞調製が可能かどうかについて検討することにしていたが、本学の細胞調製施設の規則により、患者に投与するための細胞以外の細胞の調製は細胞調製施設では行えなかった。そのため、代替として実験室での Large scale 培養の検討を行った。これにより、CD20CAR-T 細胞は臨床スケールで培養可能であることが分かった。

D. 考察

CAR の標的抗原が腫瘍組織以外に発現していると、CAR がその抗原を標的として正常組織をも攻撃することが懸念される。そのため CAR の標的抗原は腫瘍組織以外に発現がないことが極めて厳密に求められてきた。そのためになかなか新しい CAR の標的抗原の同定はこれまで困難であった。一方で、これまで抗体療法の標的としての腫瘍特異抗原の探索は広く行われてきたが、その場合には腫瘍特異性と同時にその抗原が腫瘍において高発現していることが求められてきた。こちらも同様になかなか新規に良い標的抗原は出てこなかった。

本研究の結果から、CAR は抗体の認識しうる範囲よりも低発現の標的でも十分認識しうることが示され、腫瘍抗原の探索範囲をこれまでよりも低発現の範囲に広げることによって新たな腫瘍抗原が得られる可能性が考えられた。そのような新しい戦略によって比較的発現の低い腫瘍抗原を CAR の標的抗原として同定出来れば、CAR の臨床応用の可能性も高まることが期待される。

抗体を CAR に加工した場合に必要な条件については詳細には分かっていない。今回我々は 5 種類の Affinity の異なる CD20CAR を作成し、比較検討した。Affinity は一定以上の強さが必要であったが、Affinity と活性化の程度との相関は閾値のある反応曲線を描いた。今後さらに何種類かの細胞内ドメインにおいて、細胞内ドメインと Affinity との組み合わせにおいて、最適なものを検討していく。

CD20CAR 遺伝子導入 T 細胞の臨床スケールの培養を病院の細胞調製室で行う予定であったが、病院規則から出来なかった。その代替として実験室での拡大培養を行った。その結果として細胞調製は技術的には可能であることが確認できた。今後、CD20CAR の最適構造が決定し、臨床試験の開始を目指していく。

E. 結論

新規 CD20-CAR を作成し、T 細胞に遺伝子導入を行った。これらの CD20-CAR+ T 細胞は CD20 を特異的に認識・傷害した。これらの細胞を用いて CD20 低発現細胞株・臨床分離検体に対する反応を検討した。極めて低発現の細胞株や臨床分離検体でも認識・傷害しうることがわかった。CAR のこういった特性を生かして、低発現であるが腫瘍特異性の極めて高い標的抗原の探索という新しい戦略が考えられた。新規 CD20CAR は CD20 低発現標的に対して有効であることが示唆された。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況

（予定を含む）

1. 特許取得

ヒト化抗 CD20 キメラ抗原レセプター　発明者：寺倉精太郎、渡邊慶介　権利者：名古屋大学　産業財産権の種類、番号：特願 2013-234784、出願年月日：2013 年 11 月 13 日

2. 実用新案登録

なし

3. その他

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

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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V. 研究成果の刊行物・別刷

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Letter to the Editor

Successful unrelated cord blood transplantation for adult acquired aplastic anemia using reduced intensity conditioning without ATG

Acquired aplastic anemia (aAA) patients who are transfusion-dependent and who have failed or relapsed after immunosuppressive therapy need further treatment. In cases in which a human leukocyte antigen (HLA)-identical sibling donor is not available, the use of alternative donor including HLA-matched unrelated donor and unrelated cord blood (CB) are commonly considered, but this strategy is associated with worse outcomes [1]. Because of the abundant availability of acceptable CB units, the use of cord blood transplantation (CBT) has been increasing. Although the use of CBT in patients with aAA has recently been evaluated [2], relatively little information is available on how to achieve proper engraftment with a reduced intensity conditioning (RIC) regimen in aAA patients undergoing CBT. We describe here three adult patients with aAA who underwent transplantation with unrelated CB after a RIC regimen without ATG. The patient characteristics are shown in Table 1. All three patients received single-unit CB containing more than 2.2×10^7/kg of total nucleated cell (TNC) with no more than two of six HLA-mismatches. The conditioning regimen consisted of six doses of fludarabine (Flu) 30 mg/m^2, two doses of cyclophosphamide (CY) 60 mg/kg and total body irradiation (TBI) 2 Gy × 2 with no use of anti-thymocyte globulin (ATG). The graft-versus-host disease (GVHD) prophylaxis regimen was a combination of short-term methotrexate (15, 10, and 10 mg/m^2 on days 1, 3, 6, respectively) and tacrolimus. Because patient #3 had a high titer of anti-HLA antibody, a CB unit that was not cross-reactive with this antibody was chosen.

All three patients rapidly exhibited sustained CB engraftment (Table 2). Chimerism analyses of the CD3$^+$ fraction using various numbers of tandem repeats showed initial full-donor conversion from the first point of analysis in all patients. No secondary graft-failure was observed. Acute GVHD was observed in patient #2 (skin only stage 2, Grade I) and resolved spontaneously. Chronic GVHD was observed in patient #3 (skin, oral involvement) and symptoms resolved quickly after the administration of 0.5 mg/kg oral prednisolone. The regimen was generally well tolerated, and no significant organ damage or severe toxicity occurred. The patients remain alive without transfusion dependence at 68, 44 and 9 months, with Karnofsky scores of 70% (due to postherpetic neuralgia), 100% and 100%, respectively.

Here we report three CBT recipients who received successful single-unit CBT after a RIC regimen. All three patients exhibited sustained full donor-type hematopoiesis without further intervention to increase donor-type chimerism. The conditioning regimen included 180 mg/m^2 Flu and 120 mg/kg CY with 2 Gy × 2 TBI, which may be regarded as a relatively strong regimen in terms of immunosuppressive and cytotoxic ability. Thus, one might think this regimen too potent for the induction of sustained engraftment of CB. However, Liu et al. reported that RIC regimen, consisting of Flu 120 mg/m^2, CY 1200 mg/m^2 (equivalent to 40 mg/kg if the patient's body weight was 50 kg) and rabbit ATG 30 mg/kg, was not sufficiently potent enough to induce engraftment after CBT in patients with aAA. They reported two early deaths and 16 graft-failures among the 18 CBT recipients conditioned with the above regimen [3]. Thus, it is reasonable to use a CY dose >40 mg/kg, and further study to determine the optimal CY dose between 40 and 120 mg/kg is warranted.

To ensure rapid and proper CB engraftment, graft cell contents, such as TNC, CD34$^+$ cell count and CD8$^+$ cell count, are important factors [4]. In Western countries, ATG is commonly used as the conditioning regimen for CBT. Nevertheless, the use of ATG will decrease lymphocytes, including graft-facilitating CD8$^+$ lymphocytes, which may lead to attenuation of total potency for the facilitation of engraftment in exchange for the beneficial effect of reducing the incidence of severe acute GVHD. Indeed, only one of seven CB recipients for aAA who received ATG-containing regimen achieved engraftment in a previous retrospective study in Japan [5]. Thus, we replaced ATG with 4 Gy TBI in our regimen, which may be another reason for successful engraftment.

One of the biggest differences in CBT between Western countries and Japan may be the attitude toward the use of ATG. In the recent protocol of European group, two doses of ATG 2.5 mg/kg and a single agent GVHD prophylaxis are recommended [6]. To reduce the incidence of severe acute GVHD, physicians in Europe and US would be likely to use ATG more frequently, which might result in failure to observe better engraftment. In fact, it is reported that a conditioning regimen without ATG provided a low incidence of graft-failure [7]. Taken together, we believe that ATG should not be included in the conditioning regimen for CBT, not only for a single-unit CBT but also for a double-unit CBT. We also have shown the superiority of two-drug GVHD prophylaxis (including methotrexate) over single-drug prophylaxis in CBT [8]. To compensate prophylactic effect of ATG to control severe GVHD, it would be preferable to develop the GVHD prophylaxis after transplantation without ATG. Further study to determine whether or not ATG should be used in order to achieve prompt engraftment and subsequent higher quality of life and survival after RIC-CBT is warranted.

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Table 1
Patient demographics and CB unit characteristics.

Pt no.	Age/sex	BW (kg)	Disease status at transplant	Interval from diagnosis to CBT (year)	Transfusion dependency	ABO mismatch	HLA serological mismatch	HLA allele mismatch	HLA-antibody	Donor-specific antibody	TNCC (10^7/kg)	$CD34^+$ (10^5/kg)
1	48/M	51	Severe	1.2	RBC	Match	2/6	3/8	–	–	3.67	0.50
2	53/M	65	Severe	22.1	RBC/PC	Major/minor	1/6	4/8	–	–	2.79	0.44
3	37/F	51	Non-severe	26.9	RBC/PC	Major/minor	2/6	3/8	+	–	2.24	0.55

Pt, patient; M, male; F, female; BW, body weight; RBC, red blood cell concentration; PC, platelet concentration; HLA, human leukocyte antigen; TNCC, total nucleated cell count.

Table 2
Engraftment, chimerism and other outcomes.

Pt no.	Days to ANC >500/μl	Days to reticulocyte >1%	Days to plt > 20,000/μl	Days to plt > 50,000/μl	Chimerism after CBT	Acute GVHD	Chronic GVHD	Other complications	Survival, mo	KS (%)
1	19	30	25	191	Day 20, 95% donor	No	No	Postherpetic neuralgia	Alive, 68	70
2	21	28	37	44	Day 19, 100% donor	Grade I (skin 2)	No	Polymyalgia rheumatica	Alive, 44	100
3	22	37	32	43	Day 25, 100% donor	No	Yes (skin, oral)	No	Alive, 9	100

Pt, patient; ANC, absolute neutrophil count;plt, platelet; KS, Karnofsky score.

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Conflict of interest

All authors declare that there are no competing financial interests.

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Identification of a novel HLA-A*24:02-restricted adenovirus serotype 11-specific CD8+ T-cell epitope for adoptive immunotherapy

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ABSTRACT

Subgroup B adenovirus serotype 11 (Ad11) occasionally causes fatal infections in immunocompromised patients. The present study describes a novel Ad11 epitope presented by HLA-A*24:02 that could be used for adoptive immunotherapy. Ten synthetic Ad11 hexon protein-derived nonamer peptides that bound to HLA-A*24:02 were selected by a computer algorithm and MHC stabilization assay. Stimulation of peripheral blood mononuclear cells from HLA-A*24:02+ donors with each of these synthetic peptides induced peptide-specific CD8+ T-cells for three peptides. Testing the reactivity of these peptide-specific CD8+ T-cells against various target cells confirmed that peptide TYFNLGNKF is naturally processed in Ad11-infected cells and is presented by HLA-A*24:02. Emergence of TYFNLGNKF-specific CD8+ T-cells coincided with the clearance of adenoviruses in a patient with Ad11 disease. Importantly, TYFNLGNKF-specific CD8+ T-cells were suggested to be not serotype cross-reactive. The novel HLA-A*24:02-restricted Ad11 epitope could be used for anti-Ad11 adoptive immunotherapy and to monitor immunity to Ad11 using MHC tetramers.

1. Introduction

Adenoviruses (AdV) cause lethal infections in immunocompromised hosts such as hematopoietic stem cell transplantation (HSCT) and chemotherapy recipients (Chakrabarti et al., 2002; Leen et al., 2006a; Yokose et al., 2009). Although antiviral agents, such as ribavirin and cidofovir, have been used for the treatment of AdV infection, their efficacy is limited by weak intrinsic activity against viruses and by toxicity (Ison, 2006; Lindemans et al., 2010). Furthermore, reconstitution of AdV-specific T-cells is required for the control of AdV infection (Feuchtinger et al., 2005; Heemskerk et al., 2005). These observations have led to the development of adoptive T-cell therapy for the management of AdV infection (Feuchtinger et al., 2006; Leen et al., 2006b).

There are several different approaches to generate virus-specific T-cells for adoptive therapy. In previous reports, peripheral blood mononuclear cells (PBMCs) were stimulated with the lysate of AdV-infected cells or with adenoviral vector-transduced cells to generate AdV-specific T-cells (Feuchtinger et al., 2004; Leen et al., 2004a). However, the clinical use of these strategies is complicated by the concerns associated with transferring live viral particles to patients who are immunocompromised. Another method to generate virus-specific T-cells is to stimulate PBMCs with immunogenic peptides derived from viral proteins. This method is advantageous in that synthetic peptides can be readily produced under good manufacturing practice conditions. Furthermore, the feasibility of this approach has been documented in clinical studies in which cytomegalovirus (CMV)-pp65 peptide-specific cytotoxic

Abbreviations: AdV, adenoviruses; Ad11, adenovirus serotype 11; BIMAS, Bioinformatics and Molecular Analysis Section; CM, culture medium; CMV, cytomegalovirus; CTLs, cytotoxic T-cells; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; E:T, effector to target; FBS, fetal bovine serum; HC, hemorrhagic cystitis; HSCT, hematopoietic stem cell transplantation; IFN-γ, interferon-γ; IL-2, interleukin-2; K562/A*24:02, K562 cells transduced with HLA-A*24:02; LCLs, Epstein-Barr virus-transformed B-lymphoblastoid cell lines; LYA, LYANSAHAL; mAb, monoclonal antibody; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; TCRs, T cell receptors; TYF, TYFNLGNKF; VYS, VYSGSIPYL.

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T-cells (CTLs) were given therapeutically or prophylactically to HSCT recipients (Meij et al., 2012; Micklethwaite et al., 2007).

The wide clinical application of AdV-specific T-cells generated by stimulating PBMCs with immunogenic peptides requires knowledge of T-cell epitopes restricted by prevalent MHC molecules. Previous studies that have identified T-cell epitopes of AdV have focused exclusively on the subgroup C AdV (Leen et al., 2004b, 2008; Tang et al., 2006; Zandvliet et al., 2010). Subgroup B AdV serotype 11 (Ad11) is a major pathogen for hemorrhagic cystitis (HC) (Akiyama et al., 2001; Miyamura et al., 1989; Mori et al., 2012) and occasionally causes disseminated infection with fatal outcomes in immunocompromised patients (Taniguchi et al., 2012). However, T-cell epitopes of Ad11 have not been identified. Therefore, the goal of the following study was to identify a novel T-cell epitope of Ad11 presented by HLA-A*24:02, which is one of the most common HLA class I molecule in many ethnic groups (60% in Japanese population, 20% in Caucasians, and 12% in Africans) (Gomi et al., 1999).

2. Materials and methods

2.1. Donor and patient specimens

PBMCs and serum from HLA-A*24:02+ healthy volunteer donors and a patient with Ad11-associated HC were obtained after informed consent. In addition, urine was collected from a patient with Ad11-associated HC. Measurement of AdV DNA in the patients' serum and urine was performed by real-time polymerase chain reaction, as previously described (Funahashi et al., 2010).

2.2. Cell lines

Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (LCLs) were generated by infection of PBMCs from healthy donors with concentrated EBV-containing supernatants of cultured B95-8 cells (Leen et al., 2004b). T2-A24 cells, the transporter associated with antigen processing-deficient B and T hybrid cell line T2 transfected with the HLA-A*24:02 gene, were kindly supplied by Dr. Y. Akatsuka (Aichi Cancer Center Research Institute, Nagoya, Japan). K562 cells were transduced with retroviruses that encode CD80 and CD86 and were selected to >90% purity by cell sorting for expression of these co-stimulatory ligands. CD80 and $CD86^+$ K562 were then transduced with retroviruses that encode a full-length HLA-A*24:02 (Phoenix-Ampho System; Orbigen) and sorted twice to obtain cells of >95% purity that expressed HLA-A*24:02 (named K562/A*24:02). Cell lines were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS). For preparation of peptide-pulsed LCLs, K562, and K562/A*24:02, the cells were washed once, resuspended in RPMI-1640 medium, and pulsed with the corresponding synthetic peptide at 5 μg/ml at room temperature for 2 h. The cells were then washed once and used in stimulation assays.

2.3. Infection of K562 and K562/A*24:02 with AdV

Ad11 isolated from patients was used in the experiments. K562/A*24:02 were infected with Ad11 at a multiplicity of infection of 100 and used for enzyme-linked immunosorbent assay (ELISA) 72 h after infection. Infection was confirmed by documentation of AdV hexon antigen expression by flow cytometry.

2.4. Peptides

A computer-based program (Bioinformatics and Molecular Analysis Section (BIMAS), HLA peptide binding predictions; http://www-bimas.cit.nih.gov/molbio/hla_bind/) was used to identify potential HLA-A24-binding peptides within the Ad11 hexon protein. The nonamer peptides with a score exceeding 100 were selected and synthesized. HLA-A24-binding peptide, QYDPVAALF, derived from the human CMV-pp65 protein (Kuzushima et al., 2001), and TYFSLNNKF, derived from the human AdV serotype 5 hexon protein (Leen et al., 2004b), were also synthesized. All peptides were synthesized by Medical & Biological Laboratories (Nagoya, Japan).

2.5. MHC stabilization assay

All candidate peptides were tested for their capacity to bind to HLA-A24 molecules on the surface of T2-A24 cells as described previously (Kuzushima et al., 2001). Briefly, T2-A24 cells (3×10^5 cells) were incubated with 200 μL RPMI-1640 medium containing 0.1% FBS, 5×10^{-5} M β-mercaptoethanol (Sigma), 3 μg/ml human β2-microglobulin (Sigma), and each of the peptides at a concentration of 10 μM at 37 °C for 16 h. Following the incubation, surface HLA-A24 molecules were stained with the anti HLA-A23/A24 monoclonal antibody (mAb) and anti-mouse FITC-labeled antibodies. Expression was measured by flow cytometry, and mean fluorescence intensity (MFI) was recorded. Percent MFI increase was calculated as follows: percent MFI increase = (MFI with the given peptide − MFI without peptide)/(MFI without peptide) × 100.

2.6. Generation and expansion of peptide-specific $CD8^+$ T-cells

PBMCs obtained from healthy volunteers were placed at a concentration of 2×10^6 cells per tube in a 14 ml polypropylene tube with 1 ml of RPMI-1640 medium with 10% human serum [referred to as culture medium (CM)] and directly stimulated with peptides at a concentration of 1 μg/ml. At day 3, CM was added to a final volume of 2 ml and supplemented with 25 IU/ml recombinant human interleukin-2 (IL-2) (R&D Systems, Minneapolis, MN). Cells were transferred to a 24-well plate at day 7, re-stimulated with peptides every 7 days, cultured until day 21 or 28, and tested by interferon-γ (IFN-γ) secretion assay for the presence of peptide-specific $CD8^+$ T-cells. To expand peptide-specific $CD8^+$ T-cells, $CD8^+$ cells producing IFN-γ in the presence of peptides were isolated using IFN-γ secretion assay, followed by expansion in the presence of OKT3 mAb (Janssen Pharmaceutical), IL-2, and feeder cells, as described previously (Sugimoto et al., 2009).

2.7. Antibodies and flow cytometric analysis

All antibodies were purchased from BD Biosciences (San Jose, CA) unless otherwise noted. The following anti-human antibodies for staining of cell surface markers and intracellular molecules were used: IFN-γ-FITC, CD3-PE-Cy5.5 (Invitrogen, Carlsbad, CA), CD8-PE, -APC, or -PerCP-Cy5.5, and HLA-class I-PE (eBioscience, San Diego, CA). PE-conjugated HLA class I tetramers folded with AdV peptides were used to stain virus-specific T cell receptors (TCRs) (Medical & Biological Laboratories). In addition, mouse anti HLA-A23/A24 (One Lambda, Canoga Park, CA) and mouse anti-AdV hexon protein (Abcam, Cambridge, UK) antibodies were used in combination with anti-mouse IgG/IgM-FITC.

The IFN-γ secretion assay was performed using the IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, cells were re-stimulated for 4 h at 37 °C in the presence or absence of peptides (1 μg/ml). Cells were incubated with IFN-γ catch reagent and cultured for 45 min at 37 °C to allow for IFN-γ secretion, followed by staining with anti-IFN-γ, CD3, and CD8 antibodies. Intracellular cytokine staining assay for IFN-γ was performed as previously described with some modifications (Terakura et al., 2012). In brief, cells were re-stimulated with peptide-pulsed

or peptide-unpulsed autologous LCLs and incubated at 37 °C for 4 h. Brefeldin A (Golgiplug, BD Biosciences) was added during the last 2.5 h of incubation to block secretion of cytokines. Subsequently, the cells were fixed, permeabilized, and stained with anti-IFN-γ and CD8 antibodies, using FIX/PERM and PERM/Wash solution (BD Biosciences). Data acquisition was performed with FACSAria or FACSCanto flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Inc., Ashland, OR).

2.8. ELISA

K562/A*24:02, those loaded with peptides, and those infected with Ad11 were dispensed at 3×10^4 cells/well into triplicate cultures in 96-well, round-bottom plates. Then, 3×10^4 of peptide-specific CD8$^+$ T-cells were added to each well, and after a 24 h of co-culture at 37 °C, IFN-γ in the supernatant was measured using an ELISA method (Endogen).

2.9. Cytotoxicity assay

K562 and K562/A*24:02 loaded with or without peptides were used as target cells in cytotoxicity assay. Target cells were labeled for 2 h with ^{51}Cr, washed twice, dispensed at 1×10^3 cells/well into triplicate cultures in 96-well, round-bottom plates, and incubated for 4 h at 37 °C with peptide-specific CD8$^+$ T-cells at various effector to target (E:T) ratios. Percent-specific lysis was calculated as follows: percent-specific lysis = (experimental cpm − spontaneous cpm) × 100/(maximum cpm − spontaneous cpm).

3. Results

3.1. Selection of potential HLA-A24-binding peptides within Ad11 hexon protein

To identify potential HLA-A24-binding peptides within amino acid sequences of the Ad11 hexon protein, the amino acid sequence of the protein was analyzed by a web-based algorithm designed to predict HLA-binding peptides, based on estimation of the half-time dissociation of the HLA-peptide complex (http://www-bimas.cit.nih.gov/molbio/hla_bind/). Ten peptides with estimated half-time dissociation scores above 100 were selected and synthesized (Table 1). Next, the binding capacities of these peptides to HLA-A*24:02 molecules were tested in MHC stabilization assays using T2-A24 cells. All 10 peptides increased the HLA-A24 expression on the cells, indicating that these peptides bound and stabilized the HLA complex on the cell surface (Table 1). Thus, all 10 peptides were included in the subsequent experiments.

Table 1
Ad11 hexon protein derived peptides predicted to bind to HLA-A24, and the results of the MHC stabilization assays.

Amino acid sequence	Start position	Score[a]	% MFI increase[b]
KYTPSNVTL	482	480	141
DYLSAANML	641	360	77
SYQLLLDSL	366	360	57
LYSNVALYL	469	280	186
VYSGSIPYL	696	200	583
LYANSAHAL	889	200	37
NYNIGYQGF	769	180	142
TYFNLGNKF	37	158	82
NYIGFRDNF	322	150	89
GYKDRMYSF	782	120	27

Ad11, adenovirus serotype 11; MFI, mean fluorescence intensity.
[a] Estimated half-time of dissociation from HLA-A24 (min).
[b] Percent MFI increase of HLA-A*24:02 molecules on T2-A24 cells.

3.2. Induction and expansion of peptide-specific CD8$^+$ T-cells from healthy donor PBMCs

To investigate the immunogenic potential of the 10 candidate peptides, HLA-A*24:02+ PBMCs from five healthy donors, of whom three were seropositive and two were seronegative for Ad11, were stimulated in vitro with each of these peptides. After 3-4 weekly stimulations, peptide-specific CD8$^+$ T-cell frequencies were determined by IFN-γ secretion assays. Of the 10 peptides tested, INF-γ-producing CD8$^+$ T-cells specific for the respective peptides were induced in the 3 peptides, TYFNLGNKF (TYF), VYS-

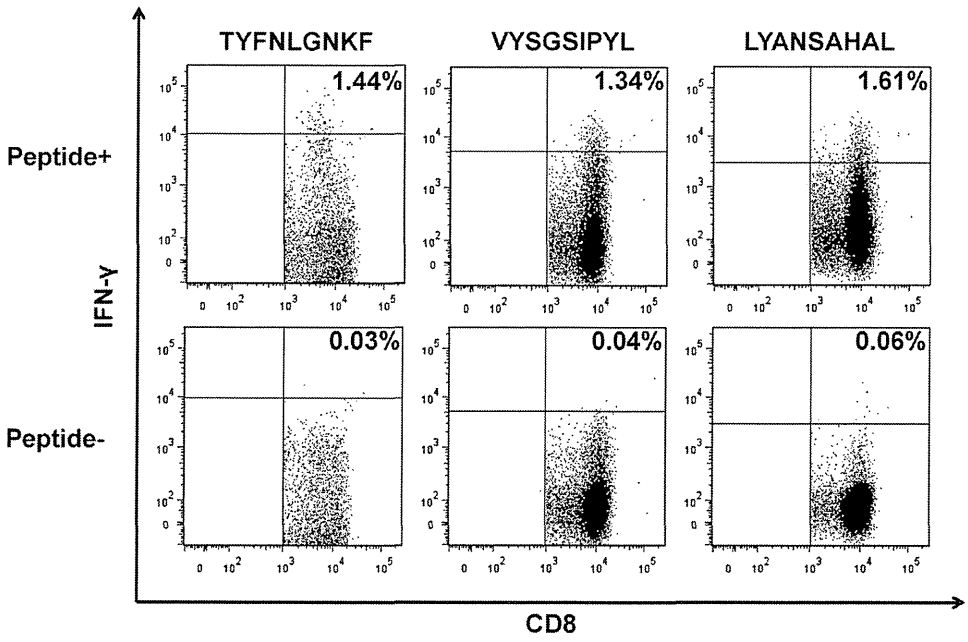

Fig. 1. Induction of peptide-specific CD8$^+$ T-cells from PBMCs of HLA-A*24:02+ healthy donors. PBMCs from five HLA-A*24:02+ healthy donors were stimulated with each of the 10 epitope candidate peptides, and frequencies of IFN-γ-producing CD8$^+$ cells were determined by IFN-γ secretion assays. The numbers in the upper right quadrants are the percentage of IFN-γ-producing cells among CD8$^+$ cells in the presence (upper row) or absence (lower row) of the peptide. Among the 10 peptides tested, 3 induced IFN-γ-producing CD8$^+$ cells.

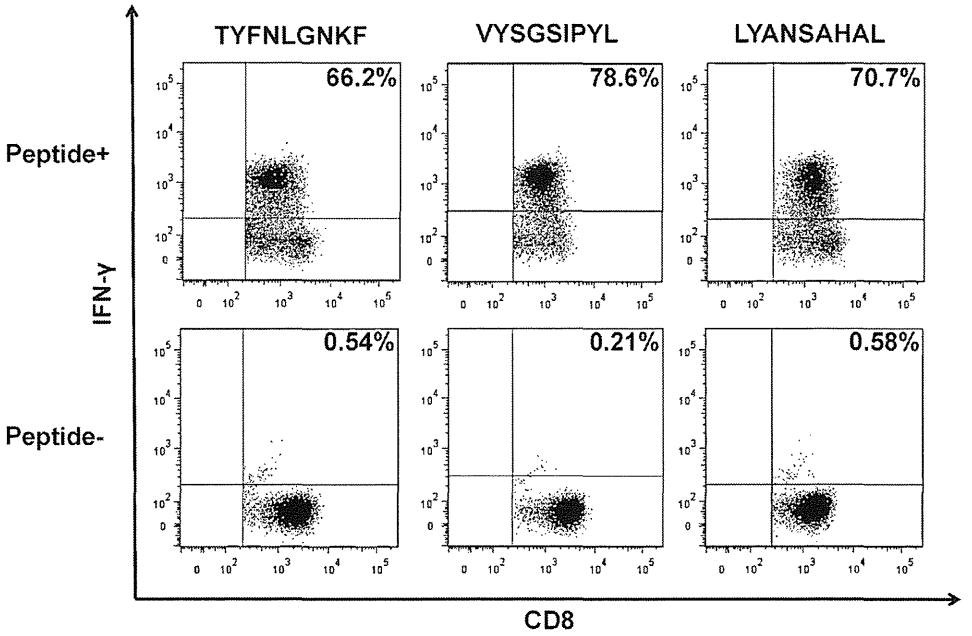

Fig. 2. Enrichment and expansion of peptide-specific CD8+ T-cells. Peptide-specific CD8+ T-cells induced by stimulating PBMCs from HLA-A*24:02+ healthy donors with epitope candidate peptides were isolated using IFN-γ secretion assay and then expanded in the presence of OKT3 mAb, IL-2, and feeder cells. Thereafter, the frequencies of IFN-γ-producing cells among CD8+ cells upon stimulation with autologous LCLs loaded with (upper row) or without (lower row) peptides were determined by intracellular IFN-γ staining assay.

GSIPYL (VYS), and LYANSAHAL (LYA) (Fig. 1). TYF-specific CD8+ T-cells were induced in all three seropositive donors, but not in the two seronegative donors. VYS- and LYA-specific CD8+ T-cells were induced in one of the two seronegative donors. Next, these INF-γ-producing CD8+ T-cells were sorted using an IFN-γ secretion assay, then expanded for subsequent analysis. After sorting and expansion, the frequencies of peptide-specific CD8+ cells among CD8+ cells determined by intracellular cytokine staining assay were above 60% for all three peptides (Fig. 2).

3.3. TYF peptide is naturally processed in Ad11-infected cells and presented in the context of HLA-A*24:02

To determine whether the epitope candidate peptides are naturally processed in Ad11-infected cells and presented by HLA-A*24:02 molecule, the expanded peptide-specific CD8+ T-cells were tested for IFN-γ production against HLA-A*24:02+ cells infected with Ad11. TYF-specific CD8+ T-cells produced significant quantities of IFN-γ against Ad11-infected or TYF peptide-loaded K562/A*24:02, but not against unmanipulated K562/A*24:02 and those loaded with an irrelevant peptide, QYDPVAALF, which is a CMV-pp65 peptide presented by HLA-A*24:02 (Fig. 3A) (Kuzushima et al., 2001). On the other hand, VYS- and LYA-specific CD8+ T-cells did not respond to Ad11-infected K562/A*24:02, indicating that VYS and LYA peptides were not naturally processed and presented on Ad11-infected cells (Fig. 3B, C). Furthermore, TYF-specific CD8+ T-cells effectively lysed TYF peptide-loaded K562/A*24:02 but not TYF peptide-loaded untransfected K562 (Fig. 4). These results indicate that TYF peptide is processed naturally in Ad11-infected cells, presented in the context of HLA-A*24:02, and is an epitope recognized by CD8+ CTLs.

3.4. TYF-specific CTLs do not recognize an HLA-A*24:02 restricted epitope of AdV serotype 5

As the newly identified epitope TYFNLGNKF was similar to the previously identified HLA-A*24:02 restricted epitope of AdV serotype 5 TYFSLNNKF (Leen et al., 2004b), we examined whether

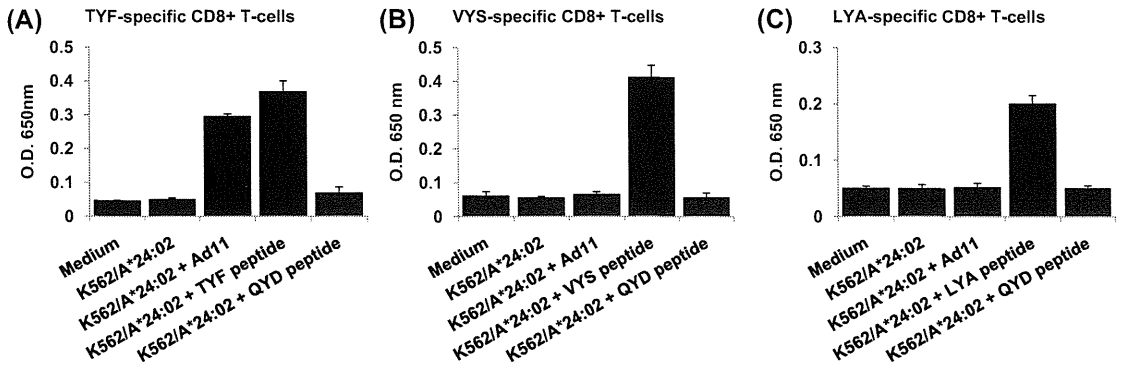

Fig. 3. TYF-specific, but not VYS- and LYA-specific, CD8+ T-cells produce IFN-γ against K562/A*24:02 infected with Ad11. Expanded TYF-specific CD8+ T-cells (A), VYS-specific CD8+ T-cells (B), and LYA-specific CD8+ T-cells (C) were incubated with K562/A*24:02, those loaded with the respective peptides, those infected with Ad11, or those loaded with an irrelevant peptide for 24 h. IFN-γ production in the supernatant was measured by an ELISA. Data are representative of two independent experiments and are the mean ± s.d. of triplicate experiments.