らに、マイナー抗原を標的とした移植後再発造血器腫瘍に対するペプチドワクチン療法、養子免疫療法は、愛知県がんセンターのヒトゲノム・遺伝子倫理委員会で承認済みの研究実施書と同意文書に基づいて、書面による同意の得られた場合のみに実施されたものである。

以上の厳格な遵守により、本研究は倫理面で問題が無かったものと考える。

C. 研究結果

①-1 新規マイナー抗原の同定法の検証：まず昨年度我々が樹立し、新たな拘束性HLA アリルとして HLA-A*0206 を報告した既知のマイナー抗原である HA-1 遺伝子（*HMHA1*）が HapMap リソースを用いて同定できるか後方視的に検証した。HLA-A*0206 の CTL-4B1 を用いて 58 種類の日本人および中国人由来の B-LCL をタイピングしたところ、37 の抗原陽性 B-LCL と 21 の抗原陰性 B-LCL が同定された。相関解析の結果、HA-1 の表現型に関連すると考えられる高い χ^2 値（52.8）を示 SNP が染色体 19q13.3 にのみ局在することが判明した（図1最上段、図2）。実際この SNP は *HMHA1* 遺伝子の intron 2 内に存在した。HA-1 をコードする SNP そのものは HapMap のデータには登録されていなかった（dbSNP データベースには登録済み）が、両者はそれぞれ隣り合った intron と exon に存在しており、仮に前向き研究として行ったとしても HA-1 エピトープの同定は容易であると推定された。

①-2 新手法を用いた前方視的な同定：

次に過去に樹立した HLA-B*4002 拘束性の CTL-3B6 と HLA-A*0206 拘束性の CTL-1B2 を用いて、未知のマイナー抗原エピトープの同定を試みた。CTL-3B6 は日本人と中国人由来の 72 種類の HapMap パネルのスクリーニングにより 36 個のマイナー抗原陽性 LCL と 14 個の陰性 LCL、22 個の判定不能 LCL の 3 群に分類した。CTL-1B2 は日本人由来の HapMap B-LCL を約半々用い、計 45 種類を細胞傷害性試験でスクリーニングし、13 の抗原陽性 B-LCL と 32 の陰性 B-LCL に選別した。CTL-4B1 の場合と同様に、HapMap の遺伝子配列データから χ^2 検定により関連する SNP を絞り込んだ。この結果、相関の強い SNP の位置を染色体 19q13.3 の位置に存在する *SLC1A5* 遺伝子まで直接絞り込むことができた（図1中段、図3）。この SNP は *SLC1A5* の 5' 非翻訳領域に存在し、その χ^2 値は最高 50 であった。確認実験として HLA-B*4002 導入 HEK293T 細胞にドナー型および患者型の *SLC1A5* cDNA を遺伝子導入し発現させたものと CTL-3B6 を反応させたところ、ドナー型を導入した場合 CTL-3B6 はインターフェロン(IFN)-γを産生しなかったが、レシピエント型を発現させたものには IFN-γを産生した。ミニ遺伝子を作成して同様な方法で抗原決定部位を絞り込んでいった結果、エピトープのアミノ酸配列は *SLC1A5* の exon 1 部分でコードされる AEATANGGLAL であった（図5A、B）。相関解析で同定された SNP が 5'非翻訳領域に存在し、エピトープは exon 1 であったことから、本法は有効な手段と考えられた。

さらに、HLA-A*0206 拘束性の CTL-1B2 のスクリーニングを実施した。日本人由来の HapMap B-LCL を用い、計 42 種類を細胞傷害性試験でスクリーニングし、13 の抗原陽性 B-LCL と 29 の陰性 B-LCL に選別した。相関解析では、4q13.1 の UGT2B17 遺伝子に存在する SNP に χ^2 値 44 のピークがみられた（図1下段、図4）。過去に UGT2B17 が HLA-A*2902 拘束性のマイナー抗原をコード

し、マイナー抗原が生成される理由としてドナーにおける本遺伝子の欠損が報告されていたため、PCR を用いて抗原陽性および陰性 B-LCL について UGT2B17 遺伝子の有無を検討した。その結果、ドナー型 SNP をもつ個人が相関して UGT2B17 遺伝子を欠損していることがわかり、ドナーだけが本遺伝子をホモで欠失する場合に患者が本遺伝子を有していると抗原性が発現する機序が判明した。詳細な遺伝子内マッピングの結果、*UGT2B17* 遺伝子の exon 6 上に CTL エピトープ CVATMIFMI が同定された（図5 C,D,E）。(亀井美智・南谷泰仁、**Blood**, *in press*)。

② ペプチドワクチン療法臨床試験：

HLA-A24 拘束性 ACC-1^{Y} および本年度より加わった ACC-1^{C}、HLA-B44 拘束性 ACC-2^{D}、HLA-A*0201/A*0206 拘束性 HA-1^{H} ペプチドは GMP グレードで合成された後、当センターの細胞調製施設にて無菌的に溶解・分注後、−30℃で凍結保存された。

本臨床試験に適応のある患者の検索は、研究協力に同意した施設にて HLA-A2、A24、B44 のいずれかをもつ症例が同種移植を受けた際に、マイナー抗原の遺伝子型もタイピングすることで行った。集計の結果、平成 21 年 2 月末の段階で 60 例がタイピング検査を受け、この中で 13 例（22%）が4種類のマイナー抗原のうち、少なくとも1つについて GVL 方向の不適合を有していた。うち最近の2例が、再発治療（PTCL-u 症例）および再発予防（ハイリスク T-ALL）目的でワクチン接種を投与した。使用したマイナー抗原はそれぞれ ACC-1^{C}、HA-1^{H} であった。

PTCL-u 症例は移植後 1 年以上経過後の鼠経部再発で、腫瘤の形成を認めた。初回容量である 30 マイクログラムのワクチンを隔週で投与したが、3回投与したところで腫瘍が進行したため投与を中止した。3回以上投与可能例は主要評価項目の判定は可能としており、本例はワクチン局所の発赤以外、GVHD も含め有害事象は認めなかった。

T-ALL 症例は移植後 104 日目から 30 マイクログラムのワクチンの投与を開始し、予定の5回の接種を終了した。本例も局所の発赤以外、有害事象は認めなかった。

2例のワクチン投与前、各ワクチン投与後の末梢血をテトラマーおよび IFN-γ ELISPOT 法にてワクチンの免疫誘導能に関し検討を行ったが、2例とも前後で有意な変動は認められなかった。

D. **考察**

今回新たに開発した HapMap 計画で収集されたリソースを用いる相関解析法は、マイナー抗原頻度が5%以上 95%以下であれば HapMap に登録されている各民族 90 以下の B-LCL をタイピングすればほぼ抗原遺伝子が同定できることがシミュレーションにより示されている。実際、新規に同定した抗原は 45 程度の B-LCL をタイピングしただけで抗原遺伝子まで絞り込むことが可能であった。しかも CTL-1B2 が認識する UGT2B17 は遺伝子欠損型であり、このようなものが SNP タイピングで見つかるということは、本法がいかにパワフルな方法であるかを示している。

従来の同定法は cDNA ライブラリーのスクリーニングの他、昨年度我々が報告した抗原陽性・陰性の B-LCL から抽出した DNA プールを SNP アレイで解析方法であった。後者は抗原遺伝子同定の迅速化に有効ではあったが、依然 DNA のプール化、SNP アレイでのタイピングが必要であった。今回の

方法は公的なリソースである HapMap の試料・データセットを利用するもので、世界各国のマイナー抗原研究者が容易に追試可能である。また、B-LCL の表現型を別の視点から分類できれば、マイナー抗原のみならず薬剤感受性遺伝子の多型などの同定にも利用出来る可能性がある。

なお本法においてさらに新規の２マイナー抗原の同定にほぼ成功している。これらは共同研究先である米国 Fred Hutchinson 癌研究所で養子免疫療法として患者に実際に投与された CTL が認識するマイナー抗原であり、今後その臨床経過との関係を明らかにし、論文として投稿予定である。さらに、現在用いている ^{51}Cr を使った古典的な細胞傷害性試験は依然として時間制限因子であるため、今後をさらにアッセイ法を改良し、迅速な抗原同定法を完成させる予定である。

マイナー抗原ペプチドワクチンによる免疫療法は、移植後白血病の再発や、予防に有用な選択的 GVL を引き起こすことができると推測され、我々はその安全性、有用性について臨床試験を開始した。マイナー抗原は非自己抗原であるため、アロ免疫を誘導することで強い GVL 効果が期待できる反面、移植が必要であることと、ドナー患者間で利用可能なマイナー抗原の GVL 方向不適合が必要な点などの制約もある。しかし、今後もドナーソースが非血縁ドナーや臍帯血に置き換わることといえ、同種造血細胞移植の有用性に変りはなく、その最終治療である移植後の再発を抑える特異的免疫療法の開発は、再発後の患者の QOL、コスト負担などを考慮すれば重要と考える。

E. 結論

公的リソースである HapMap の試料とゲノムデータを利用した新規のマイナー抗原遺伝子同定法を開発し、実際に抗原遺伝子がほぼピンポイントに同定できることを示した。これにより、マイナー抗原の同定がさらに加速されると考えられた。以上をもとに、今後免疫療法の対象となる抗原の蓄積をはかるとともに、臨床試験を通じてその安全性・有用性を示したい。

G. 研究発表

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H. **知的財産権の出願・登録状況**

特になし

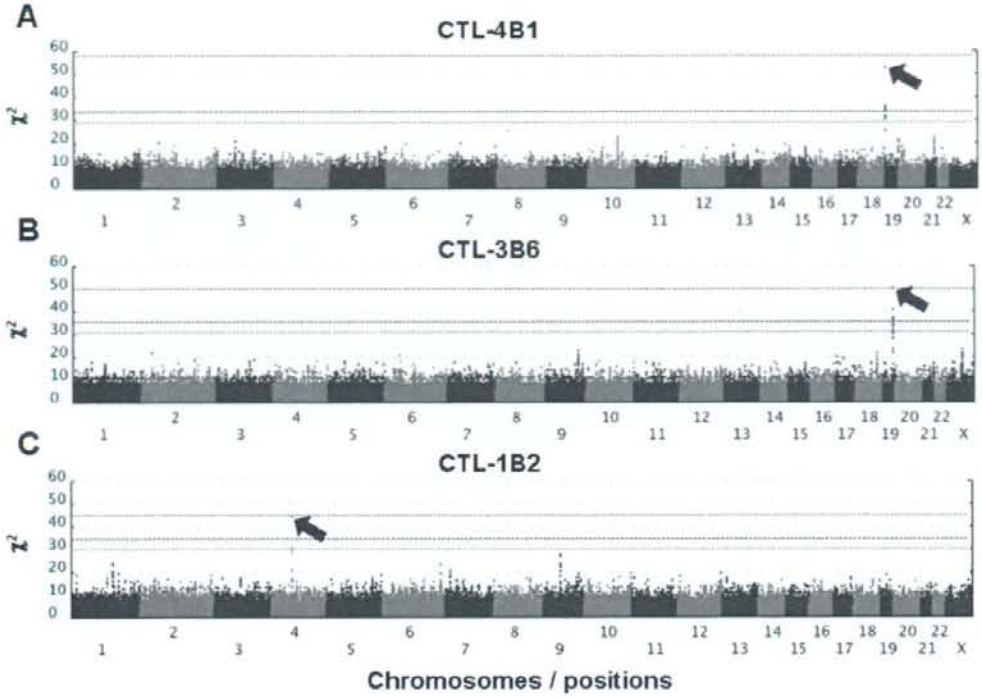


図1　3つの CTL によってタイピングされた HapMap B-LCL のゲノムデータをもとに算出された染色体と χ^2 値のプロット

赤矢印の部分に χ^2 が 40 を越える、きわめて相関の高い単一もしくは数個の SNP 群が各 CTL クローンあたり 1 カ所のみ見いだされた。

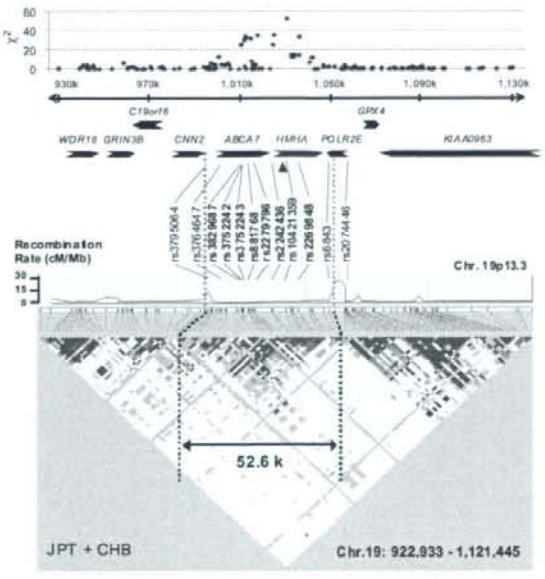


図2　後方視的検討による HapMap 法による HA-1 遺伝子の同定

HLA-A*0206 を導入した HapMap の JPT および CHB の B-LCL を CTL-4B1 の細胞傷害能でタイピングした。図 1 最上段で示された高い χ^2 値を示したゲノム部分の拡大図を示す。青色の rs#で表される SNP が比較的高い相関（χ^2 値）を示し、赤色のものが最高値を示した。これは HA-1 をコードする *HMHA1* 遺伝子の intron 2 に存在していた。

図3　HapMap 法による前方視的な新規マイナー抗原遺伝子の同定（CTL-3B6）

HLA-B*4002 を導入した HapMap の JPT および CHB の B-LCL を CTL-3B6 の細胞傷害能でタイピングした。図1中段で示された高い χ^2 値を示したゲノム部分の拡大図を示す。図2と同様に青色の rs#で表される SNP が比較的高い相関（χ^2 値）を示し、赤色のものが最高値を示した。これは CTL-3B6 が認識する抗原をコードする *SLC1A5* 遺伝子の 5'非翻訳領域に存在しており、抗原は exon 1 にコードされていた。

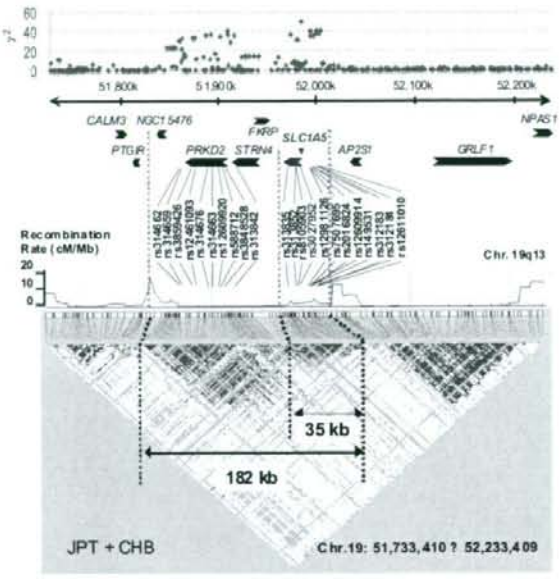
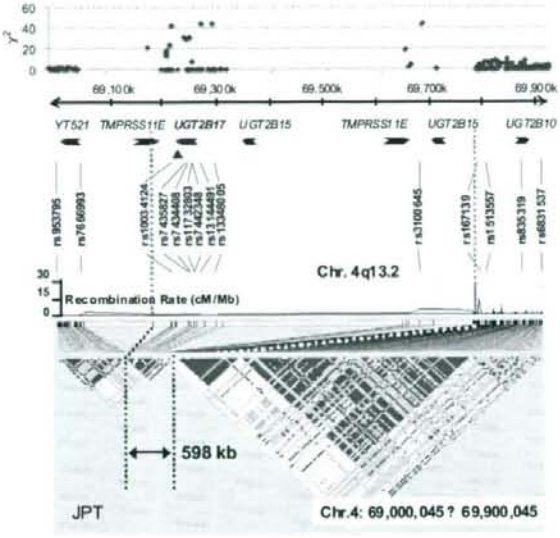


図4　HapMap 法による前方視的な新規マイナー抗原遺伝子の同定（CTL-1B2）

HLA-A*0206 を導入した HapMap の JPT の B-LCL を CTL-1B2 の細胞傷害能でタイピングした。図1下段で示された高い χ^2 値を示したゲノム部分の拡大図を示す。図2と同様に青色の rs#で表される SNP が比較的高い相関（χ^2 値）を示す。これらはすべて UGT2B17 付近にマッピングされており、CTL-1B2 が認識する抗原は *UGT2B17* 遺伝子にコードされると考えられた。ゲノム PCR により、本遺伝子が欠損する個人では抗原性がなく、存在する場合には抗原性があったため、ミニ遺伝子によるマッピングで UGT2B17 内でエピトープを検索した。



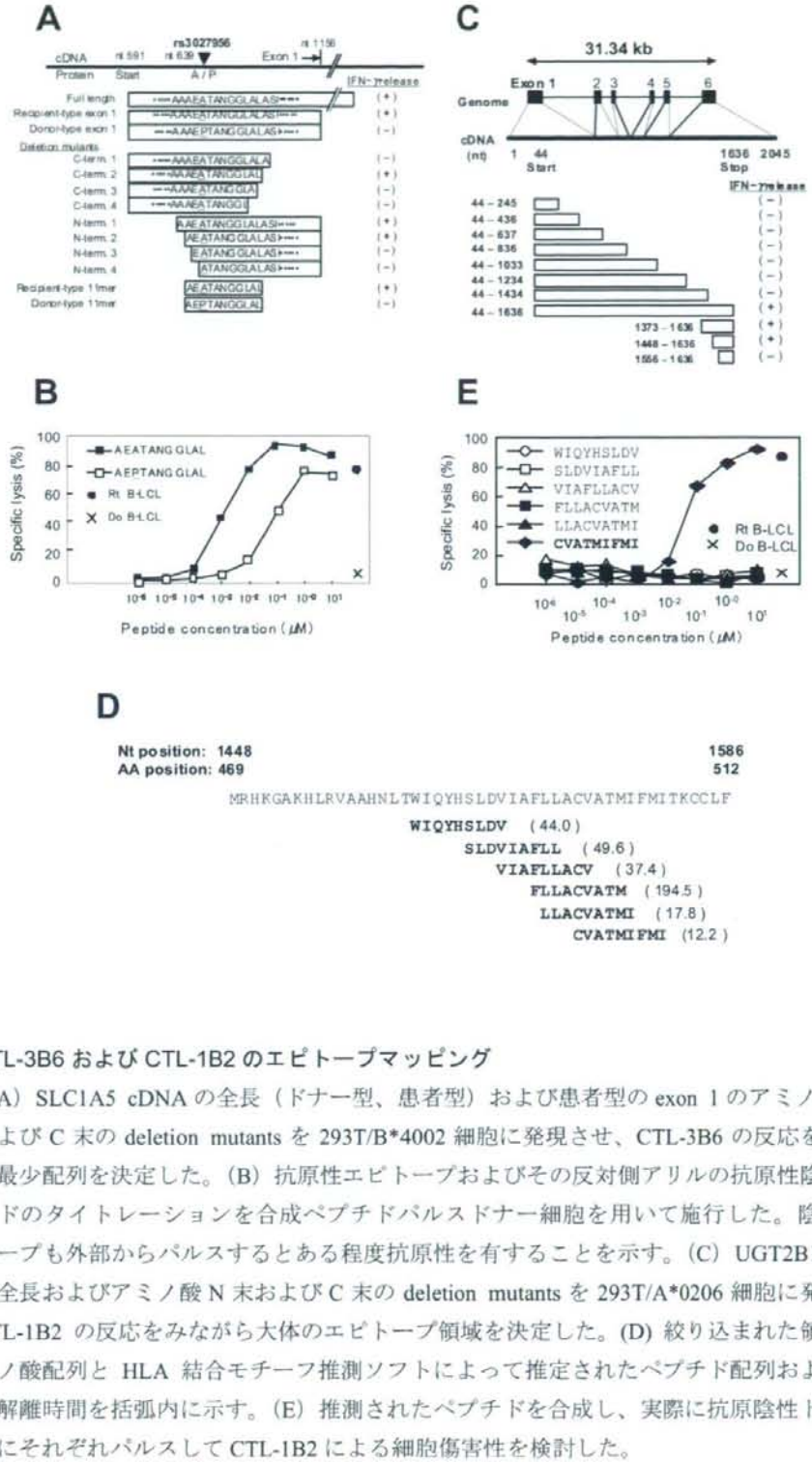


図5　CTL-3B6 および CTL-1B2 のエピトープマッピング

（A）SLC1A5 cDNA の全長（ドナー型、患者型）および患者型の exon 1 のアミノ酸 N 末および C 末の deletion mutants を 293T/B*4002 細胞に発現させ、CTL-3B6 の反応をみながら最少配列を決定した。（B）抗原性エピトープおよびその反対側アリルの抗原性陰性ペプチドのタイトレーションを合成ペプチドパルスドナー細胞を用いて施行した。陰性エピトープも外部からパルスするとある程度抗原性を有することを示す。（C）UGT2B17cDNA の全長およびアミノ酸 N 末および C 末の deletion mutants を 293T/A*0206 細胞に発現させ、CTL-1B2 の反応をみながら大体のエピトープ領域を決定した。(D) 絞り込まれた領域のアミノ酸配列と HLA 結合モチーフ推測ソフトによって推定されたペプチド配列およびその半解離時間を括弧内に示す。（E）推測されたペプチドを合成し、実際に抗原陰性ドナー細胞にそれぞれパルスして CTL-1B2 による細胞傷害性を検討した。

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）
分担研究報告書

同種造血幹細胞移植における移植免疫反応の解明とその細胞治療への応用

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研究要旨：非血縁者間骨髄移植においてドナーと患者間の HLA 抗原の違いが移植成績に大きな影響を与えており、HLA-C, DPB1 座の不適合が白血病の移植後の再発の頻度を低め（移植片対白血病効果：GVL 効果）、HLA-A, B, DRB1, DQB1 座の違いは移植後の再発に影響しないことが判明した。白血病の病型別の解析では、急性骨髄性白血病（AML）と慢性骨髄性白血病（CML）において HLA-DPB1 抗原の違いが GVL 効果に関与していた。これらの知見はＧＶＬの機序解明への道を開くものであり、これらを標的とする特異的細胞免疫療法開発の基礎データとして重要である。

A. 研究目的

HLA-A, B, C, DRB1, DQB1, DPB1 遺伝子型適合度と臨床成績、とくに白血病再発との関連(GVL 効果)を解析することにより、HLA 型適合度に基づいたドナー選択の基礎データを作成する。

B. 研究方法

HLA-A, B, C, DRB1, DQB1, DPB1 の DNA タイピングがされ非血縁者間骨髄移植を実施された白血病 4643 症例を対象にした。T 細胞除去法を用いた症例と海外ドナー症例は除外した。

統計解析は Cox regression models による多変量解析法を使用し、変数として各ＨＬＡ座における不適合な組み合わせ症例の再発リスク（HR）を HLA 座適合な症例と比較した。他のＨＬＡ座の不適合度、患者・ドナーの年齢、性、性適合、疾患、移植病期、TBI の有無、GVHD 予防法などにより adjust した。

C. 研究結果

（１）HLA-C, DPB1 座の不適合が白血病の移植後の再発の頻度を低め（移植片対白血病効果:GVL 効果）、HLA-A, B, DRB1, DQB1 座の違いは移植後の再発に影響しないことが判明した（表１、図１）。

（２）白血病の病型別の解析では、急性骨髄性白血病（AML）と慢性骨髄性白血病（CML）において HLA-DPB1 抗原/座の違いが GVL 効果に関与していた（表２）。

D.考察

4643 ペアーという多数例で多変量解析とその結果の検証を行ったことにより確かな結果を得ることができた。HLA-C と HLA-DPB1 のドナーと患者の HLA 座の違いにより GVL 効果を生じることが明確になった。白血病病型により GVL 効果が異なることは、GVL の標的抗原の表出が病型により異なることを示唆している。

E.結論

HLA 座不適合と再発との関連を解析し、た。今後、GVL 効果の作用機序解明により特異的同種細胞療法を開発するための基礎的所見を得ることができた。

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

なし

表１．HLA-C, DPB1 座の不適合が移植後白血病の再発の頻度を低め（移植片対白血病効果）、HLA-A, B, DRB1, DQB1 座の違いは影響しない

	All diseases		
	N	HR (95% CI)	*P*
HLA-A matched	4018	1.00 (Ref.)	
HLA-A 1 allele mismatched	597	1.00 (0.82-1.22)	0.99
HLA-A 2 alleles mismatched	28	0.79 (0.28-2.28)	0.67
HLA-B matched	4351	1.00 (Ref.)	
HLA-B 1 allele mismatched	288	1.06 (0.79-1.41)	0.7
HLA-B 2 alleles mismatched*	4	-	-
HLA-C matched	3308	1.00 (Ref.)	
HLA-C 1 allele mismatched	1212	0.68 (0.58-0.80)	<0.001
HLA-C 2 alleles mismatched	123	0.43 (0.24-0.75)	0.003
HLA-DRB1 matched	3718	1.00 (Ref.)	
HLA-DRB1 1 allele mismatched	866	0.93 (0.74-1.18)	0.56
HLA-DRB1 2 alleles mismatched	59	1.18 (0.53-2.63)	0.68
HLA-DQB1 matched	3597	1.00 (Ref.)	
HLA-DQB1 1 allele mismatched	958	1.12 (0.90-1.40)	0.30
HLA-DQB1 2 alleles mismatched	88	0.73 (0.35-1.52)	0.40
HLA-DPB1 matched	1584	1.00 (Ref.)	
HLA-DPB1 1 allele mismatched	2190	0.80 (0.70-0.92)	0.002
HLA-DPB1 2 alleles mismatched	869	0.62 (0.51-0.75)	<0.001

HR indicates hazard ratio and CI, confidence interval.

Each group was compared with the matched group in each locus after adjusting for other matching status of HLA, sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high and diseases other than leukemia), GVHD prophylaxis, (CSP vs. FK), ATG (ATG vs. no ATG) and preconditioning (TBI vs. non-TBI).

*Comprehensive analysis could not be performed due to the small number of cases.

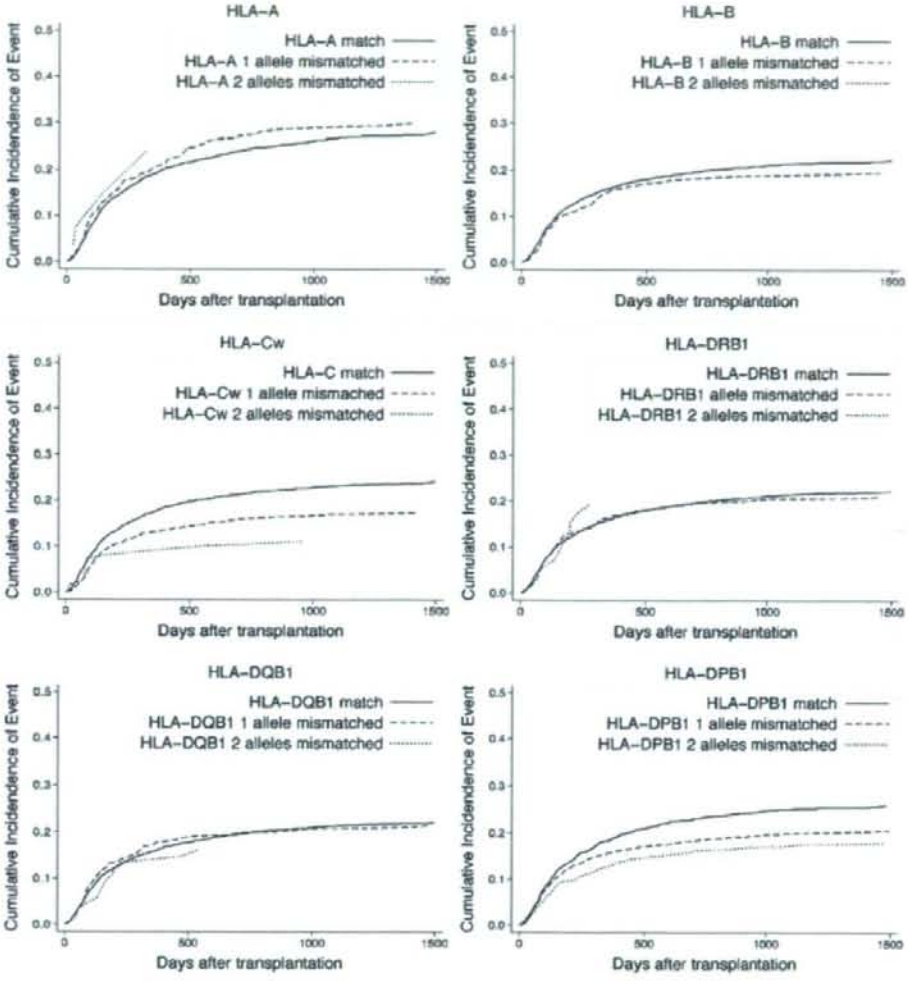
表2．急性骨髄性白血病と慢性骨髄性白血病においてHLA-DPB1抗原/座の違いがGVL効果に関与している

		aGVHD		Relapse		OS	
All diseases	N	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
HLA-DPB1 matched	864	1.00 (Ref.)		1.00 (Ref.)		1.00 (Ref.)	
HLA-DPB1 1 allele mismatched	808	1.34 (1.03-1.74)	0.028	0.83 (0.68-1.01)	0.068	0.96 (0.83-1.12)	0.62
GVL mismatch combination	258	1.18 (0.81-1.73)	0.375	0.47 (0.33-0.67)	<0.001	0.75 (0.59-0.94)	0.012
ALL	N	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
HLA-DPB1 matched	250	1.00 (Ref.)		1.00 (Ref.)		1.00 (Ref.)	
HLA-DPB1 1 allele mismatched	263	1.56 (0.96-2.54)	0.067	0.85 (0.6-1.19)	0.33	1.10 (0.85-1.43)	0.48
GVL mismatch combination	80	1.27 (0.63-2.57)	0.5	0.75 (0.45-1.26)	0.28	0.95 (0.65-1.39)	0.8
AML	N	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
HLA-DPB1 matched	308	1.00 (Ref.)		1.00 (Ref.)		1.00 (Ref.)	
HLA-DPB1 1 allele mismatched	264	1.47 (0.9-2.39)	0.13	0.83 (0.61-1.14)	0.26	0.95 (0.74-1.23)	0.72
GVL mismatch combination	89	1.25 (0.62-2.5)	0.54	0.44 (0.24-0.78)	0.006	0.71 (0.48-1.06)	0.1
CML	N	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
HLA-DPB1 matched	176	1.00 (Ref.)		1.00 (Ref.)		1.00 (Ref.)	
HLA-DPB1 1 allele mismatched	162	1.25 (0.74-2.14)	0.41	0.69 (0.40-1.20)	0.19	0.93 (0.65-1.33)	0.69
GVL mismatch combination	54	1.13 (0.51-2.47)	0.66	0.14 (0.03-0.55)	0.005	0.50 (0.25-0.98)	0.041

OS indicates overall survival; HR, hazard ratio; CI, confidence interval; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia and CML, chronic myeloid leukemia

Each group was compared with the HLA-DPB1 matched group. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high and diseases other than leukemia), GVHD prophylaxis, (CSP vs. FK), ATG (ATG vs. no ATG) and preconditioning (TBI vs. non-TBI).

図1．HLA-C, DPB1 座の不適合が移植後白血病の再発の頻度を低め（移植片対白血病効果）、HLA-A, B, DRB1, DQB1 座の違いは影響しない



III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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IV. 研究成果の刊行物・別刷

Aurora-A kinase: a novel target of cellular immunotherapy for leukemia

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Aurora-A kinase (Aur-A) is a member of the serine/threonine kinase family that regulates the cell division process, and has recently been implicated in tumorigenesis. In this study, we identified an antigenic 9–amino-acid epitope (Aur-$A_{207-215}$: YLILEYAPL) derived from Aur-A capable of generating leukemia-reactive cytotoxic T lymphocytes (CTLs) in the context of HLA-A*0201. The synthetic peptide of this epitope appeared to be capable of binding to HLA-A*2402 as well as HLA-A*0201 molecules. Leukemia cell lines and freshly isolated leukemia cells, particularly chronic myelogenous leukemia (CML) cells, appeared to express Aur-A abundantly. Aur-A–specific CTLs were able to lyse human leukemia cell lines and freshly isolated leukemia cells, but not normal cells, in an HLA-A*0201–restricted manner. Importantly, Aur-A–specific CTLs were able to lyse $CD34^+$ CML progenitor cells but did not show any cytotoxicity against normal $CD34^+$ hematopoietic stem cells. The tetramer assay revealed that the Aur-$A_{207-215}$ epitope–specific CTL precursors are present in peripheral blood of HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia, but not in healthy individuals. Our results indicate that cellular immunotherapy targeting Aur-A is a promising strategy for treatment of leukemia. (Blood. 2009;113: 66-74)

Introduction

Cellular immunotherapy for malignancies targeting various tumor-associated antigens has been developed.[1,2] Recently, some attractive target antigens recognized by leukemia-reactive cytotoxic T lymphocytes (CTLs), such as WT1 and PR1, have been discovered and phase 1/2 clinical studies of cancer immunotherapy targeting these antigens have been conducted; however, the clinical response against hematologic malignancies remains unsatisfactory.[3,4] To establish effective cancer immunotherapy, identification of target antigens that are recognized efficiently by tumor-specific CTLs is necessary. Antigens that can serve as ideal targets recognizable by tumor-specific CTLs need to have several essential characteristics. First, their expression should be limited to, or abundant in, tumor cells rather than normal cells. Second, the antigens should be efficiently processed in tumor cells and expressed on the cell surface in context with common HLA molecules. Third, target antigens should play an important role in tumorigenesis and/or progression of malignancies, because their expression is essential for tumor survival.

Aurora-A kinase (Aur-A) is a member of the serine/threonine kinase family, and the *Aur-A* gene is located at chromosome 20q13, a region frequently amplified in breast cancer.[5] Aur-A is mainly expressed in the G_2/M phase of the cell cycle and regulates mitotic cell division in normal cells.[6-8] Among normal tissues, Aur-A is expressed exclusively in testis, but in various kinds of cancer it is aberrantly overexpressed, and associated with poor prognosis.[9-14] Overexpression of Aur-A determined by amplification of *Aur-A* mRNA has also been widely observed in hematologic malignancies.[15-19] Aur-A overexpression has been linked with centrosome amplification, aneuploidy, and chromosome instability.[20,21] Furthermore, ectopic overexpression of Aur-A efficiently transforms immortalized rodent fibroblasts.[9,20] These data strongly suggest that *Aur-A* is one of the fundamental cancer-associated genes and a potential target for cancer treatment. In addition, previous reports have demonstrated that silencing of the gene encoding Aur-A in cancer cells results in inhibition of their growth and enhancement of the cytotoxic effect of anticancer agents.[22] Therefore the development of small molecules with an Aur-A–inhibitory function may make it possible to reduce or block the oncogenic activity of Aur-A. On the basis of this concept, clinical studies using Aur-A inhibitors for cancer treatment are now under way; however, their clinical efficacy is still unknown.[23-27] The biologic characteristics of Aur-A mentioned above suggest that it is an ideal target for tumor-specific CTLs, and that cancer immunotherapy targeting Aur-A could be feasible. In this study, therefore, we attempted to verify the feasibility of cellular immunotherapy for leukemia targeting Aur-A.

Methods

Synthetic peptides

Candidate peptides derived from Aur-A with high binding affinity for the HLA-A*0201 or HLA-A*2402 molecule were predicted algorithmically by the BIMAS program (http://www-bimas.cit.nih.gov/molbio/hla_bind/). On the basis of these data, peptides with favorable binding affinity for the HLA-A*0201 or HLA-A*2402 molecule were selected and synthesized (Thermo Electron; Greiner Bio-One, Tokyo, Japan). Amino acid sequences

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Table 1. Binding affinities of synthetic peptides

HLA	Position	Length, mer	Sequence	Score	Fluorescence index
A*0201	Aur-$A_{271-279}$	9	KIADFGWSV	3911	0.93
A*0201	Aur-A_{63-71}	9	KLVSSHKPV	243	0.23
A*0201	Aur-$A_{207-215}$	9	YLILEYAPL	147	1.47
A*0201	$WT1_{7-15}$	9	DLNALLPAV	12	0.06
A*0201	$CMVpp65_{495-503}$	9	NLVPMVATV	160	1.71
A*2402	Aur-$A_{207-215}$	9	YLILEYAPL	6	0.99
A*2402	$WT1_{7-15}$	9	DLNALLPAV	0.18	0.02
A*2402	$WT1_{235-243}Y$	9	CYTWNQMNL	200	4.5

The binding affinities of synthetic peptides for HLA molecules were predicted by computer algorithms available on the National Institutes of Health BIMAS website (http://www-bimas.cit.nih.gov/molbio/hla_bind). The binding affinities of synthetic peptides for HLA molecules were evaluated by MHC stabilization assay as detailed in "HLA peptide–binding assay."

of the peptides used in this study are listed in Table 1. All the peptides were synthesized with a purity exceeding 80%.

HLA peptide–binding assay

Binding affinity of peptides for the HLA-A*0201 or HLA-A*2402 molecule was assessed by an HLA-A*0201 or HLA-A*2402 stabilization assay as described previously.[28,29] Briefly, the HLA-A*0201–positive cell line (T2) or the *HLA-A*2402* gene–transfected T2 cell line (T2-A24) was plated in 24-well plates at 10^6 cells per well and incubated overnight with the candidate peptides at a concentration of 10 μM in serum-free RPMI 1640 medium. The T2 and T2-A24 cells were washed twice with phosphate-buffered saline (PBS), and then incubated with fluorescein isothiocyanate (FITC)–conjugated anti–HLA-A2 or HLA-A24 monoclonal antibody (MoAb; One Lambda, Canoga Park, CA) at 4°C for 20 minutes. The cells were washed and suspended in 1 mL PBS and analyzed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). Measurement of mean fluorescence intensity and analysis of data were done with CellQuest Software (Becton Dickinson). The fluorescence index (FI) was calculated as FI = (sample mean − background mean) / background mean.

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki.

B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus. LCLs, T2, T2-A24, and leukemia cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The *HLA-A*0201* gene–transfected C1R cell line (C1R-A*0201; kindly provided by Dr A. John Barrett, National Heart, Lung, and Blood Institute [NHLBI], Bethesda, MD) was cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) from leukemia patients and healthy volunteers, and cord blood mononuclear cells (CBMCs) from healthy donors were isolated and stored in liquid nitrogen until use. All leukemia samples contained more than 95% leukemia cells. $CD34^+$ cells from BMMCs and CBMCs were isolated using $CD34^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec, Auburn, CA). In some experiments, BMMCs and CBMCs were stained with FITC-conjugated anti-CD34 MoAb and phycoerythrin (PE)–conjugated anti-CD38 MoAb, and $CD34^+CD38^{high}$ cells and $CD34^+CD38^{low}$ cells were sorted with an EPICS ALTRA cell sorter (Beckman-Coulter, Fullerton, CA).

Generation of Aur-A peptide–specific CTL lines

Aur-A peptide–specific CTLs were generated as described previously.[30] Briefly, monocytes ($CD14^+$ mononuclear cells) were isolated from PBMCs of HLA-A*0201–positive individuals using $CD14^+$ cell-isolating MACS beads. Monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, 75 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor, 10 ng/mL recombinant human interleukin 4 (IL-4; R&D Systems, Minneapolis, MN), and 100 U/mL recombinant human tumor necrosis factor-α (Dainippon Pharmaceutical, Osaka, Japan) to generate mature dendritic cells (DCs). $CD8^+$ T lymphocytes isolated from PBMCs using $CD8^+$ cell-isolating MACS beads were plated in 96-well round-bottomed plates at 10^5 cells per well and stimulated with 10^4 autologous DCs pulsed with synthetic peptide derived from Aur-A at a concentration of 10 μM. The cells were cultured in RPMI 1640 medium supplemented with 10% human AB serum. After 7 days, the cells were restimulated with 10^4 autologous DCs pulsed with Aur-A peptide, and 10 U/mL IL-2 (Boehringer Mannheim, Mannheim, Germany) was added 4 days later. After culturing for a further 3 days (day 15 of culture), the cells were stimulated with 10^5 autologous PBMCs treated with mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan) pulsed with Aur-A peptide. Thereafter, the cells were restimulated weekly by MMC-treated autologous PBMCs pulsed with Aur-A peptide. The Aur-A peptide–specific cytotoxic activity of growing cells was examined by standard ^{51}Cr-release assay.

Cytotoxicity assays

The standard ^{51}Cr-release assays were performed as described previously.[31] Briefly, 10^4 ^{51}Cr-labeled ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, MA) target cells and various numbers of effector cells in 200 μL RPMI 1640 medium supplemented with 10% FCS were seeded into 96-well round-bottom plates. The target cells were incubated with or without synthetic peptide for 2 hours before adding the effector cells. To assess the HLA class I restriction of cytotoxicity, target cells were incubated with an anti–HLA class I framework MoAb (w6/32; ATCC, Manassas, VA) or an anti–HLA-DR MoAb (L243; ATCC) at an optimal concentration (10 μg/mL) for 1 hour before adding the effector cells. Aur-A peptide specificity of cytotoxicity was examined by cold target inhibition assay as follows. ^{51}Cr-labeled target cells (hot targets) were mixed with various numbers of ^{51}Cr-unlabeled Aur-A peptide–loaded HLA-A*0201–positive LCLs or with ^{51}Cr-unlabeled Aur-A peptide–loaded HLA-A*0201–negative LCLs (cold targets). After incubation with the effector cells for 5 hours, 100 μL supernatant was collected from each well. The percentage of specific lysis was calculated as: (experimental release cpm − spontaneous release cpm) / (maximal release cpm − spontaneous release cpm) × 100 (%).

Quantitative analysis of *Aur-A* mRNA expression

Total RNA was extracted from each sample with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. Quantitative real-time polymerase chain reaction (QRT-PCR) of *Aur-A* mRNA (Hs00269212_m1) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA (4326317E) as an internal control was performed using the TaqMan Gene Expression assay (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The expression level of *Aur-A* mRNA was corrected by reference to that of *GAPDH* mRNA, and the relative amount of *Aur-A* mRNA in each sample was calculated by the comparative ΔCt method.

Western blotting of Aur-A protein

Western blotting was performed as follows. Briefly, 10^6 cells were lysed in lysis buffer (25 mM HEPES, pH 7.5, 1% NP-40, 50 mM NaCl, 5 mM EDTA) with a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and then incubated on ice, sonicated, frozen, and thawed. After centrifugation at 12 000*g* for 15 minutes at 4°C, the supernatant was collected as the lysate. After addition of sodium dodecyl sulfate (SDS) buffer, the total cell lysates were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE), and blotted onto nitrocellulose membranes. The blots were reacted with anti–Aur-A mouse MoAb (Abcam, Cambridge, United Kingdom) followed by incubation with horseradish peroxidase–conjugated anti–mouse IgG antibody (GE Healthcare, Little Chalfont, United Kingdom). The probed proteins were visualized using an enhanced chemiluminescence system (GE Healthcare). The blotted membranes were also examined with anti–β-actin mouse MoAb (Sigma-Aldrich, St Louis, MO) to confirm that samples of equal volume had been loaded.

Detection of Aur-$A_{207\text{-}215}$–specific CTL precursors in leukemia patients and healthy individuals by tetramer assays and enzyme-linked immunospot assays

HLA-A*0201/Aur-$A_{207\text{-}215}$ peptide and HLA-A*2402/Aur-$A_{207\text{-}215}$ peptide tetramers were produced as described previously.[32,33] Briefly, recombinant HLA-A*0201 or HLA-A*2402 and the β_2-microglobulin molecule were generated by the gene-transfer method. Expression of the HLA heavy chain was limited to the extracellular domain, and the C terminus of the domain was modified by addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA-peptide complexes were folded in vitro by adding the HLA protein to β_2-microglobulin in the presence of Aur-$A_{207\text{-}215}$ (YLILEYAPL), HIV-1 p17 $Gag_{77\text{-}85}$ (SLYNTVATL), or HIV-1 $Env_{584\text{-}592}$ (RYLRDQQLL) peptide. After gel purification, the HLA complex was biotinylated using recombinant BirA enzyme (Avidity, Denver, CO), and HLA-peptide tetramers were made by mixing the biotinylated HLA with PE-labeled streptavidin (Molecular Probes, Eugene, OR) at a molar ratio of 4:1.

PBMCs from HLA-A*0201– or HLA-A*2402–positive leukemia patients and healthy individuals were seeded in 24-well plates at 1.5×10^6 per well in the presence of the Aur-$A_{207\text{-}215}$ peptide at a concentration of 10 μM in RPMI 1640 medium supplemented with 10% human AB serum and 10 U/mL IL-2. After culturing for 14 days, Aur-$A_{207\text{-}215}$–specific CTL frequencies in cultured cells were examined by tetramer staining. Cultured PBMCs were stained with FITC-conjugated anti-CD8 MoAb and the tetramer at a concentration of 20 μg/mL at 4°C for 20 minutes. After washing twice, stained cells were analyzed using a FACSCalibur and Cell Quest Software.

Enzyme-linked immunospot (ELISPOT) assays were carried out as described previously.[33] Briefly, 96-well flat-bottom MultiScreen-HA plates with a nitrocellulose base (Millipore, Bedford, MA) were coated with 10 μg/mL anti–interferon-γ (IFN-γ) MoAb (R&D Systems) and incubated overnight at 4°C. After being washed with PBS, the plates were blocked with the assay medium for 1 hour at 37°C. T2-A24 cells (5.0×10^4/well) were pulsed with Aur-$A_{207\text{-}215}$ peptide at a concentration of 10 μM or with PBS alone, and incubated in RPMI 1640 medium supplemented with 10% FCS for 1 hour at 37°C. Then, the responder cells generated were seeded into each well to mix with the target peptide–loaded T2-A24 cells, and the plates were incubated in a 5% CO_2 incubator at 37°C for 20 hours. After incubation, plates were washed vigorously with PBS containing 0.1% Tween 20. A polyclonal rabbit anti–IFN-γ antibody (Endgen, Woburn, MA) was added to each well and the plates was left for 90 minutes at room temperature, followed by exposure to peroxidase-conjugated goat anti–rabbit IgG (Zymed, San Francisco, CA) for an additional 90 minutes. To reveal IFN-γ–specific spots, 100 μL 0.1 M sodium acetate buffer (pH 5.0) containing 3-amino-9-ethylcarbazole (Sigma-Aldrich) and 0.015% H_2O_2 were added to each well. After 40 minutes, the color reaction was interrupted by washing with water, and the plates were dried. Diffuse large spots were counted under a dissecting microscope (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Results

Binding activities of Aur-A peptides for HLA-A*0201 and HLA-A*2402 molecules

The BIMAS-predicted binding scores and results of the binding assay for the HLA-A*0201 and HLA-A*2402 molecules with the 3 candidate Aur-A peptides and the positive and negative control peptides are summarized in Table 1. Among the 3 candidate Aur-A peptides, Aur-$A_{207\text{-}215}$ showed high binding affinity for HLA-A*0201 in comparison with the others. Interestingly, Aur-$A_{207\text{-}215}$ peptide appeared to be capable of binding to HLA-A*2402 as well as HLA-A*0201. These data suggest that Aur-$A_{207\text{-}215}$ peptide can elicit Aur-A–specific CTLs.

Establishment of an Aur-$A_{207\text{-}215}$ peptide–specific CTL line

By repeated stimulation of $CD8^+$ T lymphocytes with Aur-A peptide–loaded autologous DCs, as detailed in "Methods," an Aur-$A_{207\text{-}215}$ peptide–specific CTL line, designated AUR-1, was established from an HLA-A*0201–positive individual. It was possible to generate Aur-$A_{207\text{-}215}$ peptide–specific CTLs from 2 other HLA-A*0201–positive individuals; however, long-term maintenance of these CTL lines was unsuccessful. Therefore, detailed studies of the functional characteristics of Aur-A–specific CTLs were performed using AUR-1. Establishment of Aur-$A_{63\text{-}71}$–specific or Aur-$A_{271\text{-}279}$–specific stable CTL lines was unsuccessful. As shown in Figure 1, AUR-1 showed strong cytotoxicity against Aur-$A_{207\text{-}215}$ peptide–loaded autologous and allogeneic HLA-A*0201–positive LCLs but not Aur-$A_{207\text{-}215}$ peptide–unloaded HLA-A*0201–positive LCLs. AUR-1 did not show any cytotoxicity against Aur-$A_{207\text{-}215}$ peptide–loaded HLA-A*0201–negative allogeneic LCLs. Autologous LCLs loaded with other HLA-A*0201–binding peptides were not lysed by AUR-1 (data not shown). To confirm HLA-A*0201 restriction of Aur-$A_{207\text{-}215}$ peptide–specific cytotoxicity mediated by AUR-1, cytotoxic activity against the *HLA-A*0201* gene–transfectant cell line C1R-A*0201 was examined. AUR-1 was cytotoxic to C1R-A*0201 cells only in the presence of Aur-$A_{207\text{-}215}$ peptide, and this cytotoxicity was significantly attenuated by anti–HLA class I MoAb but not by anti–HLA-DR MoAb (data not shown). These data indicate that Aur-$A_{207\text{-}215}$–specific cytotoxicity of AUR-1 is restricted by HLA-A*0201.

Aur-$A_{207\text{-}215}$–specific and HLA-A*0201–restricted lysis of Aur-A–expressing leukemia cell lines by AUR-1

Aur-A mRNA expression levels in leukemia cell lines and PBMCs of healthy people as a control were assessed by the QRT-PCR method. The amount of *Aur-A* mRNA in each cell line relative to that in the chronic myelogenous leukemia (CML) cell line K562 was calculated. Similarly, Aur-A protein expression levels in leukemia cell lines and normal PBMCs were examined by Western blotting. As shown in Figure 2A, Aur-A appeared to be expressed abundantly in all the leukemia cell lines examined, including acute myelogenous leukemia (AML) and CML cell lines. In contrast, expression of Aur-A in normal PBMCs was undetectable.

AUR-1 exerted cytotoxicity against the HLA-A*0201–positive leukemia cell lines GANMO-1 and CMK11-5, but not against the HLA-A*0201–negative cell lines MEG01, KAZZ, OUN-1, and K562 (Figure 2B). As shown in Figure 2C, cytotoxicity against leukemia cell lines mediated by AUR-1 was inhibited by addition of anti–HLA class I framework MoAb but not anti–HLA-DR MoAb. The cold target inhibition assay showed that the cytotoxicity of AUR-1 against the HLA-A*0201–positive leukemia cell line was significantly abrogated