(19)日本国特許庁(JP)　(12)公表特許公報(A)　(11)特許出願公表番号
特表2004-528848
(P2004-528848A)
(43)公表日　平成16年9月24日(2004.9.24)

(51) Int.Cl.[7]	F I		テーマコード（参考）
C12N 15/09	C12N 15/00	ZNAA	4B024
A61K 35/26	A61K 35/26		4C085
A61K 39/395	A61K 39/395	E	4C087
A61P 35/00	A61K 39/395	T	4H045
C07K 14/705	A61P 35/00		

審査請求 未請求　予備審査請求 有　（全 150 頁）　最終頁に続く

(21)出願番号　特願2002-585615(P2002-585615)
(86)(22)出願日　平成14年4月30日(2002.4.30)
(85)翻訳文提出日　平成15年10月30日(2003.10.30)
(86)国際出願番号　PCT/US2002/013500
(87)国際公開番号　WO2002/088334
(87)国際公開日　平成14年11月7日(2002.11.7)
(31)優先権主張番号　60/286,981
(32)優先日　平成13年4月30日(2001.4.30)
(33)優先権主張国　米国(US)

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(54)【発明の名称】ヒト癌を処置するのに有用なキメライムノレセプター

(57)【要約】
本発明は、「ゼータカイン」と称されるキメラ膜貫通イムノレセプターであって、細胞表面に細胞外ドメインを接着することができる支持体領域に連結した可溶性レセプターリガンドを含む細胞外ドメイン、膜貫通領域および細胞内シグナリングドメインを含んで成るキメラ膜貫通イムノレセプターに関する。ゼータカインは、Ｔリンパ球の表面上で発現された場合、可溶性レセプターリガンドが特異的であるレセプターを発現するそれら特異的細胞へのＴ細胞活性を支配する。ゼータカインキメライムノレセプターは、Ｔ細胞の抗原特異性を再支配するための抗体に基づくイムノレセプターの新規伸長であり、いろいろな癌の処置への、具体的には、ヒト悪性腫瘍によって利用されるオートクリン／パラクリンサイトカインシステムによる応用を伴う。好ましい態様において、ＩｇＧのＦｃ領域に連結したＩＬ−１３Ｒα２特異的ＩＬ−１３突然変異体ＩＬ−１３（Ｅ１３Ｙ）の細胞外標的指向ドメイン、ヒトＣＤ４の膜貫通ドメイン、およびヒトＣＤ３ゼータ鎖を含む神経膠腫特異的イムノレセプターである。

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【特許請求の範囲】
【請求項１】
キメライムノレセプターであって、規定の順に配置された次の連結エレメント、
（ａ）可溶性レセプターリガンドを含む細胞外ドメイン、
（ｂ）該細胞外ドメインを細胞表面に接着することができる支持体領域、
（ｃ）膜貫通領域、および
（ｄ）細胞内シグナリングドメイン
を含むキメライムノレセプター。
【請求項２】
細胞外ドメインが、オートクリン増殖因子、パラクリン増殖因子、ケモカイン、サイトカイン、ホルモンおよび遺伝子操作された人工低分子リガンドから成る群より選択される可溶性リガンドを含む、請求項１に記載のキメライムノレセプター。
【請求項３】
支持体領域が、免疫グロブリンの定常領域、ＣＤ８および人工リンカーから成る群より選択される、請求項１に記載のキメライムノレセプター。
【請求項４】
膜貫通領域が、白血球ＣＤマーカーの膜貫通ドメインである、請求項１に記載のキメライムノレセプター。
【請求項５】
細胞内シグナリングドメインが、Ｔ細胞抗原複合体の細胞内レセプターシグナリングドメイン、ＦｃγＲIII共刺激ドメイン、ＣＤ２８、ＤＡＰ１０およびＣＤ２から成る群より選択される、請求項１に記載のキメライムノレセプター。
【請求項６】
次の連結エレメント、
（ａ）ＩＬ１３（Ｅ１３Ｙ）、
（ｂ）ＩｇＧ４定常領域、
（ｃ）ＣＤ４膜貫通ドメイン、および
（ｄ）細胞内Ｔ細胞抗原レセプターＣＤ３複合体ゼータ鎖
を規定の順に含む、請求項１に記載のキメライムノレセプター。
【請求項７】
イムノレセプターが、該イムノレセプターをコードしているＤＮＡ配列で形質転換されたＴリンパ球細胞系によって発現されている、請求項１に記載のキメライムノレセプター。
【請求項８】
ヒト癌を処置する方法であって、癌に罹患しているヒトに、請求項１～７のいずれかに記載のイムノレセプターを発現する複数の細胞を投与することを含み、ここにおいて、該イムノレセプターの可溶性レセプターリガンドが、癌特異的細胞表面レセプターに特異的である方法。
【請求項９】
癌特異的細胞表面レセプターがサイトカインレセプターである、請求項８に記載の方法。
【請求項１０】
可溶性レセプターリガンドがＩＬ－１３（Ｅ１３Ｙ）である、請求項９に記載の方法。
【発明の詳細な説明】
【技術分野】
【０００１】
技術分野
［０００１］本発明は、癌治療、およびヒト脳腫瘍および他の癌の処置における、キメライムノレセプターを発現する遺伝子修飾されたＴリンパ球の使用に関する。
【背景技術】
【０００２】
発明の背景

［０００２］原発性脳腫瘍は、若年成人の癌関連死亡率の第三の主因であり、小児では第二の主因であり、しかも小児科学的および老年医学的双方の集団において発病率が増加していると考えられる[１～４]。神経膠腫は、原発性脳腫瘍の最も一般的なタイプであり、米国では毎年、２０，０００例が診断され、１４，０００例の神経膠腫関連死がある[５～８]。神経膠腫は、それらの悪性作用に関して不均一（ｈｅｔｅｒｏｇｅｎｏｕｓ）であるが、それらの最も一般的且つ攻撃的な形において、未分化星細胞腫（ＡＡ－III度）および多形性神経膠芽細胞腫（ＧＢＭ－IV度）は、速やかに進行し且つほぼ一様に致死的である[９～１０]。現在利用可能な治療様式は、これら高度腫瘍に最小限の治癒的可能性しかなく、しばしば、中枢神経系中のそれらの場所に課される既に重症の病的状態を悪化させる。したがって、悪性神経膠腫の患者は、しばしば、生涯の最も生産的な時期に襲われ、頻繁に起こる知的諸能力の低下および高い疾患致命率が、これら腫瘍の特有の個人的および社会的影響の原因となっている。

【０００３】

［０００３］悪性神経膠腫の腫瘍学的処置の基礎は、切除術および放射線治療である[１１～１６]。最新の外科的および放射線治療的技法で、平均生存期間は、多形性神経膠芽細胞腫について８２週間および未分化星細胞腫について２７５週間まで増加したが、５年生存率は、多形性神経膠芽細胞腫について３～６％および未分化星細胞腫について１２．１％増加しているにすぎない[６～８]。長期生存の主な予後指標は、より若い年齢（<４０歳）および行動状態（ＫＰＳスコア　>７０）である[１７]。大きい腫瘍の９０％を超える切除術は、通常は、生活機能解剖学が許されるという条件付きで試みられる。術後放射線治療と共に用いられる場合、切除術の程度の生存期間への影響はあまり明らかではない[１８；１９]。切除術および放射線への化学療法の追加は、未分化星細胞腫または多形性神経膠芽細胞腫の患者に、最低限生存しうる利点を与えるにすぎない[２０～２３]。ニトロソ尿素は、単独でまたはプロカルバジンおよびビンクリスチンとの組合せで、公共に用いられる慣用的な薬物であり、全体の半数生存に影響することなく、１年および２年生存率を１５％改善すると考えられる。白金基剤薬物およびトポイソメラーゼ阻害剤を併用する更に攻撃的な治療方式は、研究中である[２６]。幹細胞救出での高用量化学療法の役割は、これまでのところ立証されていない[２７～２９]。

【０００４】

［０００４］約８０％の再発性腫瘍は、元の不完全に切除された腫瘍の残部をＸ線撮影によって増強することに由来する[１０；３０；３１]。再発が単一病巣であり、それらの場所で攻撃的再切除術を行いやすいという条件ならば、このアプローチは、特に、未分化星細胞腫の患者およびＫＰＳ>７０の多形性神経膠芽細胞腫患者について生存期間を延長することができる[１０]。再切除術で処置された再発性多形性神経膠芽細胞腫患者の半数生存は、３６週間である[１０；３０；３１]。近接照射療法かまたは定位放射線手術の形での放射線治療は、再切除術された再発性多形性神経膠芽細胞腫患者の生存期間を１０～１２週間だけ延長することができる。再発性疾患の設定での化学療法の使用は、この患者集団でのその効力が立証されていないので、利用可能な臨床試験の状況にあるはずである。

【０００５】

［０００５］悪性神経膠腫の引き続き悲惨な予後は、遺伝子治療（ＴＫ自殺、腫瘍増殖因子レセプターのアンチセンス阻害、条件致死ウイルスベクター）、免疫療法（抗体、腫瘍細胞ワクチン、イムノトキシン、活性リンパ球の養子移入）、および抗血管新生アプローチが含まれるがこれに制限されるわけではない新規な治療的実体の臨床的研究を促している[３３～４０]。悪性神経膠腫に有効なアジュバント療法の開発に向けられた挑戦の多様性には、正常な脳実質中への腫瘍細胞の広範な浸潤性成長、免疫応答の発生を減衰させるこれら腫瘍から作られる可溶性因子の能力、および中枢神経系（ＣＮＳ）中に治療薬を投与する場合の臨床的に意味のある治療的比率を決定する難しさが含まれる。新規な治療薬の早期臨床評価は、この患者集団において明らかに必要とされている。

【０００６】

［０００６］最近、トランスフェリンのレセプターおよび増殖因子は、デリバリーシステ

ムとしての毒素またはラジオヌクレオチドに抱合されたこれらレセプターのリガンドを利用した実験的神経膠腫治療薬の論題となっている[41]。このアプローチの特異性は、正常な脳と比較される、神経膠腫細胞上の標的レセプターの独特の発現または過剰発現に頼っている。興味深いことに、免疫系によって利用されるインターロイキンの若干のレセプター複合体は、神経膠腫、特に、高親和性ＩＬ－１３レセプターによって発現される[42~48]。免疫系によって利用されるＩＬ－１３レセプター三分子複合体は、ＩＬ－１３Ｒα１、ＩＬ－４Ｒβおよびγｃから成るが、これとは異なり、神経膠腫細胞は、ＩＬ－４Ｒβまたはγｃについての必要条件とは無関係にＩＬ－１３を結合することができる特有のＩＬ－１３Ｒα２鎖を過剰発現する[44;49;50]。そのホモログＩＬ－４と同様に、ＩＬ－１３は、ＣＮＳの外に多面的（pleotrophic）免疫調節活性を有する。どちらのサイトカインも、Ｂリンパ球によるＩｇＥ生産を刺激し且つマクロファージによる前炎症性サイトカイン生産を抑制する。ＣＮＳ内のＩＬ－１３の免疫生物学は、ほとんど知られていない。

【０００７】

［０００７］放射性標識ＩＬ－１３でのオートラジオグラフィーを用いた Debinski et al. による詳細な研究は、研究されたほとんど全ての悪性神経膠腫組織上に豊富なＩＬ－１３結合を示している[42;45;46;48]。更に、その結合は、腫瘍切片内でおよび単細胞分析により、きわめて均一である[46;48]。ヒト神経膠腫細胞系へのＩＬ－１３結合のスキャッチャード分析は、平均して１７，０００～２８，０００の結合部位／細胞を示している[45]。ＩＬ－１３Ｒα２　mRNAに特異的なプローブを用いた分子分析では、全てのＣＮＳ解剖学的位置において正常な脳エレメントによる神経膠腫特異的レセプターの発現を示すことができない[42;43]。更に、放射性標識ＩＬ－１３でのオートラジオグラフィーでは、ＣＮＳにおける検出可能な特異的ＩＬ－１３結合を示すことができなかったことから、共有されるＩＬ－１３Ｒα１／ＩＬ－４β／γｃレセプターは、ＣＮＳにおいて検出可能なレベルで発現されることもないということが示唆された[46]。これら知見は、別個に、非病理学的脳切片についてＩＬ－１３Ｒα１およびＩＬ－４βに特異的な抗体での免疫組織化学的技法を用いて証明された[54]。したがって、ＩＬ－１３Ｒα２は、これまでに記載された神経膠腫についての最も特異的で且つ遍在的に発現される細胞表面標的としてある。

【０００８】

［０００８］ＣＮＳにおけるＩＬ－１３Ｒα２の神経膠腫特異的発現を利用する戦略として、ＩＬ－１３サイトカインの分子コンストラクトであって、種々の細胞毒素（シュードモナス属（Pseudomonas）外毒素およびジフテリア（Diptheria）毒素）がそのカルボキシル末端に融合しているものが記載されてきている[55~58]。ＩＬ－１３レセプターへの結合でのこれら毒素のインターナリゼーションは、これら融合タンパク質の選択的毒性の根拠である。これら毒素は、in vitro の神経膠腫細胞に対してピコモル濃度で強力な細胞傷害性を示す[55]。免疫欠損マウスにおけるヒト頭蓋内神経膠腫異種移植片は、毒性が認められることなく、ＩＬ－１３－毒素融合タンパク質の腫瘍内注射によって排除されうる[55]。これら研究は、ＩＬ－１３に支配されたイムノトキシンを悪性神経膠腫に局所的に利用する臨床研究の開始を支持する。

【０００９】

［０００９］しかしながら、広く発現されるＩＬ－１３Ｒα１／ＩＬ－４β／γｃレセプター複合体へのＩＬ－１３に基づく細胞毒素の結合は、ＣＮＳ外の正常組織に不都合な毒性を媒介する可能性があり、したがって、これら薬剤の全身投与を制限する。ＩＬ－１３は、分子レベルで充分に調べられていて、個々のレセプターサブユニットと連結するのに重要であるこのサイトカインの構造ドメインは、地図に示されている[55;58]。結果として、ＩＬ－１３中の選択されたアミノ酸置換は、このサイトカインとそのレセプターサブユニットとの連結に予想可能な作用をする。ＩＬ－１３のαヘリックスＡ、具体的にはアミノ酸１３におけるアミノ酸置換は、ＩＬ－４βと連結するその能力を破壊し、それによって、ＩＬ－１３Ｒα１／ＩＬ－４β／γｃレセプターへのＩＬ－１３の親和性を選択

的に５倍だけ減少させる[55;57;58]。驚くべきことに、ＩＬ－１３Ｒα２への突然変異体ＩＬ－１３（Ｅ１３Ｙ）の結合は、保存されただけでなく、野生型ＩＬ－１３に相対して５０倍増加した。したがって、最小限に変更されたＩＬ－１３類似体は、神経膠腫細胞へのＩＬ－１３の特異性および親和性をＩＬ－１３Ｒα２への選択的結合によって同時に、ＩＬ－１３Ｒα１／ＩＬ－４β／γｃレセプターを有する正常組織に相対して増加させることができる。

【００１０】

［００１０］悪性神経膠腫は、（１）大部分の患者が、切除術および放射線治療で最低限の疾患重症度状態に達する、および（２）ＣＮＳの境界内のこれら腫瘍の解剖学的位置が、エフェクター細胞の直接的局所投与を可能にさせることから、免疫療法介入にとってきわめて魅力のある臨床的実体である。少なくとも二つの病理学的研究は、悪性神経膠腫における血管周囲リンパ球性浸潤の程度が、改善された予後と相関するということを示している[59~61]。動物モデルシステムが確立されており、そこで、リンホカイン活性化キラー（ＬＡＫ）細胞ではないが神経膠腫特異的Ｔ細胞は、大脳内に植え込まれた神経膠腫の後退を媒介することができる[62~71]。Ｔ細胞は、ＬＡＫ細胞とは異なり、脳実質中に浸潤する能力を有するので、原発性腫瘍から遠く離れていることがありうる浸潤性腫瘍細胞を標的にすることができる。これら知見にもかかわらず、神経膠腫が、主に、免疫抑制性サイトカイン（ＴＧＦ－β２）およびプロスタグランジンの精巧さによって、免疫破壊を積極的に覆すという実質的な証拠があり、これが、神経膠腫反応性Ｔ細胞応答の誘導／増幅を阻害する[72~74]。これら知見は、in vivo 応答を生じることについての腫瘍に媒介される制限を克服する戦略としての養子療法のための ex vivo で増大した抗神経膠腫エフェクター細胞の評価を促している。

【００１１】

［００１１］悪性神経膠腫切除術腔への ex vivo 活性化リンパ球の投与を伴う少なくとも１０種類の試験研究が、これまでに報告されている[75~85]。エフェクター細胞タイプ（ＬＡＫ、ＴＩＬ、アロ反応性（alloreactive）ＣＴＬ）の種類、それらの不均一組成／患者間の組成の変動、および神経膠腫標的に対するこれらエフェクター細胞のしばしば穏当な in vitro 反応性にもかかわらず、これら研究は、事例の長期生存者を含む再発性／難治性疾患の患者において、総計して約５０％の応答率を報告している。これら研究は、神経膠腫への細胞性免疫療法の優れた臨床的作用が、均一できわめて強力なエフェクター細胞で予想されうるという前提を支持する。

【００１２】

［００１２］これら試験研究は、悪性神経膠腫の患者の切除術腔中への、Ｔ細胞増殖因子であるインターロイキン－２（ＩＬ－２）および ex vivo 活性化リンパ球の直接的投与の安全性および許容性についても報告している[75;76;78;82;86~92]。広範囲の個々の細胞用量（＞１０^{9} 個細胞／用量）、更には、高い累積細胞用量（＞２７ｘ１０^{9} 個細胞）でさえも、毒性は穏当であり、典型的には、II度またはそれ未満の一時的頭痛、悪心、嘔吐および発熱から成る。上記のように、これら研究は、ｒｈＩＬ－２の同時投与も用いて、転移したリンパ球の in vivo 生存を支持した。リンパ球と同時にかまたはリンパ球投与後に逐次的に与えられる多重用量は、４８時間毎に供給されるＩＬ－２の１２用量コースについて、１．２ｘ１０^{6} ＩＵ／用量と同程度に高い用量で許容された。

【００１３】

［００１３］上に概説された知見に基づいて、神経膠腫免疫療法で用いられるリンパ球エフェクター細胞の抗腫瘍力価を改善する戦略は開発中である。一つのアプローチは、表皮増殖因子レセプター（ＥＧＦＲ）結合性ドメインを利用する神経膠腫標的と一緒に、抗ＣＤ３ドメインによってＴリンパ球を同時限局化し且つ活性化することができる二重特異性抗体を利用している[93~96]。自己リンパ球と組み合わせたこの二重特異性抗体での予備臨床実験は、Ｔ細胞が、切除術腔中において現場で活性化されるということを示唆している。しかしながら、脳実質内の浸潤性腫瘍細胞に標的指向することは、このアプローチ

の潜在的に有意の限界である。Ｔ細胞は、神経膠腫によって発現される標的抗原に特異的である場合、有意に増加した抗神経膠腫活性を有すると考えられる。Ｔリンパ球が反応性である腫瘍抗原をコードしている、ＳＡＲＴ－１遺伝子を含めた増加する多数のヒト遺伝子がクローン化されているが、これは、ほぼ７５％の高度神経膠腫によって発現されると考えられる[97]。樹状細胞に基づく in vitro 細胞培養技術、更には、テトラマーに基づくＴ細胞選択技術は両方とも、養子両方のための抗原特異的Ｔ細胞の単離を実行可能にしている。ＳＡＲＴ－１のような抗原は、制限性ＨＬＡ対立遺伝子の場合にＴ細胞によって認識されるので、抗原特異的アプローチは、一般的な神経膠腫患者集団に広く応用可能であるようにこれら抗原を提示することができる抗原および制限性ＨＬＡ対立遺伝子の数の実質的な増大を必要とするであろう。

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【００１４】

［００１４］Ｔ細胞抗原レセプター複合体ゼータ鎖の細胞内シグナリングドメインに融合した細胞外一本鎖抗体（ｓｃＦｖＦｃ）から成るように遺伝子操作されたキメラ抗原レセプター（ｓｃＦｖＦｃ：ζ）は、Ｔ細胞中で発現された場合、モノクローナル抗体の特異性に基づく抗原認識を再支配する能力を有する[98]。腫瘍細胞表面エピトープについて標的特異性を有するｓｃＦｖＦｃ：ζレセプターの設計は、それが既存の抗腫瘍免疫に頼っていないので、養子療法のための抗腫瘍免疫エフェクター細胞を生じる概念上魅力のある戦略である。これらレセプターは、それらがＭＨＣ非依存様式で抗原を結合するという点で「普遍的」であり、したがって、一つのレセプターコンストラクトを用いて、抗原陽性腫瘍を有する患者集団を処置することができる。ヒト腫瘍に標的指向するためのいくつかのコンストラクトは、参考文献に記載されており、Ｈｅｒ２／Ｎｅｕ、ＣＥＡ、ＥＲＲＢ－２、ＣＤ４４ｖ６、および腎細胞癌上で選択的に発現されるエピトープに特異性を有するレセプターが含まれる[98〜104]。これらエピトープは全て、キメラＴ細胞レセプターによってｓｃＦｖ結合に接近可能な細胞表面部分であるという共通の特性を共有している。in vitro 研究は、ＣＤ４＋およびＣＤ８＋双方のＴ細胞エフェクター機能が、これらレセプターによって引き起こされうるということを示している。更に、動物モデルは、養子移入されたｓｃＦｖＦｃ：ζを発現するＴ細胞の、樹立された腫瘍を撲滅する能力を示している[105]。腫瘍特異的ｓｃＦｖＦｃ：ζレセプターを発現する初代ヒトＴ細胞の機能は、in vitro で評価されており、これら細胞は、特異的に、腫瘍標的を溶解し、そしてＩＬ－２、ＴＮＦ、ＩＦＮ－γおよびＧＭ－ＣＳＦを含めた一連の前炎症性サイトカインを分泌する[104]。Ｉ期試験養子療法研究は、ＨＩＶ感染個体のＨＩＶｇｐ１２０に特異的な自己ｓｃＦｖＦｃ：ζ発現性Ｔ細胞、および乳腺癌および結腸直腸腺癌を含めたいろいろな腺癌で発現されるＴＡＧ－７２に特異性を有する自己ｓｃＦｖＦｃ：ζ発現性Ｔ細胞を利用して進行中である。

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【００１５】

［００１５］City of Hope の研究者らは、ＣＤ２０＋Ｂ細胞悪性腫瘍に標的指向する目的のためのＣＤ２０特異的ｓｃＦｖＦｃ：ζレセプターコンストラクト、および神経芽細胞腫に標的指向するためのＬ１－ＣＡＭ特異的キメライムノレセプターを遺伝子操作している[106]。前臨床実験室研究は、再配列されていない染色体に組み込まれたベクターＤＮＡの単コピーを含有し且つＣＤ２０特異的ｓｃＦｖＦｃ：ζレセプターを発現するＣＤ８＋ＣＴＬクローンを、健康個体およびリンパ腫患者から単離し且つ増大させる実行可能性を示している[107]。これを達成するために、ＣＭＶ即時／初期プロモーターの転写制御下にあるキメラレセプター配列およびＳＶ４０初期プロモーターの転写制御下にあるＮｅｏＲ遺伝子を含有する精製直鎖状プラスミドＤＮＡを、エレクトロポレーションと称される手順である、短時間電流への細胞およびＤＮＡの暴露によって、活性化されたヒト末梢血単核細胞中に導入した。Fred Hutchinson Cancer Research Center, Seattle, Washington におけるＦＤＡ認可臨床試験で現在用いられている選択法、クローニング法および増大法を利用して、ＣＤ２０特異的細胞溶解活性を有する遺伝子修飾されたＣＤ８＋ＣＴＬクローンを、６人の健康な志願者各々から、１５回の別個のエレクトロポレーション手順で生じている。これらクローンは、ヒトＣＤ２０＋リンパ腫細胞系の一団と一緒に共培

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養された場合、増殖し、特異的に標的細胞を溶解し、そして刺激されてサイトカインを生産する。

【発明の開示】

【発明が解決しようとする課題】

【００１６】

発明の要旨

［００１６］本発明は、「ゼータカイン」と称されるキメラ膜貫通イムノレセプターであって、細胞表面に細胞外ドメインを接着すること（tethering）ができる支持体領域に連結した可溶性レセプターリガンドを含む細胞外ドメイン、膜貫通領域および細胞内シグナリングドメインを含んで成るものに関する。ゼータカインは、Ｔリンパ球の表面上で発現された場合、可溶性レセプターリガンドが特異的であるレセプターを発現するそれら細胞へのＴ細胞活性を支配する。ゼータカインキメライムノレセプターは、Ｔ細胞の抗原特異性を再支配するための抗体に基づくイムノレセプターの新規伸長であり、いろいろな癌の処置に、具体的には、ヒト悪性腫瘍によって利用されるオートクリン／パラクリンサイトカインシステムによって応用される。

【００１７】

［００１７］悪性神経膠腫および腎細胞癌によるＩＬ－１３Ｒα２の腫瘍限定発現を細胞免疫療法の標的として利用した一つの好ましい態様において、ＩＬ－１３Ｒα２に選択的高親和性結合を有するＩＬ－１３サイトカインの突然変異体ＩＬ－１３（Ｅ１３Ｙ）は、ＩＬ－１３Ｒα２発現性腫瘍細胞へのＴ細胞抗原特異性を再支配することができるＩ型膜貫通キメライムノレセプターに変換されている。ゼータカインのこの態様は、ヒトＩｇＧ４Ｆｃに融合した細胞外ＩＬ－１３（Ｅ１３Ｙ）、膜貫通ＣＤ４および細胞内Ｔ細胞抗原レセプターＣＤ３複合体ゼータ鎖から成る。細胞表面上でレセプターを選択的に発現するいろいろなガン細胞タイプのいずれかに特異的であり、その選択的リガンドが知られているまたは遺伝子操作されうる類似のイムノレセプターを生じることができる。

【００１８】

［００１８］このようなイムノレセプターを発現するように安定して形質転換されたヒトＴ細胞のバルク系統（bulk lines）およびクローンは、それらが特異的であるガン細胞タイプの再支配された細胞溶解を示すが、非標的細胞に対しては無視しうる毒性を示す。このような遺伝子操作されたＴ細胞は、神経膠腫のような処置するのが難しい癌を含めた悪性腫瘍のための強力且つ選択的な療法である。

【課題を解決するための手段】

【００１９】

詳細な説明

［００２８］遺伝子操作され再支配されたＴ細胞での腫瘍標的指向に理想的な細胞表面エピトープは、単に腫瘍細胞上において均一様式でおよび同じ診断の患者集団内の全ての腫瘍上において発現されると考えられる。腫瘍細胞膜からの標的分子のモジュレーションおよび／または流出（shedding）は、再支配されたＴ細胞認識のための特定の標的エピトープの有用性に影響を与えることもありうる。これまでのところ、「理想的な」腫瘍特異的エピトープは僅かしか定義されていないが、二次エピトープは、臨界的正常組織での発現不足かまたは腫瘍での相対的過剰発現に基づいて標的とされてきている。悪性神経膠腫の場合、この癌の処置のためのＴ細胞の腔内投与は、ＣＭＳ外の他の組織による発現においてより小さいストリンジェンシーで、正常ＣＮＳではないが腫瘍細胞上で発現されるものへの標的エピトープの増大を可能にする。ＣＭＳ外の組織の交差反応性による毒性に関する問題は、（ａ）腔内投与経路に基づくＣＮＳにおける細胞の隔絶（sequestration）、および（ｂ）典型的に全身投与される細胞用量と比較して低い投与細胞数によって軽減される。

【００２０】

［００２９］ＩＬ－１３Ｒα２レセプターは、悪性神経膠腫について最も遍在的且つ特異的な細胞表面標的として突出している[47]。感受性のオートラジオグラフィーおよび免疫

組織化学的研究では、ＣＮＳにおいてＩＬ－１３レセプターを検出することができない[46;48]。更に、神経膠腫に限定されたＩＬ－１３Ｒα２レセプターを選択的に結合するＩＬ－１３サイトカインの突然変異は、ＣＮＳ外のＩＬ－１３Ｒα１／ＩＬ－４β＋正常組織に対するＩＬ－１３に向けられた治療薬の不都合な反応性に対する更にもう一つの防護手段である[55;57]。神経膠腫ＩＬ－１３Ｒα２に標的指向することの潜在的有用性、順に、ＣＤ４ＴＭおよびＣＤ３ゼータの細胞質テイルに融合するヒトＩｇＧ４Ｆｃによって原形質膜に接着した細胞外ＩＬ－１３突然変異体サイトカイン（Ｅ１３Ｙ）から成る、Ｔ細胞の特異性を再支配するための新規な遺伝子操作されたキメライムノレセプターの設計および試験。このキメライムノレセプターは、「ＩＬ－１３ゼータカイン」という呼称を与えられている。ＩＬ－１３Ｒα２レセプター／ＩＬ－１３（Ｅ１３Ｙ）レセプターーリガンド対は、概して、ゼータカインで用いるためのレセプターーリガンド対の適合性を理解し且つ評価するための優れた指針である。理想的なゼータカインは、ＩＬ－１３（Ｅ１３Ｙ）の性状（独特の癌細胞表面レセプターへの特異性、それが天然に存在する可溶性細胞シグナル分子に由来することによる in vivo 安定性、同じ理由での低免疫原性）を有する細胞外可溶性レセプターリガンドを含む。可溶性レセプターリガンドは、細胞外環境においてより安定であると考えられ、非抗原性であり、そしてより選択的であるという点で、抗体フラグメント（ｓｃＦｖＦｃイムノレセプターなど）または細胞接着分子の先行技術使用にまさる明確な利点としての可溶性レセプターリガンドの使用。

【００２１】

［００３０］本発明によるキメライムノレセプターは、順に、細胞内レセプターシグナリングドメインに連結した膜貫通ドメインによって、細胞表面に可溶性レセプターリガンドを接着している細胞外支持体領域に連結したこのリガンドを含んで成る細胞外ドメインを含む。適当な可溶性レセプターリガンドの例には、オートクリンおよびパラクリン増殖因子、ケモカイン、サイトカイン、ホルモン、および必要な特異性を示す遺伝子操作された人工低分子リガンドが含まれる。天然のリガンド配列は、特定の標的細胞へのそれらの特異性を増加させるように遺伝子操作することもできる。特定のゼータカインで用いるための可溶性レセプターリガンドの選択は、標的細胞の性質、および神経膠腫に対する使用に好ましいリガンドであるＩＬ－１３（Ｅ１３Ｙ）分子に関して上に論及された品質によって左右される。適当な支持体領域の例には、免疫グロブリンの定常（Ｆｃ）領域、ヒトＣＤ８∝、および標的細胞上に結合しているレセプターへの向上した接近のために標的指向部分を細胞表面から離れて移動させるのに役立つ人工リンカーが含まれる。好ましい支持体領域は、ＩｇＧ（ＩｇＧ４など）のＦｃ領域である。適当な膜貫通ドメインの例には、白血球ＣＤマーカーの膜貫通ドメイン、好ましくは、ＣＤ８の膜貫通ドメインが含まれる。細胞内レセプターシグナリングドメインの例は、Ｔ細胞抗原レセプター複合体のもの、好ましくは、ＣＤ３のゼータ鎖、更には、ＦｃγRIII共刺激シグナリングドメイン、ＣＤ２８、ＤＡＰ１０、ＣＤ２単独またはＣＤ３ゼータを含む系列である。

【００２２】

［００３１］ＩＬ－１３ゼータカイン態様において、Ｅ１３Ｙアミノ酸置換を有するヒトＩＬ－１３ｃＤＮＡは、ＰＣＲスプライスオーバーラップ伸長によって合成した。完全長さＩＬ－１３ゼータカインコンストラクトは、ＰＣＲスプライスオーバーラップ伸長によって組み立てたが、これは、ヒトＧＭ－ＣＳＦレセプターα鎖リーダーペプチド、ＩＬ－１３（Ｅ１３Ｙ）－Ｇｌｙ－Ｇｌｙ－Ｇｌｙ、ヒトＩｇＧ４　Ｆｃ、ヒトＣＤ４ＴＭ、およびヒト細胞質ゼータ鎖から成る。このｃＤＮＡコンストラクトを、ヒト Elongation Factor－１αプロモーター（Invivogen, San Diego）の転写制御下において修飾ｐＭＧプラスミドの多重クローニング部位中に連結した。この発現ベクターは、ＣＭＶ即時／初期プロモーターから、トランスフェクタントの in vitro 選択のためのハイグロマイシンホスホトランスフェラーゼ活性とガンシクロビルでの細胞の in vivo 除去（ablation）のためのＨＳＶチミジンキナーゼ活性とを一つの分子内に一緒にしている融合タンパク質ＨｙＴＫをエンコードしているＨｙＴＫｃＤＮＡを同時発現する。グリコシル化の阻害剤であるツニカマイシンと一緒にプレインキュベートされた全細胞 Jurkat 溶解産物の、抗ゼー

タ抗体プローブでのウェスタンブロットは、予想される無傷の５６－ｋＤａキメラレセプタータンパク質が発現されるということを示した。このレセプターは、本来のＩＬ－１３サイトカインの翻訳後修飾と一致して強くグリコシル化される[108]。抗ヒトＩＬ－１３および抗ヒトＦｃ特異的抗体を含むＩＬ－１３ゼータカイン＋ Jurkat 細胞のフローサイトメトリー分析は、Ｉ型膜貫通タンパク質としてのＩＬ－１３ゼータカインの細胞表面発現を確証した。

【００２３】

［００３２］City of Hope で開発され確立されたヒトＴ細胞遺伝子修飾法を用いて[107]、ＩＬ－１３ゼータカインキメライムノレセプターを発現する初代ヒトＴ細胞クローンを、前臨床機能特性決定のために生じている。ＩＬ－１３ゼータカイン＋ＣＤ８＋ＣＴＬクローンは、ex vivo 増大培養において強い増殖活性を示す。増大したクローンは、４時間クロム放出検定において、ヒトＩＬ－１３Ｒα２＋神経膠芽細胞腫細胞系に対する再支配された細胞溶解活性を示す。この細胞溶解活性レベルは、Ｔ細胞上でのゼータカイン発現レベルおよび神経膠腫標的細胞上のＩＬ－１３Ｒα２レセプター密度レベルと相関する。死滅の他に、ＩＬ－１３ゼータカイン＋クローンは、サイトカイン分泌（ＩＦＮ－γ、ＴＮＦ－α、ＧＭ－ＣＳＦ）について活性化される。活性化は、神経膠腫細胞上のＩＬ－１３Ｒα２レセプターとＩＬ－１３ゼータカインの相互作用によって特異的に媒介されたが、不適切なキメライムノレセプターを発現するＣＴＬクローンは、神経膠腫細胞に応答しないからであるし、しかも活性化は、Ｔ細胞トランスフェクタント上のＩＬ－１３および神経膠腫標的細胞上のＩＬ－１３Ｒα２に対する遮断抗体または可溶性ＩＬ－１３の培養物への添加によって、用量依存方式で阻害されうるからである。最後に、ＩＬ－１３ゼータカイン発現性ＣＤ８＋ＣＴＬクローンは、培養物中の神経膠腫細胞によって刺激された場合に増殖する。強力な抗神経膠腫エフェクター活性を有するＩＬ－１３ゼータカイン＋ＣＴＬクローンは、正常ＣＮＳへの限られた側副損傷を含む悪性神経膠腫に対して有意の臨床的活性を有するであろう。

【００２４】

［００３３］本発明によるイムノレセプターは、当該技術分野において知られているいずれの手段によっても生じることができるが、好ましくは、それは、組換えＤＮＡ技術を用いて生じる。キメラレセプターのいくつかの領域をエンコードしている核酸配列は、分子クローニングの標準的な技法（ゲノムライブラリースクリーニング、ＰＣＲ、プライマー補助連結、位置指定突然変異誘発等）によって製造し且つ完全なコーディング配列に組み立てることができる。得られたコーディング領域を、好ましくは、発現ベクター中に挿入し且つ用いて、適当な発現宿主細胞系、好ましくは、Ｔリンパ球細胞系、最も好ましくは、自己Ｔリンパ球細胞系を形質転換する。イムノレセプターの発現のための第三者は、Ｔ細胞系／クローン、形質転換された体液または外因性の（xerogenic）免疫学的エフェクター細胞系を得た。ＮＫ細胞、マクロファージ、好中球、ＬＡＫ細胞、ＬＩＫ細胞、およびこれら細胞に分化する幹細胞を用いることもできる。好ましい態様において、リンパ球は、白血球搬出法によって患者から得られ、そして自己Ｔ細胞を、ゼータカインを発現するように形質導入し且つ臨床的に許容しうる手段によって患者に投与し戻して、抗癌治療を達成する。

【００２５】

［００３４］治療的作用に適当な用量は、好ましくは、一連の投薬サイクルで約１０^{6}～約１０^{9}個細胞／用量であると考えられる。好ましい投薬計画は、用量を段階的に増加させる、０日目に約１０^{7}個細胞で始め、５日目までに約１０^{8}個細胞の標的用量まで漸増増加させる４回の１週間投薬サイクルから成る。適当な投与方式には、静脈内、皮下、腔内（例えば、レザバー接近装置（reservoir-access device）による）、腹腔内、および腫瘍塊中への直接注射が含まれる。

【００２６】

［００３５］次の実施例は、本発明の一つの態様を単に詳しく説明するためにある。

【実施例１】

【００２７】
実施例１：イムノレセプターコーディング配列の構築
［００３６］本発明によるイムノレセプターのコーディング配列を、ＩＬ１３（Ｅ１３Ｙ）コーディング配列の de novo 合成により、次のプライマーを用いて構築した（イムノレセプターのコーディング配列および発現ベクターの構築を示すフローチャートについては、図８を参照されたい）。
【００２８】
【化１】
IL13P1:

EcoRI
TAT**GAATTC**ATGGCGCTTTTGTTGACCACGGTCATTGCTCTCACTTGCC
TTGGCGGCTTTGCCTCCCCAGGCCCTGTGCCTCCCTCTACAGCCCTCAG
GTAC [SEQ ID NO. 1]

IL13P2:
GTTGATGCTCCATACCATGCTGCCATTGCAGAGCGGAGCCTTCTGGTTC
TGGGTGATGTTGACCAGCTCCTCAATGAGGTACCTGAGGGCTGTAGAG
GGAG [SEQ ID NO. 2]

IL13P3:
CTCTGGGTCTTCTCGATGGCACTGCAGCCTGACACGTTGATCAGGGATT
CCAGGGCTGCACAGTACATGCCAGCTGTCAGGTTGATGCTCCATACCAT
GC [SEQ ID NO. 3]

IL13P4:
CCTCGATTTTGGTGTCTCGGACATGCAAGCTGGAAAACTGCCCAGCTGA
GACCTTGTGCGGGCAGAATCCGCTCAGCATCCTCTGGGTCTTCTCGATG
GC [SEQ ID NO. 4]

IL13P5:

BamHI
TC**GGATCC**TCAGTTGAACCGTCCCTCGCGAAAAAGTTTCTTTAAATGTA
AGAGCAGGTCCTTTACAAACTGGGCCACCTCGATTTTGGTGTCTCGG
[SEQ ID NO. 5]

［００３７］最終配列（４１７ｂｐ）を、ＥｃｏＲＩ－ＢａｍＨＩで末端消化し、そしてプラスミドｐＳＫ（stratagene, LaJolla, CA）中に連結してライゲーション（ligation）３１２＃３とした。ライゲーション３１２＃３を、突然変異誘発させて（stratagene キット、製造者の取扱説明書による）、欠失したヌクレオチドを、プライマー５’：ＩＬ１３　３１２＃３ｍｕｔ５－３
【００２９】
【化２】
(CAACCTGACAGCTGGCATGTACTGTGCAGCCCTGGAATC [SEQ ID NO. 6])

および３’：ＩＬ１３　３１２＃３ｍｕｔ３－５
【００３０】
【化３】
(GATTCCAGGGCTGCACAGTACATGCCAGCTGTCAGGTTG [SEQ ID NO. 7])

、および鋳型としてのライゲーション３１２＃３を用いて固定して、ライゲーション３４８＃１（ＩＬ１３ゼータカイン／ｐＳＫ）を形成した。
【００３１】
［００３８］コーディング Human ＧＭ－ＣＳＦＲα鎖 Signal Peptide（ｈｓｐ）コーディング配列を、標準的なＰＣＲスプライスオーバーラップ伸長によってＩＬ１３（Ｅ１３Ｙ）の５’末端に融合した。このｈｓｐ配列（１０１ｂｐ）は、鋳型ライゲーション３０１＃１０（ｈｓｐ／ｐＳＫ）（ヒトＴ細胞ｃＤＮＡからのヒトＧＣＳＦレセプターα鎖リーダー配列）から、プライマー５’：１９ｈｓｐ５’
【００３２】
【化４】
(ATC**TCTAGA**GCCGCCACCATGCTTCTCCTGGTGACAAGCCTTC [SEQ ID NO. 8])

（ＸｂａＩ部位を太字で強調）および３’：ｈｓｐ－ＩＬ１３ＦＲ
【００３３】
【化５】
(GAGGGAGGCACAGGGCCTGGGATCAGGAGGAATG [SEQ ID NO. 9])

を用いて得た。ＩＬ－１３配列（３７１ｂｐ）は、プライマー５’：ｈｓｐ－ＩＬ１３ＦＦ
【００３４】
【化６】
(CATTCCTCCTGATCCCAGGCCCTGTGCCTCCCTC [SEQ ID NO. 10])

および３’：ＩＬ１３－ＩｇＧ４ＦＲ
【００３５】
【化７】
(GGGACCATATTTGGACTCGTTGAACCGTCCCTCGC [SEQ ID NO. 11])

、および鋳型としてのライゲーション３１２＃３を用いて得た。融合は、このようにして得られた１０１ｂｐ　ｈｓｐ配列および３７１ｂｐ　ＩＬ１３配列、およびプライマー５’：１９ｈｓｐ５’および３’：ＩＬ１３－ＩｇＧ４ＦＲを用いて行って、４３８ｂｐ融合ｈｓｐ－ＩＬ１３配列を生じた。
【００３６】
［００３９］ＩｇＧ４Ｆｃ領域ＩｇＧ４ｍ：ゼータをコードしている配列を、このｈｓｐ－ＩＬ１３融合配列の３’末端に同じ方法を用いて融合した。ＩｇＧ４ｍ：ゼータ配列（１１１９ｂｐ）は、プライマー５’：ＩＬ１３－ＩｇＧ４ＦＦ
【００３７】
【化８】
(GCGAGGGACGGTTCAACGAGTCCAAATATGGTCCC [SEQ ID NO. 12])

および３’：ＺｅｔａＮ３’
【００３８】
【化９】
(AT**GCGGCCGC**TCAGCGAGGGGGCAGG [SEQ ID NO. 13])

（ＮｏｔＩ部位を太字で強調）を用い、鋳型として配列Ｒ９．１０（ＩｇＧ４ｍＺｅｔａ／ｐＳＫ）を用いて得た。この１１１９ｂｐ　ＩｇＧ４ｍ：ゼータ配列を、ｈｓｐ－ＩＬ１３融合配列に、鋳型としてのそれぞれの配列、およびプライマー５’：１９ｈｓｐ５’および３’：ＺｅｔａＮ３’を用いて融合して、１５２２ｂｐ　ｈｓｐ－ＩＬ１３－ＩｇＧ４ｍ：ゼータ融合配列を生じた。その両末端を、ＸｂａＩ－ＮｏｔＩで消化し、ライゲーション３５１＃７としてｐＳＫ中に連結して、プラスミドＩＬ１３ゼータカイン／ｐＳＫ（４４６４ｂｐ）を生じた。

【実施例２】

【００３９】

実施例２：発現ベクターの構築

［００４０］ＩＬ１３ゼータカインコーディング配列を含有する発現ベクターは、実施例１で得られたＩＬ１３ゼータカイン／ｐＳＫを、ＸｂａＩ－ＮｏｔＩで消化し、クレノウ（Klenow）を含むブラント末端を生じ、そして得られたフラグメントをプラスミドｐＭＧ＾Ｐａｃ（Invirogen）（ＳｇｒＡＩで開き、クレノウでブラントにし、ＳＡＰでの脱リン酸化によって最初に製造される）中に連結して、プラスミドＩＬ１３ゼータカイン／ｐＭＧを生じることによって生成した。図８を参照されたい。ＩＬ１３ゼータカイン／ｐＭＧのハイグロマイシン耐性領域を、ＮｏｔＩ－ＮｈｅＩでの消化によって除去し、そしてプラスミドＣＥ７Ｒ／ＨｙＴＫ－ｐＭＧ（Jensen, City of Hope）からＮｏｔＩ－ＮｈｅＩでの消化によって得られる選択／自殺融合ＨｙＴＫによって置き換えて、発現ベクターＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧ（６７８５ｂｐ）を生じた。このプラスミドは、Human Elongation Factor－１αプロモーター（ｈＥＦ１ｐ）を６～５４９塩基に、ＩＬ１３ゼータカインコーディング配列を６９２～２１８５塩基に、シミアンウイルス４０後期（Simian Virus 40 Late）ポリアデニル化シグナル（ＬａｔｅＳＶ４０ｐＡＮ）を２２３２～２５００塩基に、最小の大腸菌（Ｅ．coli）複製起点（Ｏｒｉ　ＣｏｌＥ１）を２５０１～３２４７塩基に、合成ポリＡおよび Pause 部位（ＳｐＡＮ）を３２４８～３４３４塩基に、極初期（Immeate-early）ＣＭＶエンハンサー／プロモーター（ｈＣＭＶ－１Ａｐｒｏｍ）を３４５５～４０７７塩基に、ハイグロマイシン耐性チミジンキナーゼコーディング領域融合（ＨｙＴＫ）を４２５９～６３３４塩基に、そしてウシ成長ホルモンポリアデニル化シグナルおよび転写ポーズ（ＢＧｈ　ｐＡｎ）を６３３５～６６３３塩基に含む。このプラスミドは、ＰａｃＩ線状化部位を３２３５～３２４２塩基に有する。ｈＥＦ１ｐおよびＩＬ１３ゼータカインエレメントは、ＩＬ１３ゼータカイン／ｐＭＧから得、残りのエレメントは、ＣＥ７Ｒ／ＨｙＴＫ－ｐＭＧから（ＨｙＴＫエレメントを除いて、最終的には親プラスミドｐＭＧ＾Ｐａｃから）得た。要するに、ＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧは、ｈＥＦ１プロモーターからＩＬ１３ゼータカイン遺伝子、およびＣＭＶ－１ＡプロモーターからＨｙＴＫ融合を発現する修飾ｐＭＧ主鎖である。プラスミドＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧの地図は、図９で明らかである。

【実施例３】

【００４０】

実施例３：イムノレセプターの発現

［００４１］発現されたコンストラクトの統合性の評価を、グリコシル化の阻害剤であるツニカマイシンの存在下または不存在下で培養された Jurkat Ｔ細胞安定トランスフェクタント[107]に由来する全細胞溶解産物の抗ゼータ抗体でプローブされたウェスタンブロットによって最初に表した。図１。Jurkat Ｔ細胞安定トランスフェクタント（Jurkat－ＩＬ１３－ｐＭＧバルク系統（bulk line））は、ＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧ発現ベクターを Jurkat Ｔ細胞にエレクトロポレーション後、陽性トランスフェクタントの選択および増大を行うことによって得た。Jurkat－ＩＬ１３－ｐＭＧバルク系統から2×10^{6}個／ウェルの細胞を、２４ウェルプレート中に５μｇ／ｍｌ、１０μｇ／ｍｌまたは２０μｇ／ｍｌのツニカマイシンと一緒にまたは不含でプレーティングした。そのプレートを３７℃で２２時間インキュベートした。細胞を各々のウェルから採取し、各試料をＰＢＳで洗浄し、そして１錠／１０ｍｌ Complete Protease Inhibitor Cocktail

（Boehringer Mannheim, Indianapolis, IN）を含有する５０μｌのＲＩＰＡ緩衝液（ＰＢＳ，１％ＮＰ４０，０．５％デオキシコール酸ナトリウム，０．１％ＳＤＳ）中に再懸濁させた。試料を氷上で３０分間インキュベート後、２１ゲージ針のシリンジでの吸引によって破壊し、次に、氷上で更に３０分間インキュベート後、４℃において１４，０００ｒｐｍで２０分間遠心分離した。遠心分離された溶解産物上澄み試料を採取し、減圧条件下において等容量の試料用緩衝液中で沸騰させた後、１２％アクリルアミドゲル上でＳＤＳ－ＰＡＧＥ電気泳動を行った。ニトロセルロースへの転移後、膜を４℃でＯ／Ｎ乾燥させた。翌朝、膜を、Ｔ－ＴＢＳ（トリス緩衝化生理食塩水ｐＨ８．０中の０．０２％トゥイーン２０）中に０．０４ｇｍ／ｍｌの脱脂粉乳を含有する Blotto 溶液中で１時間ブロックした。次に、膜を、１μｇ／ｍｌ濃度の一次マウス抗ヒトＣＤ３ζモノクローナル抗体（Pharmingen, San Diego, CA）と一緒に２時間インキュベートし、洗浄後、１：３０００希釈（Blotto 溶液中）のヤギ抗マウスＩｇＧアルカリ性ホスファターゼ抱合二次抗体（Bio-Rad ImmunoStar Kit, Hercules, CA）と一緒に１時間インキュベートした。展開前に、膜をＴ－ＴＢＳ中で更に４回洗浄後、３ｍｌのホスファターゼ基質溶液（Biorad ImmunoStar Kit, Hercules, CA）と一緒に室温で５分間インキュベートした。次に、膜をプラスチックで覆い、ｘ線フィルムに露出させた。野生型ヒトＩＬ－１３の既知のグリコシル化パターンと一致して、発現されたＩＬ－１３（Ｅ１３Ｙ）ゼータカインの電気泳動移動度は、ツニカマイシンの存在下で発現された場合に約５４ｋＤａのアミノ酸主鎖まで減少する、強くグリコシル化されたタンパク質を示している。

【００４１】

［００４２］ＩＬ－１３（Ｅ１３Ｙ）ゼータカインは、フィコエリトリン（ＰＥ）抱合抗ヒトＩＬ１３モノクローナル抗体およびフルオレセインイソチオシアネート（ＦＩＴＣ）抱合マウス抗ヒトＦｃ（ガンマ）フラグメント特異的$Ｆ（ａｂ'）_2$抗体でのトランスフェクタントのフローサイトメトリー分析によって証明されるように、ホモダイマーＩ型膜貫通タンパク質として細胞表面に移行する。図２。Jurkat ＩＬ１３ゼータカイン－ｐＭＧトランスフェクタントを、細胞表面キメラレセプター発現の分析のために、抗ヒトＦｃ（ＦＩＴＣ）抗体（Jackson ImmunoResearch, West Grove, PA）、リコンビナントヒトＩＬ１３Ｒα２／ヒトＩｇＧ１キメラ（Ｒ＆Ｄ Systems, Minneapolis, MN）、次に、ＦＩＴＣ抱合抗ヒトＩｇＧ１モノクローナル抗体（Sigma, St.Louis, MO）および抗ＩＬ１３（ＰＥ）抗体（Becton Dickinson, San Jose, CA）で染色した。健康ドナーの初代細胞も、ＦＩＴＣに抱合された抗ＣＤ４、抗ＣＤ８、抗ＴＣＲおよびイソタイプ対照モノクローナル抗体（Becton Dickinson, San Jose, CA）で染色して、細胞表面表現型を評価した。各々の染色には、１０^6個の細胞を洗浄し、２％ＦＣＳ、０．２ｍｇ／ｍｌ　$ＮａＮ_3$および５μｌの抗体原液を含有する１００μｌのＰＢＳ中に再懸濁させた。４℃で３０分間インキュベーション後、細胞を２回洗浄し、そして二次抗体で染色するかまたは、１％パラホルムアルデヒドを含有するＰＢＳ中に再懸濁させ、ＦＡＣＳCaliber サイトメーターで分析した。

【実施例４】

【００４２】

実施例４：ＩＬ１３Ｒα２レセプターへのＩＬ１３（Ｅ１３Ｙ）ゼータカインの結合

［００４３］ヒトＩｇＧ４Ｆｃによって細胞膜に接着したＩＬ－１３（Ｅ１３Ｙ）（すなわち、ＩＬ１３（Ｅ１３Ｙ）ゼータカイン）は、可溶性ＩＬ１３Ｒα２－Ｆｃ融合タンパク質を用いたフローサイトメトリー分析によって評価されるように、その標的ＩＬ１３Ｒα２レセプターに結合することができる。図３。クローン化されたヒトＰＢＭＣ　ＩＬ１３ゼータカイン－ｐＭＧトランスフェクタントは、ＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧ発現ベクターをＰＢＭＣにエレクトロポレーション後、陽性トランスフェクタント$^{10^7}$の選択および増大を行うことによって得た。ＩＬ１３ゼータカイン$^+$ＣＴＬクローン細胞を、細胞表面キメラレセプター発現の分析のために、フルオレセインイソチオシアネート（ＦＩＴＣ）抱合マウス抗ヒトＦｃ（ガンマ）フラグメント特異的$Ｆ（ａｂ'）_2$（Jackson ImmunoResearch, West Grove, PA）、リコンビナントヒトＩＬ１３Ｒα２／ヒト

ＩｇＧ１キメラ（Ｒ＆Ｄ Systems, Minneapolis, MN）、次に、ＦＩＴＣ抱合抗ヒトＩｇＧ１モノクローナル抗体（Sigma, St.Louis, MO）およびフィコエリトリン（ＰＥ）抱合抗ヒトＩＬ１３モノクローナル抗体（Becton Dickinson, San Jose, CA）で染色した。健康ドナーの初代細胞も、ＦＩＴＣに抱合された抗ＣＤ４、抗ＣＤ８、抗ＴＣＲおよびイソタイプ対照モノクローナル抗体（Becton Dickinson, San Jose, CA）で染色して、細胞表面表現型を評価した。各々の染色には、$１０^{６}$個の細胞を洗浄し、２％ＦＣＳ、０．２ｍｇ／ｍｌ　$ＮａＮ_{３}$および５μｌの抗体を含有する１００μｌのＰＢＳ中に再懸濁させた。４℃で３０分間インキュベーション後、細胞を２回洗浄し、そして二次抗体で染色するかまたは、１％パラホルムアルデヒドを含有するＰＢＳ中に再懸濁させ、ＦＡＣＳCaliberサイトメーターで分析した。

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【００４３】

［００４４］次に、初代ヒトＴ細胞の代理抗原レセプターとしてのＩＬ－１３（Ｅ１３Ｙ）ゼータカインの免疫生物学を評価した。初代ヒトＴ細胞に、プラスミド発現ベクターをエレクトロポレーションした。陽性形質転換細胞を、ハイグロマイシンで選択し、制限希釈でクローン化後、ＯＫＴ３、ＩＬ－２および照射支持細胞での循環刺激サイクルによって増大させた。次に、ウェスタンブロットおよびＦＡＣＳによってＩＬ－１３ゼータカイン発現を示したクローンを、４時間クロム放出検定において、種々の$ＩＬ－１３Ｒα２^{+}$／$ＣＤ２０^{-}$神経膠腫細胞系（Ｕ２５１，ＳＮ－Ｂ１９，Ｕ１３８）および$ＩＬ１３α^{-}$／$ＣＤ２０^{+}$Ｂ細胞リンパ球系（Daudi）に対する機能性評価を行った。これら試験は、ＩＬ１３ゼータカインが、神経膠腫細胞に特異的であった細胞溶解活性を与えたということ（図４ａ）、およびこの特異的細胞溶解活性が、神経膠腫細胞についてクラスとして存在しているということ（図４ｂ）を示した。ＭＪ－ＩＬ１３－ｐＭＧクローンの細胞溶解活性は、$^{５１}Ｃｒ$で標識されたＳＮ－Ｂ１９、Ｕ２５１およびＵ１３８神経膠腫細胞系（ＩＬ１３α２＋／ＣＤ２０－）および Daudi（ＣＤ２０＋／ＩＬ１３α２－）を標的として用いることによって検定した。ＭＪ－ＩＬ１３エフェクターを、刺激後８～１２日目に検定した。エフェクターを採取し、洗浄し、検定用培地中に再懸濁させた。$２．５ｘ１０^{５}$個、$１．２５ｘ１０^{５}$個、$２．５ｘ１０^{４}$個および$５ｘ１０^{３}$個のエフェクターを、９６ウェルＶ底微量滴定プレート中において$５ｘ１０^{３}$個の標的細胞と一緒に三重に、３７℃で４時間培養した。インキュベーション後、細胞不含上澄みの１００μｌアリコートを採取し、その上澄み中の$^{５１}Ｃｒ$をγカウンターで検定した。特異的細胞溶解％は、次のように計算した。

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【００４４】

【数１】

$$\frac{（実験^{51}Cr放出）－（対照^{51}Cr放出）}{（最大^{51}Cr放出）－（対照^{51}Cr放出）} \times 100$$

対照ウェルは、標的細胞単独の存在下でインキュベートされた標的細胞を含有した。最大$^{５１}Ｃｒ$放出は、２％ＳＤＳの存在下において標識された標的細胞によって放出された$^{５１}Ｃｒ$を測定することによって決定した。約４０％のＩＬ－１３（Ｅ１３Ｙ）$ゼータカイン^{+}$　$ＴＣＲα／β^{+}$リンパ球から成る安定してトランスフェクションされたヒトＴ細胞のバルク系統は、４時間クロム放出検定において、$１３Ｒα２^{+}$神経膠腫標的に特異的な再支配された細胞溶解を示したが（２５：１のＥ：Ｔ比で＞５０％の特異的溶解）、ＩＬ－$１３Ｒα２^{-}$標的に対しては無視しうる活性を示した（２５：１のＥ：Ｔ比で＜８％の特異的溶解）。抗ＩＬ－１３抗体への高レベル結合に基づいて選択されたＩＬ－１３（Ｅ１３Ｙ）$ゼータカイン^{+}$　$ＣＤ８^{+}$　$ＴＣＲα／β^{+}$　ＴＣＬクローンも、再支配されたＩＬ１３Ｒα２特異的神経膠腫細胞死滅を示す。図４ｂ。

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【００４５】

［００４５］ＩＬ１３ゼータカイン発現性$ＣＤ８^{+}$　ＴＣＬクローンは、培養物中の神経膠腫細胞によって刺激された場合に活性化し、増殖する。図５～７。ＩＬ１３ゼータカイン

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を発現するＭＪ－ＩＬ１３－ｐＭＧ　Ｃ１．Ｆ２反応細胞を、in vitro のＩＦＮγ、ＧＭ－ＣＳＦおよびＴＮＦα生産のレセプターに媒介される誘発（triggering）について評価した。２ｘ１０^{6}個の反応細胞を、２４ウェル組織培養プレート中において２ｘ１０^{5}個の照射刺激細胞（Daudi, Fibroblasts, Neuroblastoma １０ＨＴＢ、および神経膠芽細胞腫Ｕ２５１）と一緒に２ｍｌの全量で共培養した。遮断用ラット抗ヒトＩＬ１３モノクローナル抗体（Pharmingen, San Diego, CA）、リコンビナントヒトＩＬ１３（Ｒ＆Ｄ Systems, Minneapolis, MN）およびＩＬ１３Ｒα２特異的ヤギＩｇＧ（Ｒ＆Ｄ Systems, Minneapolis, MN）を、Ｕ２５１刺激細胞のアリコート（２ｘ１０^{5}個／ｍｌ）に、１ｎｇ／ｍｌ、１０ｎｇ／ｍｌ、１００ｎｇ／ｍｌおよび１μｇ／ｍｌの濃度で加え、３０分後、反応細胞を加えた。プレートを３７℃で７２時間インキュベートし、その後、培養物上澄みを採取し、小分けし、－７０℃で貯蔵した。ＩＦＮγ、ＧＭ－ＣＳＦおよびＴＮＦαについてのＥＬＩＳＡ検定は、Ｒ＆Ｄ Systems（Minneapolis, MN）キットを製造者の取扱説明書によって用いて行った。試料は、未希釈の、または１：５または１：１０に希釈された二重ウェルで調べた。展開されたＥＬＩＳＡプレートを、マイクロプレートリーダーで評価し、サイトカイン濃度は、標準曲線からの外挿によって決定した。結果は、ピコグラム／ｍｌとして報告され、神経膠腫刺激細胞によるサイトカイン生産について強い活性化を示している。図５、図６。

【００４６】
［００４６］最後に、ＩＬ１３ゼータカイン$^{+}$ ＣＤ８$^{+}$ ＴＣＬのＩＬ－２非依存増殖を、神経膠腫刺激細胞との共培養で認めたが（図７ａ）、ＩＬ１３Ｒα２刺激細胞では認められなかった。増殖は、ｒｈＩＬ－１３抗体の添加によって阻害され（図７ｂ）、認められた増殖が、ＩＬ１３Ｒα２神経膠腫細胞特異的レセプターへのゼータカインの結合に依存したということが示された。

【実施例５】
【００４７】
実施例５：治療的使用に適したＩＬ１３ゼータカイン$^{+}$ Ｔ細胞の製造
［００４７］単核細胞を、ヘパリン化全血から、臨床等級 Ficoll（Pharmacia, Uppsula, Sweden）上での遠心分離によって分離する。ＰＢＭＣを滅菌リン酸緩衝化生理食塩水（Irvine Scientific）中で２回洗浄し、そしてＲＰＭＩ１６４０ＨＥＰＥＳ、１０％熱失活ＦＣＳおよび４ｍＭ　Ｌ－グルタミンから成る培地中に懸濁させる。患者ＰＢＭＣ中に存在するＴ細胞を、Orthoclone ＯＫＴ３の培養物（３０ｎｇ／ｍｌ）への添加によってポリクローナル活性化する。次に、細胞培養物を、研究対象指定インキュベーター中において脱気されたＴ７５組織培養フラスコ中でインキュベートする。培養の開始後２４時間に、ｒｈＩＬ－２を２５Ｕ／ｍｌで加える。

【００４８】
［００４８］培養の開始後３日目に、ＰＢＭＣを採取し、遠心分離し、低張性エレクトロポレーション用緩衝液（Eppendorf）中に２０ｘ１０^{6}個細胞／ｍｌで再懸濁させる。実施例３による２５μｇのプラスミドＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧを、４００μｌの細胞懸濁液と一緒に、滅菌０．２ｃｍエレクトロポレーション用キュベットに加える。各々のキュベットに、２５０Ｖ／４０μｓの単一電気パルスを施し、再度ＲＴで１０分間インキュベートする。生存している細胞をキュベットから採取し、プールし、２５Ｕ／ｍｌのｒｈＩＬ－２を含有する培地中に再懸濁させる。フラスコを患者指定組織培養インキュベーター中に入れる。エレクトロポレーション後３日目に、ハイグロマイシンを０．２ｍｇ／ｍｌの最終濃度で細胞に加える。エレクトロポレーションされたＰＢＭＣを、４８時間毎に培地およびＩＬ－２の補足を行いながら全１４日間培養する。

【００４９】
［００４９］エレクトロポレーションされたＯＫＴ３活性化患者ＰＢＭＣからのハイグロマイシン耐性ＣＤ８＋ＣＴＬのクローニングは、培養１４日目に開始する。簡単にいうと、生存しうる患者ＰＢＭＣを、３０ｎｇ／ｍｌのＯＫＴ３および５０Ｕ／ｍｌのｒｈＩＬ－２を含有する２００ｍｌ容量の培地中の１００ｘ１０^{6}個の低温保存された（cyropres

erved）照射支持ＰＢＭＣおよび２０ｘ１０^{6}個の照射ＴＭ－ＬＣＬの混合物に加える。このマスターミックスを、各ウェルに０．２ｍｌが入る１０個の９６ウェルクローニング用プレート中にプレーティングする。プレートを、蒸発損失を減少させるようにアルミニウム箔で包み、患者指定組織培養インキュベーター中に入れる。培養１９日目、各ウェルに、０．２ｍｇ／ｍｌの最終濃度のためのハイグロマイシンを入れる。ウェルを、３０日目に細胞成長について倒立顕微鏡での可視化によって調べ、陽性ウェルに再刺激のための印を付ける。

【００５０】

［００５０］細胞成長のある各々のクローニングウェルの内容物を、個別に、２５ｍｌの組織培養培地中に５０ｘ１０^{6}個の照射ＰＢＭＣ、１０ｘ１０^{6}個の照射ＬＣＬおよび３０ｎｇ／ｍｌのＯＫＴ３を含有するＴ２５フラスコに移す。再刺激後１日目、３日目、５日目、７日目、９日目、１１日目および１３日目に、フラスコに５０Ｕ／ｍｌのｒｈＩＬ－２および１５ｍｌの新鮮培地を入れる。刺激サイクルの５日目に、フラスコに０．２ｍｇ／ｍｌのハイグロマイシンも補足する。播種後１４日目に、細胞を採取し、計数し、そして５０ｍｌの組織培養培地中に１５０ｘ１０^{6}個の照射ＰＢＭＣ、３０ｘ１０^{6}個の照射ＴＭ－ＬＣＬおよび３０ｎｇ／ｍｌのＯＫＴ３を含有するＴ７５フラスコ中で再刺激する。フラスコに、上に概説されるｒｈＩＬ－２およびハイグロマイシンの培養物への添加物を入れる。

【００５１】

［００５１］療法で可能性のある使用のための増大に選択されるＣＴＬを、ＣＲＢ－３００６に収容されるＦＡＣＳCalibur での免疫蛍光法によって、ＦＩＴＣ抱合モノクローナル抗体ＷＴ／３１（ａβＴＣＲ）、Ｌｅｕ２ａ（ＣＤ８）およびＯＫＴ４（ＣＤ４）を用いて分析して、必要なクローン表現型（αβＴＣＲ＋、ＣＤ４－、ＣＤ８＋およびＩＬ１３＋）を確認する。臨床的使用のためのクローンの選択の判定基準には、イソタイプ対照ＦＩＴＣ／ＰＥ抱合抗体と比較される均一なＴＣＲαβ＋、ＣＤ４－、ＣＤ８＋およびＩＬ１３＋が含まれる。プラスミドベクター染色体組込みの単一部位は、サザンブロット分析によって確認する。遺伝子修飾されたＴ細胞クローンからのＤＮＡは、プラスミドベクターに特異的なＤＮＡプローブでスクリーニングされるであろう。プラスミドベクター中のＨｙＴＫに特異的なプローブＤＮＡは、フルオレセイン抱合ｄＵＴＰでのランダムプライミングにより、製造者の取扱説明書（Amersham, Arlington Hts, IL）によって合成する。Ｔ細胞ゲノムＤＮＡを、標準的な技法によって単離する。Ｔ細胞クローンからの１０マイクログラムのゲノムＤＮＡを、３７℃で一晩消化後、０．８５％アガロースゲル上で電気泳動によって分離する。次に、ＤＮＡを、アルカリ性キャピラリー転移法を用いてナイロンフィルター（BioRad, Hercules, CA）に移す。フィルターとプローブとを、１０μｇ／ｍｌのサケ精子ＤＮＡ（Sigma）を含有する０．５Ｍ　Na_2PO_4、ｐＨ７．２、７％ＳＤＳ中において６５℃で一晩ハイブリッド形成させる。次に、フィルターを、４０ｍＭ　Na_2PO_4、ｐＨ７．２、１％ＳＤＳ中において６５℃で４回洗浄後、化学発光ＡＰ抱合抗フルオレセイン抗体（Amersham, Arlington Hts, IL）を用いて可視化する。クローン選択の判定基準は、ベクターバンドに特有の単一バンドである。

【００５２】

［００５２］ＩＬ－１３ゼータカインの発現は、キメラレセプタータンパク質を抗ゼータ抗体で検出するウェスタンブロット法によって確認する。トランスフェクションされたＴ細胞クローンの全細胞溶解産物は、１錠／１０ｍｌ Complete Protease Inhibitor Cocktail（Boehringer Mannheim）を含有する１ｍｌのＲＩＰＡ緩衝液（ＰＢＳ，１％ＮＰ４０，０．５％デオキシコール酸ナトリウム，０．１％ＳＤＳ）中での２ｘ１０^{7}個の洗浄された細胞の溶解によって生じる。氷上で８０分間インキュベーション後、遠心分離された全細胞溶解産物上澄みのアリコートを採取し、減圧条件下において等容量のローディング緩衝液中で沸騰させた後、成形済み１２％アクリルアミドゲル（BioRad）上でＳＤＳ－ＰＡＧＥ電気泳動を行う。ニトロセルロースへの転移後、膜を、０．０７ｇｍ／ｍｌの脱脂粉乳を含有するブロット（blotto）溶液中で２時間ブロックする。膜を、Ｔ－ＴＢＳ（ト

リス緩衝化生理食塩水ｐＨ８．０中の０．０５％トゥイーン２０）中で洗浄後、１μｇ／ｍｌ濃度の一次マウス抗ヒトＣＤ３ζモノクローナル抗体８Ｄ３（Pharmingen, San Diego, CA）と一緒に２時間インキュベートする。Ｔ－ＴＢＳ中で更に４回洗浄後、膜を、１：５００希釈のヤギ抗マウスＩｇＧアルカリ性ホスファターゼ抱合二次抗体と一緒に１時間インキュベートする。展開前に、膜をＴ－ＴＢＳ中ですすぎ洗浄後、３０ｍｌの「ＡＫＰ」溶液（Promega, Madison, WI）で製造者の取扱説明書によって展開させる。クローン選択の判定基準は、キメラゼータバンドの存在である。

【００５３】

［００５３］ＩＬ－１３ゼータカインキメライムノレセプターを発現するＣＤ８＋細胞傷害性Ｔ細胞クローンは、ＨＬＡに制限されない様式でキメラレセプターと細胞表面標的エピトープとの相互作用後、ヒト神経膠芽細胞腫標的細胞を認識し且つ溶解する。標的ＩＬ－１３Ｒα２エピトープ発現およびクラスＩ　ＭＨＣ非依存認識のための必要条件は、各々のａβＴＣＲ＋、ＣＤ８＋、ＣＤ４－、ＩＬ－１３ゼータカイン＋ＣＴＬクローンを、ＩＬ－１３Ｒα２＋ Daudi 細胞トランスフェクタントおよびＩＬ－１３Ｒα２－ Daudi 細胞に対して検定することによって確認されるであろう。Ｔ細胞エフェクターを、ＯＫＴ３で刺激後１２～１４日目に検定する。エフェクターを採取し、洗浄し、検定用培地中に再懸濁させる。ＩＬ－１３Ｒα２を発現する Daudi 細胞トランスフェクタント。２．５ｘ１０^{5}個、１．２５ｘ１０^{5}個、０．２５ｘ１０^{5}個および０．０５ｘ１０^{5}個のエフェクターを、Ｖ底微量滴定プレート（Costar, Cambridge, MA）中において５ｘ１０^{3}個の標的細胞と一緒に三重に３７℃で４時間プレーティングする。遠心分離およびインキュベーション後、細胞不含上澄みの１００μＬアリコートを採取し、計数する。特異的細胞溶解％は、次のように計算する。

【００５４】

【数２】

$$\frac{(\text{実験}^{51}\text{Cr放出})-(\text{対照}^{51}\text{Cr放出})}{(\text{最大}^{51}\text{Cr放出})-(\text{対照}^{51}\text{Cr放出})}\times 100$$

対照ウェルは、検定用培地中でインキュベートされた標的細胞を含有する。最大^{51}Ｃｒ放出は、２％ＳＤＳで溶解された標的細胞の^{51}Ｃｒ含有量を測定することによって決定する。クローン選択の判定基準は、５：１のＥ：Ｔ比におけるＩＬ－１３Ｒα２＋ Daudi トランスフェクタントの＞２５％の特異的溶解、および同じＥ：Ｔ比における親 Daudi の＜１０％の溶解である。

【００５５】

実施例６：ＩＬ－１３ゼータカイン発現性Ｔ細胞を用いたヒト神経膠腫の処置。

［００５４］ＩＬ－１３ＲゼータカインキメライムノレセプターおよびＨｙＴＫを発現するように実施例５によって遺伝子修飾されたＴ細胞クローンを、次について選択する。

【００５６】

ａ．フローサイトメトリーによって決定される細胞表面表現型ＴＣＲα／β^{+}、ＣＤ４$^{-}$、ＣＤ８$^{+}$、ＩＬ－１３$^{+}$。

ｂ．サザンブロットによって示される、染色体に組み込まれたプラスミドベクターＤＮＡの単コピーの存在。

【００５７】

ｃ．ウェスタンブロットによって検出されるＩＬ－１３ゼータカインタンパク質の発現。

ｄ．４時間クロム放出検定におけるヒトＩＬ－１３Ｒα２$^{+}$標的の特異的溶解。

【００５８】

ｅ．in vitro 成長についての外因性ＩＬ－２への依存。

ｆ．マイコプラズマ、真菌、細菌の無菌状態および＜５ＥＵ／ｍｌの内毒素レベル。

ｇ．ガンシクロビルへのクローンの in vitro 感受性。

【００５９】

［００５５］末梢血単核細胞は、患者から白血球搬出法によって、好ましくは、最初の切除手術から回復後およびステロイドの漸減および／またはそれらの最も最近の全身化学療法から少なくとも３週間の時点に得る。標的白血球搬出単核細胞収量は、$５ｘ１０^{9}$個であり、ハイグロマイシン耐性細胞溶解性Ｔ細胞クローンの標的数は、ex vivo 増大のための全ての品質管理パラメーターを満たす少なくとも５個のクローンが識別されることを期待して２５である。クローンを低温保存し、そしてＸ線連続撮影および臨床検査によって患者を監視する。疾患進行の再発が示される場合、患者に、腫瘍切除腔にＴ細胞を供給するためのレザバー接近装置（Omaya レザバー）の配置および／または再切除を行う。外科手術からの回復およびステロイドの漸減後、適用可能ならば、その患者でＴ細胞療法を開始する。

【００６０】

［００５６］その患者に、少なくとも４回の１週間サイクルの療法を与える。最初のサイクル中、細胞用量上昇は、０日目に１０^{7}個の初期細胞用量から、次に３日目の$５ｘ１０^{7}$個、５日目の１０^{8}個の標的用量まで続く。サイクル２は、サイクル１の開始から１週間程度の早期に開始する。ＭＲＩにおいて残存する疾患で腫瘍退行を示している患者には、サイクル３および４の後、１週間の残余／再実行の反復から成る７週目以降に開始する療法の追加のコースが与えられてよいが、但し、これら処置は、疾患進行またはＣＲが、Ｘ線撮影評価に基づいて得られるような時点まで充分に許容される（＜３度の最大毒性）という条件付きである。

【００６１】

［００５７］細胞用量は、類似した患者集団[７５～８５]に供給される腔内ＬＡＫ細胞（１０^{9}個までの個々の細胞用量および$２．７５ｘ１０^{10}$個程度に高い累積細胞数が安全に投与されている）、ex vivo 増大ＴＩＬ（最小毒性について報告される１０^{9}個細胞／用量まで）およびアロ反応性リンパ球（累積細胞用量について１０^{8}個の細胞用量で始めて$５１．５ｘ１０^{8}$個まで）を用いた研究で与えられる用量未満の少なくとも対数である。このプロトコールで考えられるようなより低い細胞用量の根本的理由は、以前に利用されたエフェクター細胞集団の穏当な反応性プロフィールと比較して増加したＩＬ－１３ゼータカイン＋ＣＴＬクローンの in vitro 反応性／抗腫瘍力価に基づいている。低用量反復投薬は、単回多量細胞数点滴で生じるかもしれない潜在的に危険な炎症性応答を免れるのに好ましい。各々の注入は、単一Ｔ細胞クローンから成るであろう。同じクローンを、患者の処置コースの間中投与するであろう。Ｔ細胞投与の当日に、増大したクローンを、無菌的に、５０ｃｃのＰＢＳ中で２回洗浄することによって処理後、医薬用保存剤不含規定生理食塩水中に、患者供給用の細胞用量を２ｍｌで生じる容量で再懸濁させる。Ｔ細胞を５～１０分間にわたって点滴する。２ｍｌのＰＦＮＳフラッシュは、Ｔ細胞後５分間にわたって投与するであろう。療法への応答は、脳ＭＲＩ＋／－ガンドリニウムにより、分光法で評価する。

【００６２】

［００５８］神経膠腫切除腔中へのＴ細胞投与について予想される副作用は、典型的には、自己限定性悪心および嘔吐、発熱、および既存の神経学的欠損の一時的悪化から成る。これら毒性は、分泌されたサイトカインの作用との組合せでＴ細胞によって媒介される腫瘍床中の局所炎症／水腫双方によることがありうる。これら副作用は、典型的には、一時的であり且つII度未満の重症度である。たとえ患者が一層重症の毒性を経験するとしても、デカドロンは単独でまたはガンシクロビルとの組合せで、炎症過程を減衰させ且つ注入された細胞を除去するであろうと考えられる。細菌または真菌で汚染されている細胞製品の不注意による注入は、重篤なまたは生命を危うくする毒性を媒介する可能性がある。細胞製品の充分な注入前培養は、汚染された組織培養フラスコを識別し且つこの可能性を最小限にするために行う。再注入当日に、培養液のグラム染色、更には、内毒素レベルを実施する。

【００６３】

［００５９］ＩＬ－１３Ｒα２の発現についての充分な分子分析は、この分子が、ＣＮＳ

の場合[44;46;48;54]に腫瘍特異的であるということを示している。更に、実証可能なＩＬ－１３Ｒα２発現を含む唯一のヒト組織は、精巣であると考えられる[42]。この発現の腫瘍精巣制限パターンは、種々のヒト癌、特に、黒色腫および腎細胞癌[109～111]によって発現される腫瘍抗原（すなわち、ＭＡＧＥ、ＢＡＧＥ、ＧＡＧＥ）の増加する数を連想させる。ワクチンおよび養子Ｔ細胞療法での臨床経験は、このクラスの抗原が、精巣の同時自己免疫攻撃を伴うことなく、全身腫瘍免疫療法に利用されうるということを示している[112～114]。おそらくは、これは、無傷の血液精巣関門の作用および精巣内の免疫学的に特権的な環境を選択的に反映している。突然変異体ＩＬ－１３標的指向部分の申し分のない特異性にもかかわらず、毒性は、理論的には、細胞が全身循環中に充分な数で出て行き且つＩＬ－１３Ｒα１／ＩＬ－４βレセプターを発現する組織を認識する場合に可能性がある。この間接的な危険、更には、点滴されたＴ細胞が、ある患者では腫瘍床中の過剰すぎる炎症応答を媒介することがありうるという可能性を考慮して、ガンシクロビルでの in vivo 除去に感受性のＴ細胞を与えるＨｙＴＫ遺伝子をクローンに装備する[115～118]。ガンシクロビル自殺は、患者内Ｔ細胞用量上昇戦略との組合せで、可能性のある危険を最小限にして、関与するものを探求するのに役立つ。

【００６４】

［００６０］療法に関連した副作用（頭痛、発熱、悪寒、悪心等）には、その状態に適当な確立された処置を用いて対処する。処置している医師の意見で、患者をかなり医学的に危険にさせるいずれか新しい３度またはいずれか４度の処置関連毒性が認められる場合、その患者にガンシクロビルを与える。非経口投与されるガンシクロビルは、分割される１０ｍｇ／ｋｇ／日で１２時間毎に投与する。１４日間コースが処方されるであろうが、その時間間隔で症状の消散が得られないならば、延長してよい。ガンシクロビルでの処置は、ＩＬ－１３ゼータカイン$^{+}$ＨｙＴＫ$^{+}$ＣＤ８$^{+}$ＣＴＬクローンの除去をもたらす。患者は、最初の７２時間のガンシクロビル療法のために監視する目的で入院すべきである。症状が４８時間以内にガンシクロビルに応答しない場合、コルチコステロイドおよびシクロスポリンが含まれるがこれに制限されるわけではない追加の免疫抑制薬を、処置している医師の判断で加えてよい。毒性が重症である場合、処置している医師の判断で、ガンシクロビルと一緒にデカドロンおよび／または他の免疫抑制薬を、より早期に用いる。

【００６５】

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【００６６】

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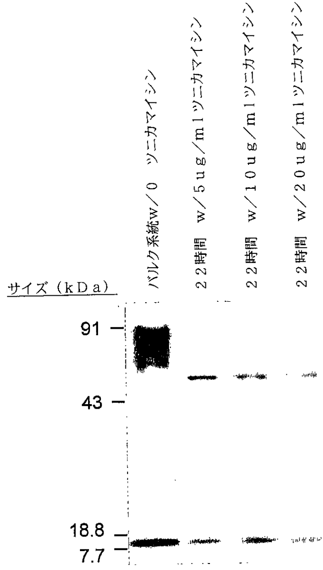
【図面の簡単な説明】

【００７７】

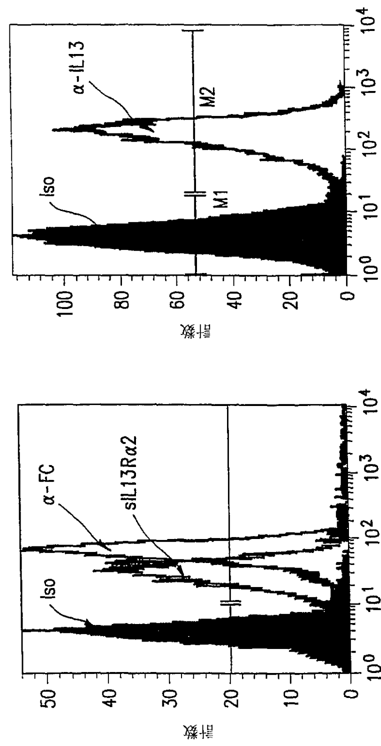
【図１】［００１９］図１：ＩＬ１３ゼータカインキメライムノレセプターは、Jurkat Ｔ細胞中で無傷のグリコシル化タンパク質として発現されるということを示しているウェ

スタンブロットの結果。
【図２】［００２０］図２：発現されたＩＬ１３ゼータカインキメライムノレセプターは、Ｉ型膜貫通タンパク質として細胞表面に移行するということを示しているフローサイトメトリー分析の結果。
【図３】［００２１］図３：代表的な一次ヒトＩＬ１３ゼータカイン$^{+}$ＣＴＬクローンの細胞表面表現型を示しているフローサイトメトリー分析の結果。
【図４Ａ】［００２２］図４：（ａ）ＩＬ１３ゼータカイン$^{+}$ＣＴＬクローンが、神経膠腫特異的な再支配された細胞溶解活性を獲得したことを示しているクロム放出検定の結果。
【図４Ｂ】図４：（ｂ）一次ヒトＩＬ１３ゼータカイン$^{+}$ＣＤ８$^{+}$ＣＴＬクローンによる抗神経膠腫細胞溶解活性のプロフィールは、神経膠腫細胞で一般的に認められたことを示しているクロム放出検定の結果。
【図５】［００２３］図５：ＩＬ１３ゼータカイン$^{+}$ＣＴＬクローンは、神経膠腫刺激性細胞によってサイトカイン生産について活性化されるということを示している、サイトカイン生産の in vitro 刺激の結果。
【図６】［００２４］図６：抗ＩＬ１３Ｒ　ＭａｂおよびｒｈＩＬ１３によるサイトカイン生産についてのＩＬ１３ゼータカイン$^{+}$ＣＴＬ活性化の特異的阻害を示している、サイトカイン生産の in vitro 刺激の結果。
【図７Ａ】［００２５］図７：（ａ）ＩＬ１３ゼータカイン$^{+}$ＣＤ８$^{+}$ＣＴＬ細胞は、神経膠腫刺激物質との共培養で増殖するということを示している増殖研究の結果。
【図７Ｂ】図７：（ｂ）神経膠腫に刺激されたＩＬ１３ゼータカイン$^{+}$ＣＤ８$^{+}$ＣＴＬ細胞増殖のｒｈＩＬ－１３による阻害を示している増殖研究の結果。
【図８Ａ】［００２６］図８：ＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧの構築のフローチャート。
【図８Ｂ】図８：ＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧの構築のフローチャート。
【図８Ｃ】図８：ＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧの構築のフローチャート。
【図９】［００２７］図９：ＩＬ１３ゼータカイン／ＨｙＴＫ－ｐＭＧのプラスミド地図。

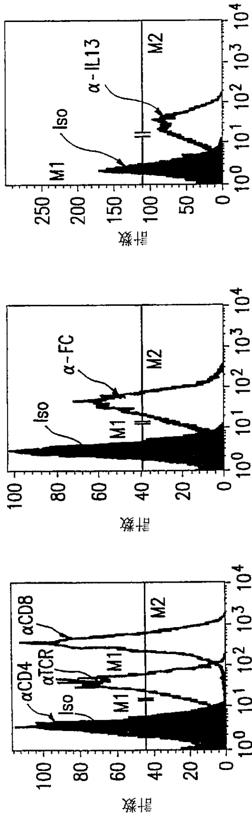
【図１】



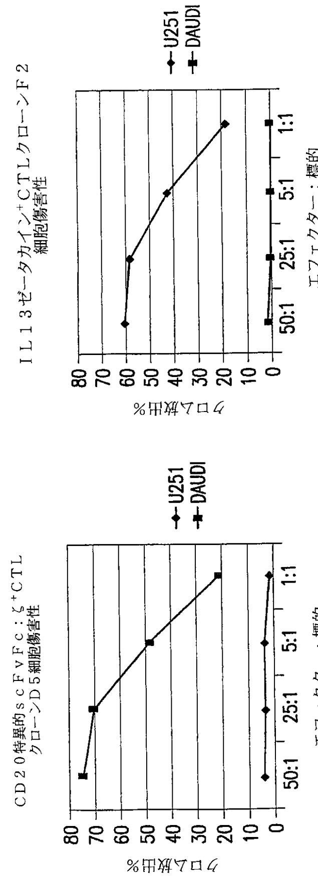
【図２】



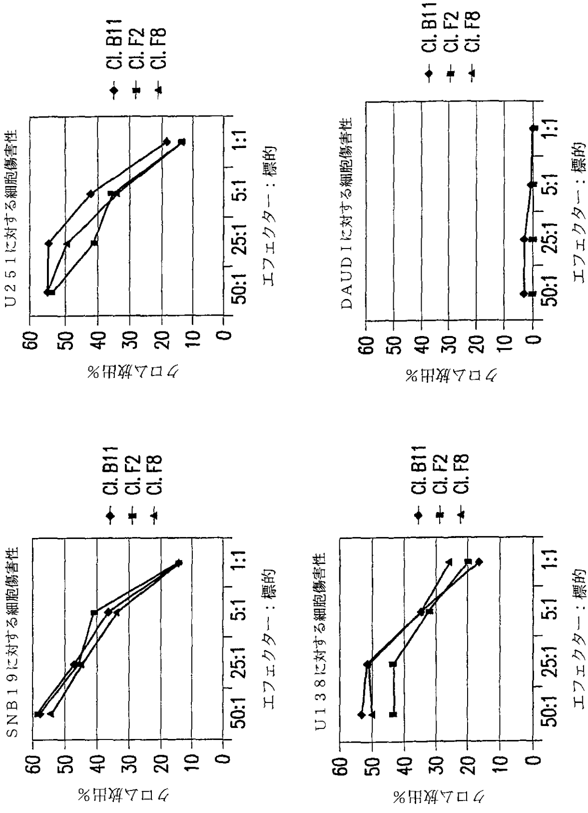
【図３】



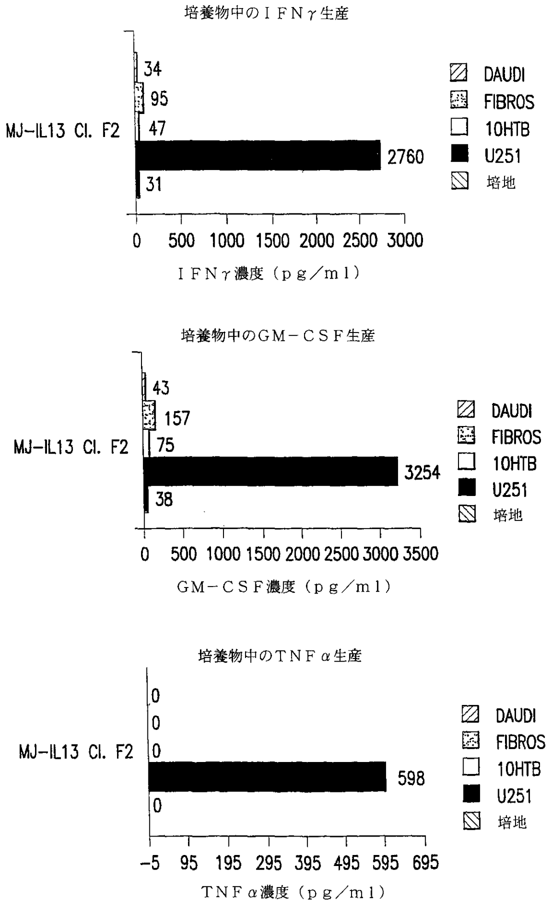
【図４Ａ】



【図４Ｂ】



【図５】



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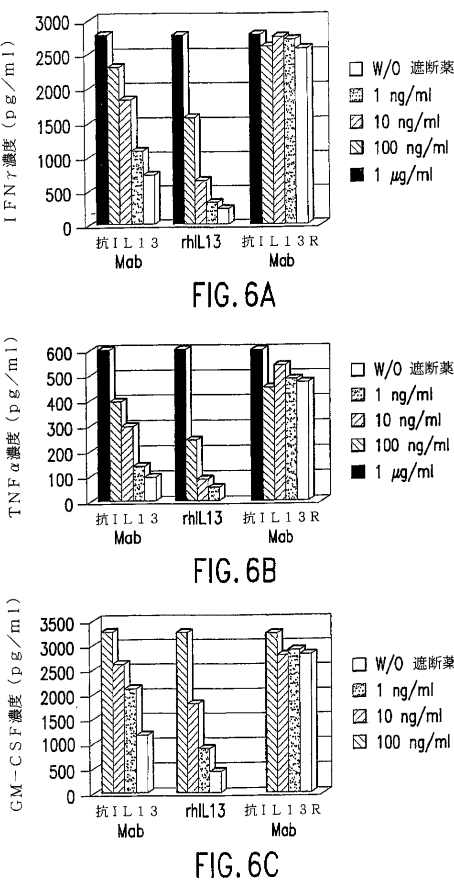
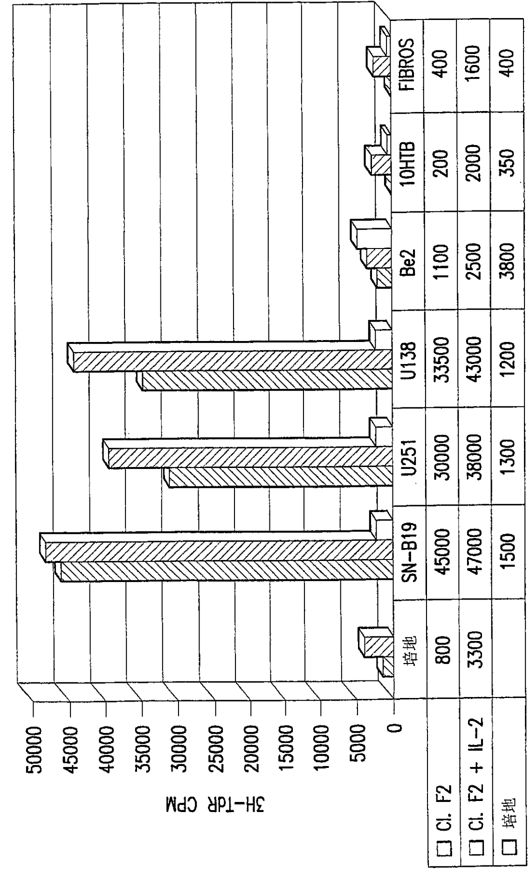


FIG.6A

FIG.6B

FIG.6C

【図７Ａ】



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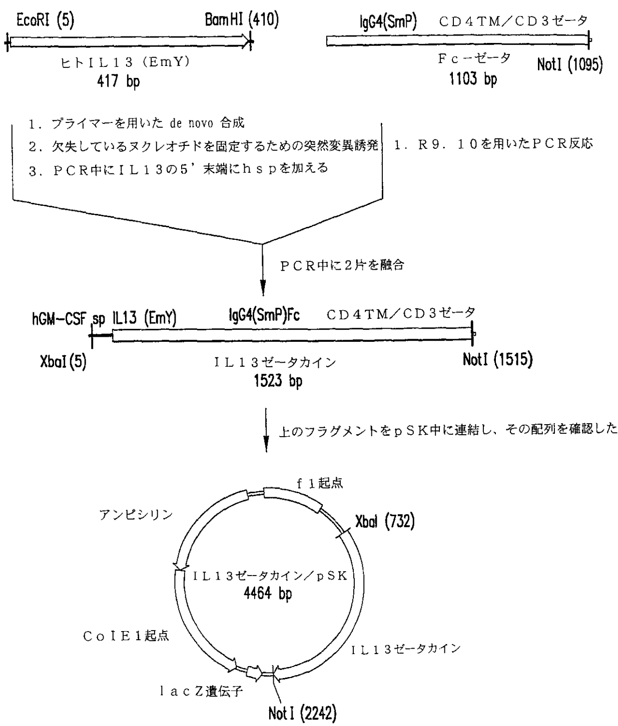
Cl. F2 + IL-2	3300	47000	38000	43000	2500	2000	1600
培地		1500	1300	1200	3800	350	400

【図８Ａ】



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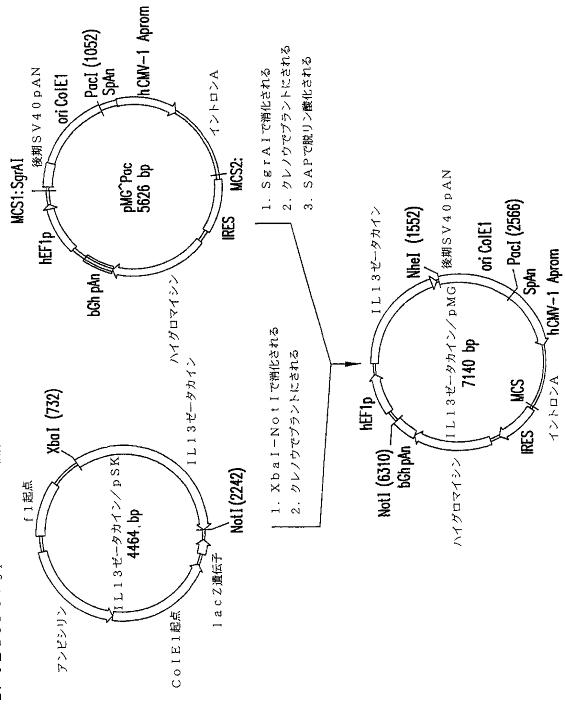

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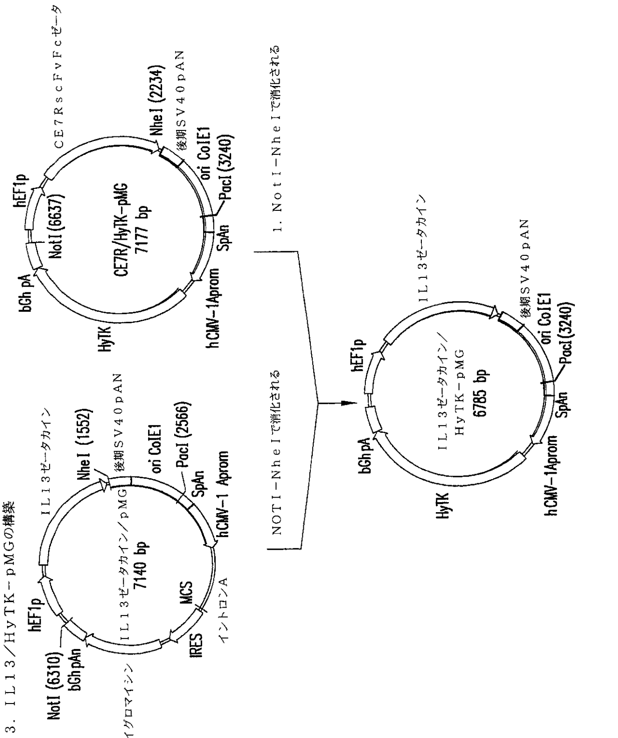

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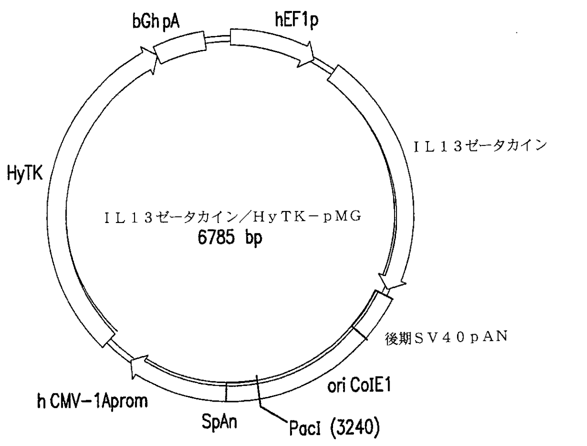

【国際公開パンフレット】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 November 2002 (07.11.2002)

PCT

(10) International Publication Number
WO 02/088334 A1

(51) International Patent Classification[7]: C12N 5/06, 15/00, 5/00, 5/08, C07H 21/04, A01N 65/00

(21) International Application Number: PCT/US02/13500

(22) International Filing Date: 30 April 2002 (30.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/286,981 30 April 2001 (30.04.2001) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/088334 A1

(54) Title: CHIMERIC IMMUNORECEPTOR USEFUL IN TREATING HUMAN CANCERS

(57) Abstract: The present invention relates to chimeric transmembrane immunoreceptors, named "zetakines", comprised of an extracellular domain comprising a soluble receptor ligand linked to a support region capable of tethering the extracellular domain to a cell surface, a transmembrane region and an intracellular signalling domain. Zetakines, when expressed on the surface of T lymphocytes, direct T cell activity to those specific cells expressing a receptor for which the soluble receptor ligand is specific. Zetakine chimeric immunoreceptors represent a novel extension of antibody-based immunoreceptors for redirecting the antigen specificity of T cells, with application to treatment of a variety of cancers, particularly via the autocrin/paracrine cytokine systems utilized by human maligancy. In a preferred embodiment is a glioma-specific immunoreceptor comprising the extracellular targetting domain of the IL-13Rα2-specific IL-13 mutant IL-13(E13Y) linked to the Fc region of IgG, the transmembrane domain of human CD4, and the human CD3 zeta chain.

CHIMERIC IMMUNORECEPTOR USEFUL IN TREATING HUMAN CANCERS

TECHNICAL FIELD

[0001] This invention relates to cancer therapy, and the use of genetically-modified T-lymphocytes expressing chimeric immunoreceptors in the treatment of human brain tumors and other cancers.

BACKGROUND OF THE INVENTION

[0002] Primary brain tumors are the third leading contributor to cancer-related mortality in young adults, are the second leading contributor in children, and appear to be increasing in incidence both in the pediatric and geriatric population[1-4]. Gliomas are the most common type of primary brain tumors; 20,000 cases are diagnosed and 14,000 glioma-related deaths occur annually in the United States[5-8]. Gliomas are heterogeneous with respect to their malignant behavior and, in their most common and aggressive forms, anaplastic astrocytoma (AA-grade III) and glioblastoma multiforme (GBM-grade IV), are rapidly progressive and nearly uniformly lethal[9; 10]. Currently available therapeutic modalities have minimal curative potential for these high-grade tumors and often exacerbate the already severe morbidities imposed by their location in the central nervous system. Thus patients with malignant glioma are often struck in the most productive period of their lives; frequent deterioration of mental faculties and a high case:fatality ratio contribute to the unique personal and social impact of these tumors.

[0003] The cornerstones of oncologic management of malignant glioma are resection and radiation therapy[11-16]. With modern surgical and radiotherapeutic techniques the mean duration of survival has increased to 82 weeks for glioblastoma multiforme and 275 weeks for anaplastic astrocytoma, although 5-year survival rates have only increased from 3 to 6% for glioblastoma multiforme and 12.1% for anaplastic astrocytoma[6-8]. The major prognostic indicators for prolonged survival are younger age (<40yrs) and performance status (KPS score >70)[17]. Resections of >90% of bulky tumors are usually attempted provided that vital functional anatomy is spared. When used in conjunction with post-operative radiation therapy, the impact of extent of resection on duration of survival is less clear[18; 19]. The addition of chemotherapy to resection and radiation provides only marginal survival advantage to patients with anaplastic astrocytoma or glioblastoma multiforme[20-23]. Nitrosureas alone or in combination with procarbazine and vincristine are the conventional drugs used in the community and appear to improve the 1-year and 2-year survival rates by 15% without impacting on the overall median survival[24; 25]. More aggressive regimens incorporating platinum-based drugs and topoisomerase inhibitors are under investigation[26]. The role of high-dose chemotherapy with stem cell rescue has not been substantiated to date[27-29].

[0004] Approximately 80% of recurrent tumors arise from radiographically enhancing remnants of the original incompletely resected tumor[10; 30; 31]. Provided recurrences are unifocal and amenable in their location to aggressive re-resection, this approach can extend survival duration, particularly for patients with anaplastic astrocytoma and those glioblastoma multiforme patients with a KPS >70.[10] The median survival of recurrent glioblastoma multiforme patients treated with re-resection is 36 weeks[10; 30; 31]. Radiation therapy in the form of either brachytherapy or stereotactic radiosurgery may extend the duration of survival in re-resected recurrent glioblastoma multiforme patients by only 10-12 weeks[32]. The use of chemotherapy in the setting of recurrent disease should be in the context of available clinical trials, as its efficacy in this patient population is unsubstantiated.

[0005] The continued dismal prognosis of malignant glioma has prompted the clinical investigation of novel therapeutic entities, including, but not limited to: gene therapy (TK-suicide, antisense inhibition of tumor growth factor receptors, conditionally lethal viral vectors), immunotherapy (antibody, tumor cell vaccines, immunotoxins, adoptive transfer of activated lymphocytes), and anti-angiogenesis approaches[33-40]. The multiplicity of challenges faced in the development of effective adjuvant therapies for malignant glioma include the extensive infiltrative growth of tumor cells into normal brain parenchyma, the capacity of soluble factors elaborated from these tumors to attenuate the development of immune responses, and the difficulty of establishing clinically meaningful therapeutic ratios when administering therapeutics into the central nervous system (CNS). Early clinical evaluation of novel therapeutics is clearly indicated in this patient population.

[0006] Recently, receptors for transferrin and growth factors have been the subject of experimental glioma therapeutics utilizing ligands for these receptors conjugated to toxins or radionucleotides as a delivery system[41]. The specificity of this approach relies on the unique expression or over-expression of targeted receptors on glioma cells compared to normal brain. Interestingly, some receptor complexes for interleukins utilized by the immune system are expressed by gliomas, in particular high-affinity IL-13 receptors[42-48]. Unlike the IL-13 receptor trimolecular complex utilized by the immune system, which consists of the IL-13Rα1, the IL-4Rβ, and γc, glioma cells overexpress a unique IL-13Rα2 chain capable of binding IL-13 independently of the requirement for IL-4Rβ or γc[44; 49; 50]. Like its homologue IL-4, IL-13 has pleotrophic immunoregulatory activity outside the CNS[51-53]. Both cytokines stimulate IgE production by B lymphocytes and suppress pro-inflammatory cytokine production by macrophages. The immunobiology of IL-13 within the CNS is largely unknown.

[0007] Detailed studies by Debinski et al. using autoradiography with radiolabeled IL-13 have demonstrated abundant IL-13 binding on nearly all malignant glioma tissues studied[42; 45; 46; 48]. Moreover, the binding is highly homogeneous within tumor sections and from single cell analysis[46; 48]. Scatchard analyses of IL-13 binding to human glioma cell lines reveals on average 17,000-

28,000 binding sites/cell[45]. Molecular analysis using probes specific for IL-13Rα2 mRNA fail to demonstrate expression of the glioma-specific receptor by normal brain elements in all CNS anatomic locations[42; 43]. Furthermore, autoradiography with radiolabeled IL-13 failed to demonstrate detectable specific IL-13 binding in the CNS, suggesting that the shared IL13Rα1/IL-4β/γc receptor is also not expressed at detectable levels in the CNS[46]. These findings were independently verified using immunohistochemical techniques on non-pathologic brain sections with antibodies specific for IL-13Rα1 and IL-4β[54]. Thus IL-13Rα2 stands as the most specific and ubiquitously expressed cell-surface target for glioma described to date.

[0008] As a strategy to exploit the glioma-specific expression of IL-13Rα2 in the CNS, molecular constructs of the IL-13 cytokine have been described that fuse various cytotoxins (*Pseudomonas* exotoxin and *Diptheria* toxin) to its carboxyl terminal[55-58]. Internalization of these toxins upon binding to IL-13 receptors is the basis of the selective toxicity of these fusion proteins. These toxins display potent cytotoxicity towards glioma cells *in vitro* at picomolar concentrations[55]. Human intracranial glioma xenografts in immunodeficient mice can be eliminated by intratumor injection of the IL-13-toxin fusion protein without observed toxicities[55]. These studies support the initiation of clinical investigation utilizing IL-13-directed immunotoxins loco-regionally for malignant glioma.

[0009] However, the binding of IL-13-based cytotoxins to the broadly expressed IL-13Rα1/IL-4β/γc receptor complex has the potential of mediating untoward toxicities to normal tissues outside the CNS, and thus limits the systemic administration of these agents. IL-13 has been extensively dissected at the molecular level: structural domains of this cytokine that are important for associating with individual receptor subunits have been mapped[55; 58]. Consequently, selected amino acid substitutions in IL-13 have predictable effects on the association of this cytokine with its receptor subunits. Amino acid substitutions in IL-13's alpha helix A, in particular at amino acid 13, disrupt its ability to associate with IL-4β, thereby selectively reducing the affinity of IL-13 to the IL-13Rα1/IL-4β/γc receptor by a factor of five[55; 57; 58]. Surprisingly, binding of

mutant IL-13(E13Y) to IL-13Rα2 was not only preserved but increased relative to wild-type IL-13 by 50-fold. Thus, minimally altered IL-13 analogs can simultaneously increase IL-13's specificity and affinity for glioma cells via selective binding to IL-13Rα2 relative to normal tissues bearing IL-13Rα1/IL-4β/γc receptors.

[0010] Malignant gliomas represent a clinical entity that is highly attractive for immunotherapeutic intervention since 1) most patients with resection and radiation therapy achieve a state of minimal disease burden and 2) the anatomic location of these tumors within the confines of the CNS make direct loco-regional administration of effector cells possible. At least two pathologic studies have demonstrated that the extent of perivascular lymphocytic infiltration in malignant gliomas correlates with an improved prognosis[59-61]. Animal model systems have established that glioma-specific T cells, but not lymphokine-activated killer (LAK) cells, can mediate the regression of intracerebrally implanted gliomas[62-71]. T cells, unlike LAK cells, have the capacity to infiltrate into brain parenchyma and thus can target infiltrating tumor cells that may be distant from the primary tumor. Despite these findings, there is a substantial body of evidence that gliomas actively subvert immune destruction, primarily by the elaboration of immunosuppressive cytokines (TGF-β2) and prostaglandins, which, inhibit the induction/amplification of glioma-reactive T cell responses[72-74]. These findings have prompted the evaluation of *ex vivo* expanded anti-glioma effector cells for adoptive therapy as a strategy to overcome tumor-mediated limitations of generating responses *in vivo*.

[0011] At least ten pilot studies involving the administration of *ex vivo* activated lymphocytes to malignant glioma resection cavities have been reported to date[75-85]. Despite the variety of effector cell types (LAK, TILs, alloreactive CTLs), their heterogeneous composition/variability of composition from patient to patient, and the often modest *in vitro* reactivity of these effector cells towards glioma targets, these studies, in aggregate, report an approximate 50% response rate in patients with recurrent/refractory disease with anecdotal long-term survivors. These studies support the premise that a superior clinical effect of cellular immunotherapy for glioma might be expected with homogenous highly potent effector cells.

[0012] These pilot studies also report on the safety and tolerability of direct administration of *ex vivo* activated lymphocytes and interleukin-2 (IL-2), a T cell growth factor, into the resection cavity of patients with malignant glioma[75; 76; 78; 82; 86-92]. Even at large individual cell doses (>10^9 cells/dose), as well as high cumulative cell doses (>27×10^9 cells), toxicities are modest, and typically consist of grade II or less transient headache, nausea, vomiting and fever. As noted above, these studies also employed the co-administration of rhIL-2 to support the *in vivo* survival of transferred lymphocytes. Multiple doses given either concurrently with lymphocytes or sequentially after lymphocyte administration were tolerated at doses as high as 1.2×10^6 IU/dose for 12-dose courses of IL-2 delivered every 48-hours.

[0013] Based on the findings outlined above, strategies to improve the anti-tumor potency of lymphocyte effector cells used in glioma immunotherapy are under development. One approach utilizes bi-specific antibodies capable of co-localizing and activating T lymphocytes via an anti-CD3 domain with glioma targets utilizing an epidermal growth factor receptor (EGFR) binding domain[93-96]. Preliminary clinical experience with this bi-specific antibody in combination with autologous lymphocytes suggests that T cells are activated *in situ* in the resection cavity. Targeting infiltrating tumor cells within the brain parenchyma, however, is a potentially significant limitation of this approach. T cells might have significantly increased anti-glioma activity if they are specific for target antigens expressed by gliomas. A growing number of human genes encoding tumor antigens to which T lymphocytes are reactive have been cloned, including the SART-1 gene, which appears to be expressed by nearly 75% of high-grade gliomas[97]. Both dendritic cell-based *in vitro* cell culture techniques, as well as tetramer-based T cell selection technologies are making feasible the isolation of antigen-specific T cells for adoptive therapy. Since antigens like SART-1 are recognized by T cells in the context of restricting HLA alleles, antigen-specific approaches will require substantial expansion in the number of antigens and restricting HLA alleles capable of presenting these antigens to be broadly applicable to the general population of glioma patients.

[0014] Chimeric antigen receptors engineered to consist of an extracellular single chain antibody (scFvFc) fused to the intracellular signaling domain of the T cell antigen receptor complex zeta chain (scFvFc:ζ) have the ability, when expressed in T cells, to redirect antigen recognition based on the monoclonal antibody's specificity[98]. The design of scFvFc:ζ receptors with target specificities for tumor cell-surface epitopes is a conceptually attractive strategy to generate antitumor immune effector cells for adoptive therapy as it does not rely on pre-existing anti-tumor immunity. These receptors are "universal" in that they bind antigen in a MHC independent fashion, thus, one receptor construct can be used to treat a population of patients with antigen-positive tumors. Several constructs for targeting human tumors have been described in the literature including receptors with specificities for Her2/Neu, CEA, ERRB-2, CD44v6, and epitopes selectively expressed on renal cell carcinoma[98-104]. These epitopes all share the common characteristic of being cell-surface moieties accessible to scFv binding by the chimeric T cell receptor. *In vitro* studies have demonstrated that both CD4+ and CD8+ T cell effector functions can be triggered via these receptors. Moreover, animal models have demonstrated the capacity of adoptively transferred scFvFc:ζ expressing T cells to eradicate established tumors[105]. The function of primary human T cells expressing tumor-specific scFvFc:ζ receptors have been evaluated *in vitro*; these cells specifically lyse tumor targets and secrete an array of pro-inflammatory cytokines including IL-2, TNF, IFN-γ, and GM-CSF[104]. Phase I pilot adoptive therapy studies are underway utilizing autologous scFvFc:ζ-expressing T cells specific for HIV gp120 in HIV infected individuals and autologous scFvFc:ζ-expressing T cells with specificity for TAG-72 expressed on a variety of adenocarcinomas, including breast and colorectal adenocarcinoma.

[0015] Investigators at City of Hope have engineered a CD20-specific scFvFc:ζ receptor construct for the purpose of targeting CD20+ B-cell malignancy and an L1-CAM-specific chimeric immunoreceptor for targeting neuroblastoma[106]. Preclinical laboratory studies have demonstrated the feasibility of isolating and expanding from healthy individuals and lymphoma patients CD8+ CTL clones that contain a single copy of unrearranged chromosomally integrated vector DNA and

express the CD20-specific scFvFc:ζ receptor[107]. To accomplish this, purified linear plasmid DNA containing the chimeric receptor sequence under the transcriptional control of the CMV immediate/early promoter and the NeoR gene under the transcriptional control of the SV40 early promoter was introduced into activated human peripheral blood mononuclear cells by exposure of cells and DNA to a brief electrical current, a procedure called electroporation. Utilizing selection, cloning, and expansion methods currently employed in FDA-approved clinical trials at the Fred Hutchinson Cancer Research Center, Seattle, Washington, gene modified CD8+ CTL clones with CD20-specific cytolytic activity have been generated from each of six healthy volunteers in 15 separate electroporation procedures. These clones when co-cultured with a panel of human CD20+ lymphoma cell lines proliferate, specifically lyse target cells, and are stimulated to produce cytokines.

SUMMARY OF THE INVENTION

[0016] The present invention relates to chimeric transmembrane immunoreceptors, named "zetakines," comprised of an extracellular domain comprising a soluble receptor ligand linked to a support region capable of tethering the extracellular domain to a cell surface, a transmembrane region and an intracellular signaling domain. Zetakines, when expressed on the surface of T lymphocytes, direct T cell activity to those cells expressing a receptor for which the soluble receptor ligand is specific. Zetakine chimeric immunoreceptors represent a novel extension of antibody-based immunoreceptors for redirecting the antigen specificity of T cells, with application to treatment of a variety of cancers, particularly via the autocrine/paracrine cytokine systems utilized by human malignancy.

[0017] In one preferred embodiment exploiting the tumor-restricted expression of IL-13Rα2 by malignant glioma and renal cell carcinoma as a target for cellular immunotherapy, a mutant of the IL-13 cytokine, IL-13(E13Y), having selective high-affinity binding to IL-13Rα2 has been converted into a type I transmembrane chimeric immunoreceptor capable of redirecting T cell antigen specificity to IL-13Rα2-expressing tumor cells. This embodiment of the zetakine consists of

extracellular IL-13(E13Y) fused to human IgG4 Fc, transmembrane CD4, and intracellular T cell antigen receptor CD3 complex zeta chain. Analogous immunoreceptors can be created that are specific to any of a variety of cancer cell types that selectively express receptors on their cell surfaces, for which selective ligands are known or can be engineered.

[0018] Bulk lines and clones of human T cells stably transformed to express such an immunoreceptor display redirected cytolysis of the cancer cell type to which they are specific, while showing negligible toxicity towards non-target cells. Such engineered T cells are a potent and selective therapy for malignancies, including difficult to treat cancers such as glioma.

BRIEF DESCRIPTION OF THE FIGURES

[0019] Figure 1: Results of a Western Blot showing that the IL13zetakine Chimeric Immunoreceptor is expressed as an intact glycosylated protein in Jurkat T cells.

[0020] Figure 2: Results of flow cytometric analysis showing that expressed IL13zetakine chimeric immunoreceptor trafficks to the cell-surface as a type I transmembrane protein.

[0021] Figure 3: Results of flow cytometric analysis showing the cell surface phenotype of a representative primary human IL13zetakine^{+} CTL clone.

[0022] Figure 4: Results of a chromium release assays showing (a) that the IL13zetakine^{+} CTL clone acquired glioma-specific re-directed cytolytic activity, and (b) the profile of anti-glioma cytolytic activity by primary human IL13zetakine^{+} CD8^{+} CTL clones was observed in glioma cells generally.

[0023] Figure 5: Results of *in vitro* stimulation of cytokine production, showing that IL13zetakine^{+} CTL clones are activated for cytokine production by glioma stimulator cells.

[00024] Figure 6: Results of *in vitro* stimulation of cytokine production, showing the specific inhibition of IL13zetakine^{+} CTL activation for cytokine production by anti-IL13R Mab and rhIL13.

[0025] Figure 7: Results of growth studies showing (a) that IL13zetakine$^+$ CD8$^+$ CTL cells proliferate upon co-culture with glioma stimulators, and (b) the inhibition of glioma-stimulated proliferation of IL13zetakine$^+$ CD8$^+$ CTL cells by rhIL-13.

[0026] Figure 8: Flow chart of the construction of IL13zetakine/HyTK-pMG.

[0027] Figure 9: Plasmid map of IL13zetakine/HyTK-pMG.

DETAILED DESCRIPTION

[0028] An ideal cell-surface epitope for tumor targeting with genetically-engineered re-directed T cells would be expressed solely on tumor cells in a homogeneous fashion and on all tumors within a population of patients with the same diagnosis. Modulation and/or shedding of the target molecule from the tumor cell membrane may also impact on the utility of a particular target epitope for re-directed T cell recognition. To date few "ideal" tumor-specific epitopes have been defined and secondary epitopes have been targeted based on either lack of expression on critical normal tissues or relative over-expression on tumors. In the case of malignant glioma, the intracavitary administration of T cells for the treatment of this cancer permits the expansion of target epitopes to those expressed on tumor cells but not normal CNS with less stringency on expression by other tissues outside the CNS. The concern regarding toxicity from cross-reactivity of tissues outside the CNS is mitigated by a) the sequestration of cells in the CNS based on the intracavitary route of administration and b) the low cell numbers administered in comparison to cell doses typically administered systemically.

[0029] The IL-13Rα2 receptor stands out as the most ubiquitous and specific cell-surface target for malignant glioma[47]. Sensitive autoradiographic and immunohistochemical studies fail to detect IL-13 receptors in the CNS[46; 48]. Moreover, mutation of the IL-13 cytokine to selectively bind the glioma-restricted IL-13Rα2 receptor is a further safeguard against untoward reactivity of IL-13-directed therapeutics against IL-13Rα1/IL-4β+ normal tissues outside the CNS[55; 57]. The potential utility of targeting glioma IL-13Rα2 the design and testing of a novel engineered chimeric immunoreceptor for re-directing the specificity of T cells that

consists of an extracellular IL-13 mutant cytokine (E13Y) tethered to the plasma membrane by human IgG4 Fc which, in turn, is fused to CD4TM and the cytoplasmic tail of CD3 zeta. This chimeric immunoreceptor has been given the designation of "IL-13 zetakine". The IL-13Rα2 receptor/IL-13(E13Y) receptor-ligand pair is an excellent guide for understanding and assessing the suitability of receptor-ligand pairs generally for use in zetakines. An ideal zetakine comprises an extracellular soluble receptor ligand having the properties of IL-13(E13Y) (specificity for a unique cancer cell surface receptor, *in vivo* stability due to it being derived from a naturally-occurring soluble cell signal molecule, low immunogenicity for the same reason). The use of soluble receptor ligands as distinct advantages over the prior art use of antibody fragments (such as the scFvFc immunoreceptors) or cell adhesion molecules, in that soluble receptor ligands are more likely to be stable in the extracellular environment, non-antigenic, and more selective.

[0030] Chimeric immunoreceptors according to the present invention comprise an extracellular domain comprised of a soluble receptor ligand linked to an extracellular support region that tethers the ligand to the cell surface via a transmembrane domain, in turn linked to an intracellular receptor signaling domain. Examples of suitable soluble receptor ligands include autocrine and paracrine growth factors, chemokines, cytokines, hormones, and engineered artificial small molecule ligands that exhibit the required specificity. Natural ligand sequences can also be engineered to increase their specificity for a particular target cell. Selection of a soluble receptor ligand for use in a particular zetakine is governed by the nature of the target cell, and the qualities discussed above with regard to the IL-13(E13Y) molecule, a preferred ligand for use against glioma. Examples of suitable support regions include the constant (Fc) regions of immunoglobins, human CD8∝, and artificial linkers that serve to move the targeting moiety away from the cell surface for improved access to receptor binding on target cells. A preferred support region is the Fc region of an IgG (such as IgG4). Examples of suitable transmembrane domains include the transmembrane domains of the leukocyte CD markers, preferably that of CD8.

Examples of intracellular receptor signaling domains are those of the T cell antigen receptor complex, preferably the zeta chain of CD3 also Fcγ RIII costimulatory signaling domains, CD28, DAP10, CD2, alone or in a series with CD3zeta.

[0031] In the IL-13 zetakine embodiment, the human IL-13 cDNA having the E13Y amino acid substitution was synthesized by PCR splice overlap extension. A full length IL-13 zetakine construct was assembled by PCR splice overlap extension and consists of the human GM-CSF receptor alpha chain leader peptide, IL-13(E13Y)-Gly-Gly-Gly, human IgG4 Fc, human CD4TM, and human cytoplasmic zeta chain. This cDNA construct was ligated into the multiple cloning site of a modified pMG plasmid under the transcriptional control of the human Elongation Factor-1alpha promoter (Invivogen, San Diego). This expression vector co-expresses the HyTK cDNA encoding the fusion protein HyTK that combines in a single molecule hygromycin phosphotransferase activity for *in vitro* selection of transfectants and HSV thymidine kinase activity for *in vivo* ablation of cells with ganciclovir from the CMV immediate/early promoter. Western blot of whole cell Jurkat lysates pre-incubated with tunicamycin, an inhibitor of glycosylation, with an anti-zeta antibody probe demonstrated that the expected intact 56-kDa chimeric receptor protein is expressed. This receptor is heavily glycosylated consistent with post-translational modification of the native IL-13 cytokine[108]. Flow cytometric analysis of IL-13 zetakine+ Jurkat cells with anti-human IL-13 and anti-human Fc specific antibodies confirmed the cell-surface expression of the IL-13 zetakine as a type I transmembrane protein.

[0032] Using established human T cell genetic modification methods developed at City of Hope[107], primary human T cell clones expressing the IL-13 zetakine chimeric immunoreceptor have been generated for pre-clinical functional characterization. IL-13 zetakine+ CD8+ CTL clones display robust proliferative activity in *ex vivo* expansion cultures. Expanded clones display re-directed cytolytic activity in 4-hr chromium release assays against human IL-13Rα2+ glioblastoma cell lines. The level of cytolytic activity correlates with levels of zetakine expression on T cells and IL-13Rα2 receptor density on glioma target cells. In addition to killing, IL-13 zetakine+ clones are activated for cytokine

secretion (IFN-γ, TNF-α, GM-CSF). Activation was specifically mediated by the interaction of the IL-13 zetakine with the IL-13Rα2 receptor on glioma cells since CTL clones expressing an irrelevant chimeric immunoreceptor do not respond to glioma cells, and, since activation can be inhibited in a dose-dependent manner by the addition to culture of soluble IL-13 or blocking antibodies against IL-13 on T cell transfectants and IL-13Rα2 on glioma target cells. Lastly, IL-13 zetakine-expressing CD8+ CTL clones proliferate when stimulated by glioma cells in culture. IL-13 zetakine+ CTL clones having potent anti-glioma effector activity will have significant clinical activity against malignant gliomas with limited collateral damage to normal CNS.

[0033] An immunoreceptor according to the present invention can be produced by any means known in the art, though preferably it is produced using recombinant DNA techniques. A nucleic acid sequence encoding the several regions of the chimeric receptor can prepared and assembled into a complete coding sequence by standard techniques of molecular cloning (genomic library screening, PCR, primer-assisted ligation, site-directed mutagenesis, etc.) . The resulting coding region is preferably inserted into an expression vector and used to transform a suitable expression host cell line, preferably a T lymphocyte cell line, and most preferably an autologous T lymphocyte cell line. A third party derived T cell line/clone, a transformed humor or xerogenic immunologic effector cell line, for expression of the immunoreceptor. NK cells, macrophages, neutrophils, LAK cells, LIK cells, and stem cells that differentiate into these cells, can also be used. In a preferred embodiment, lymphocytes are obtained from a patient by leukopharesis, and the autologous T cells are transduced to express the zetakine and administered back to the patient by any clinically acceptable means, to achieve anti-cancer therapy.

[0034] Suitable doses for a therapeutic effect would be between about 10^6 and about 10^9 cells per dose, preferably in a series of dosing cycles. A preferred dosing regimen consists of four one-week dosing cycles of escalating doses, starting at about 10^7 cells on Day 0, increasing incrementally up to a target dose of about 10^8 cells by Day 5. Suitable modes of administration include intravenous,

subcutaneous, intracavitary (for example by reservoir-access device), intraperitoneal, and direct injection into a tumor mass.

[0035] The following examples are solely for the purpose of illustrating one embodiment of the invention.

EXAMPLE 1: Construction of an immunoreceptor coding sequence

[0036] The coding sequence for an immunoreceptor according to the present invention was constructed by de novo synthesis of the IL13(E13Y) coding sequence using the following primers (see Fig. 8 for a flow chart showing the construction of the immunoreceptor coding sequence and expression vector):

IL13P1:

EcoRI
TAT**GAATTC**ATGGCGCTTTTGTTGACCACGGTCATTGCTCTCACTTGCC
TTGGCGGCTTTGCCTCCCCAGGCCCTGTGCCTCCCTCTACAGCCCTCAG
GTAC [SEQ ID NO. 1]

IL13P2:
GTTGATGCTCCATACCATGCTGCCATTGCAGAGCGGAGCCTTCTGGTTC
TGGGTGATGTTGACCAGCTCCTCAATGAGGTACCTGAGGGCTGTAGAG
GGAG [SEQ ID NO. 2]

IL13P3:
CTCTGGGTCTTCTCGATGGCACTGCAGCCTGACACGTTGATCAGGGATT
CCAGGGCTGCACAGTACATGCCAGCTGTCAGGTTGATGCTCCATACCAT
GC [SEQ ID NO. 3]

IL13P4:
CCTCGATTTTGGTGTCTCGGACATGCAAGCTGGAAAACTGCCCAGCTGA
GACCTTGTGCGGGCAGAATCCGCTCAGCATCCTCTGGGTCTTCTCGATG
GC [SEQ ID NO. 4]

IL13P5:

BamHI
TC**GGATCC**TCAGTTGAACCGTCCCTCGCGAAAAAGTTTCTTTAAATGTA
AGAGCAGGTCCTTTACAAACTGGGCCACCTCGATTTTGGTGTCTCGG
[SEQ ID NO. 5]

[0037] The final sequence (417bp) was end-digested with EcoRI-BamHI, and ligated into the plasmid pSK (stratagene, LaJolla, CA) as ligation 312#3. Ligation 312#3 was mutagenized (stratagene kit, per manufacturer's instructions) to fix a

deleted nucleotide using the primers 5': IL13 312#3 mut5-3 (CAACCTGACAGCTGGCATGTACTGTGCAGCCCTGGAATC [SEQ ID NO. 6]) and 3':IL13 312#3 mut3-5 (GATTCCAGGGCTGCACAG**T**ACATGCCAGCTGTCAGGTTG [SEQ ID NO. 7]), and ligation 312#3 as a template, to form ligation 348#1 (IL13zetakine/pSK).

[0038] The coding Human GM-CSFR alpha chain Signal Peptide (hsp) coding sequence was fused to the 5' end of IL13(E13Y) by standard PCR splice overlap extension. The hsp sequence (101 bp) was obtained from the template ligation 301#10 (hsp/pSK) (human GCSF receptor α-chain leader sequence from human T cell cDNA), using the primers 5':19hsp5' (ATC**TCTAGA**GCCGCCACCATGCTTCTCCTGGTGACAAGCCTTC [SEQ ID NO. 8]) (XbaI site highlighted in bold), and 3': hsp-IL13FR (GAGGGAGGCACAGGGCCTGGGATCAGGAGGAATG [SEQ ID NO. 9]). The IL-13 sequence (371 bp) was obtained using the primers 5': hsp-IL13FF (CATTCCTCCTGATCCCAGGCCCTGTGCCTCCCTC [SEQ ID NO. 10]) and 3': IL13-IgG4FR (GGGACCATATTTGGACTCGTTGAACCGTCCCTCGC [SEQ ID NO. 11]), and ligation 312#3 as template. Fusion was achieved using the 101 bp hsp sequence and 371 bp IL13 sequence thus obtained, and the primers 5': 19hsp5' and 3': IL13-IgG4FR, to yeild a 438 bp fusion hsp-IL13 sequence.

[0039] A sequence encoding the IgG4 Fc region IgG4m:zeta was fused to the 3' end of the hsp-IL13 fusion sequence using the same methods. The IgG4m:zeta sequence (1119 bp) was obtained using the primers 5': IL13-IgG4FF (GCGAGGGACGGTTCAACGAGTCCAAATATGGTCCC [SEQ ID NO. 12]) and 3': ZetaN3' (AT**GCGGCCGC**TCAGCGAGGGGGCAGG [SEQ ID NO. 13]) (NotI site highlighted in bold), using the sequence R9.10 (IgG4mZeta/pSK) as template. The 1119 bp IgG4m:zeta sequence was fused to the hsp-IL13 fusion sequence using the respective sequences as templates, and the primers 5': 19hsp5' and 3': ZetaN3', to yeild a 1522 bp hsp-IL13-IgG4m:zeta fusion sequence. The ends were digested with XbaI-NotI, and ligated into pSK as ligation 351#7, to create the plasmid IL13zetakine/pSK (4464 bp).

EXAMPLE 2: Construction of expression vector

[0040] An expression vector containing the IL13 zetakine coding sequence was created by digesting the IL13zetakine/pSK of Example 1 with XbaI-NotI, and creating blunt ends with Klenow, and ligating the resulting fragment into the plasmid pMG^Pac (Invirogen) (first prepared by opening with SgrAI, blunting with Klenow, and dephosphorylation with SAP), to yield the plasmid IL13zetakine/pMG. See Fig. 8. The hygromycin resistance region of IL13zetakine/pMG was removed by digestion with NotI-NheI, and replaced by the selection/suicide fusion HyTK, obtained from plasmid CE7R/HyTK-pMG (Jensen, City of Hope) by digestion with NotI-NheI, to create the expression vector IL13zetakine/HyTK-pMG (6785 bp). This plasmid comprises the Human Elongation Factor-1α promoter (hEF1p) at bases 6-549, the IL13zetakine coding sequence at bases 692-2185, the Simian Virus 40 Late polyadenylation signal (Late SV40pAN) at bases 2232-2500, a minimal *E. coli* origin of replication (Ori ColE1) at bases 2501-3247, a synthetic poly A and Pause site (SpAN) at bases 3248-3434, the Immeate-early CMV enhancer/promoter (h CMV-1Aprom) at bases 3455-4077, the Hygromycin resistance-Thymidine kinase coding region fusion (HyTK) at bases 4259-6334, and the bovine growth hormone polyadenylation signal and a transcription pause (BGh pAn) at bases 6335-6633. The plasmid has a PacI linearization site at bases 3235-3242. The hEF1p and IL13zetakine elements derived from IL13zetakine/pMG, and the remaining elements derived from CE7R/HyTk-pMG (and with the exception of the HyTK element, ultimately from the parent plasmid pMG^Pac). In sum, IL13zetakine/HyTK-pMG is a modified pMG backbone, expressing the IL13zetakine gene from the hEF1 promoter, and the HyTK fusion from the h CMV-1A promoter. A map of the plasmid IL13zetakine/HyTK-pMG appears in Fig. 9.

EXAMPLE 3: Expression of the immunoreceptor

[0041] Assessment of the integrity of the expressed construct was first delineated by Wester blot probed with an anit-zeta antibody of whole cell lysates derived from Jurkat T cell stable transfectants[107] cocultured in the presence or absence of

tunicamycin, an inhibitor of glycosylation. Fig. 1. Jurkat T cell stable transfectants (Jurkat-IL13-pMG bulk line) were obtained by electroporating Jurkat T cells with the IL13zetakine/HyTK-pMG expression vector, followed by selection and expansion of positive transfectants. $2x10^6$ cells from the Jurkat-IL13-pMG bulk line were plated per well in a 24-well plate with or without 5 μg/ml, 10 μg/ml, or 20 μg/ml Tunicamycin. The plate was incubated at 37°C for 22 hrs. Cells were harvested from each well, and each sample was washed with PBS and resuspended in 50 μl RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 tablet/10ml Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN). Samples were incubated on ice for 30 minutes then disrupted by aspiration with syringe with 21 gauge needle then incubated on ice for an additional 30 minutes before being centrifuged at 4°C for 20 minutes at 14,000 rpm. Samples of centrifuged lysate supernatant were harvested and boiled in an equal volume of sample buffer under reducing conditions, then subjected to SDS-PAGE electrophoresis on a 12% acrylamide gel. Following transfer to nitrocellulose, membrane was allowed to dry O/N at 4°C. Next morning, membrane was blocked in a Blotto solution containing 0.04 gm/ml non-fat dried milk in T-TBS (0.02% Tween 20 in Tris buffered saline pH 8.0) for 1 hour. Membrane was then incubated with primary mouse anti-human CD3ζ monoclonal antibody (Pharmingen, San Diego, CA) at a concentration of 1 μg/ml for 2 hours, washed, and then incubated with a 1:3000 dilution (in Blotto solution) of goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Bio-Rad ImmunoStar Kit, Hercules, CA) for 1 hour. Prior to developing, membrane was washed 4 additional times in T-TBS, and then incubated with 3 ml of phosphatase substrate solution (Biorad ImmunoStar Kit, Hercules, CA) for 5 minutes at room temperature. Membrane was then covered with plastic, and exposed to x-ray film. Consistant with the known glycosylation pattern of wild-type human IL-13, the electrophoretic mobility of expressed IL-13(E13Y) zetakine is demonstrative of a heavily glycosylated protein which, when expressed in the presence of tunicamycin, is reduced to an amino acid backbone of approximately 54 kDa.

[0042] The IL-13(E13Y) zetakine traffics to the cell surface as a homodimeric type I transmembrane protein, as evidenced by flow cytometric analysis of transfectants with a phycoerythrin (PE)-conjugated anti human-IL13 monoclonal antibody and a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Fc (gamma) fragment-specific $F(ab')_2$ antibody. Fig. 2. Jurkat IL13zetakine-pMG transfectants were stained with anti-human Fc(FITC) antibody (Jackson ImmunoResearch, West Grove, PA), recombinant human IL13Rα2/human IgG1 chimera (R&D Systems, Minneapolis, MN) followed by FITC-conjugated anti human-IgG1 monoclonal antibody (Sigma, St. Louis, MO), and an anti-IL13(PE) antibody (Becton Dickinson, San Jose, CA) for analysis of cell surface chimeric receptor expression. Healthy donor primary cells were also stained with FITC-conjugated anti-CD4, anti-CD8, anti-TCR, and isotype control monoclonal antibodies (Becton Dickinson, San Jose, CA) to assess cell surface phenotype. For each stain, 10^6 cells were washed and resuspended in 100μl of PBS containing 2% FCS, 0.2 mg/ml NaN_3, and 5 μl of stock antibody. Following a 30 minute incubation at 4°C, cells were washed twice and either stained with a secondary antibody, or resuspended in PBS containing 1% paraformaldehyde and analyzed on a FACSCaliber cytometer.

EXAMPLE 4: Binding of IL13(E13Y) zetakine to IL13Rα2 receptor

[0043] IL-13(E13Y), tethered to the cell membrane by human IgG4 Fc (i.e., IL13(E13Y) zetakine), is capable of binding to its target IL13Rα2 receptor as assessed by flow cytometric analysis using soluble IL13Rα2-Fc fusion protein. Fig. 3. Cloned human PBMC IL13zetakine-pMG transfectants were obtained by electroporating PBMC with the IL13zetakine/HyTK-pMG expression vector, followed by selection and expansion of positive transfectants[107]. $IL13zetakine^+$ CTL clonal cells were stained with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Fc (gamma) fragment-specific $F(ab')_2$ (Jackson ImmunoResearch, West Grove, PA), recombinant human IL13Rα2/human IgG1 chimera (R&D Systems, Minneapolis, MN) followed by FITC-conjugated anti human-IgG1 monoclonal antibody (Sigma, St. Louis, MO), and a phycoerythrin

(PE)-conjugated anti human-IL13 monoclonal antibody (Becton Dickinson, San Jose, CA) for analysis of cell surface chimeric receptor expression. Healthy donor primary cells were also stained with FITC-conjugated anti-CD4, anti-CD8, anti-TCR, and isotype control monoclonal antibodies (Becton Dickinson, San Jose, CA) to assess cell surface phenotype. For each stain, 10^6 cells were washed and resuspended in 100μl of PBS containing 2% FCS, 0.2 mg/ml NaN_3, and 5 μl of antibody. Following a 30 minute incubation at 4°C, cells were washed twice and either stained with a secondary antibody, or resuspended in PBS containing 1% paraformaldehyde and analyzed on a FACSCaliber cytometer.

[0044] Next, the immunobiology of the IL-13(E13Y) zetakine as a surrogate antigen receptor for primary human T cells was evaluated. Primary human T cells were electroporated with the plasmid expression vector. Positive transformants were selected with hygromycin, cloned in limiting dilution, then expanded by recursive stimulation cyles with OKT3, IL-2 and irradiated feeder cells. Clones demonstrating IL 13zetakine expression by Western blot and FACS were then subjected to functional evaluation in 4-hr chromium release assays against a variety of IL-13α2$^+$/CD20$^-$ glioma cell lines (U251, SN-B19, U138), and the IL-13α$^-$/CD20$^+$ B cell lyphocyte line Daudi). These tests showed that IL13zetakine conferred cytolytic activity that was specific for glioma cells (Fig. 4a), and that this specific cytolytic activity is present for glioma cells as a class (Fig. 4b). The cytolytic activity of MJ-IL13-pMG clones was assayed by employing ^{51}Cr-labeled SN-B19, U251, and U138 glioma cell lines (IL13α2+/CD20-) and Daudi (CD20+/IL13α2-) as targets. MJ-IL13 effectors were assayed 8-12 days following stimulation. Effectors were harvested, washed, and resuspeded in assay media: $2.5x10^5$, $1.25x10^5$, $2.5x10^4$, and $5x10^3$ effectors were cultured in triplicate at 37°C for 4 hours with $5x10^3$ target cells in 96-well V-bottom microtiter plates. After incubation, 100μl aliquots of cell-free supernatant were harvested and ^{51}Cr in the supernatants was assayed with a γ-counter. Percent specific cytolysis was calculated as follows:

$$\frac{(\text{Experimental }^{51}\text{Cr release}) - (\text{control }^{51}\text{Cr release})}{(\text{Maximum }^{51}\text{Cr release}) - (\text{control }^{51}\text{Cr release})} \times 100$$

Control wells contained target cells incubated in the presence of target cells alone. Maximum ^{51}Cr release was determined by measuring the ^{51}Cr released by labeled target cells in the presence of 2% SDS. Bulk lines of stabley transfected human T cells consisting of approximately 40% IL-13(E13Y) zetakine^{+} TCRα/β^{+} lymphocytes displayed re-directed cytolysis specific for 13Rα2^{+} glioma targets in 4-hr chromium release assays (>50% specific lysis at E:T ratios of 25:1), with negligable acitivity against IL-13Rα2^{-} targets (<8% specific lysis at E:T ratios of 25:1). IL-13(E13Y) zetakine^{+}CD8^{+}TCRα/β^{+} CTL clones selected on the basis of high-level binding to anti-IL-13 antibody also display redirected IL13Rα2-specific glioma cell killing. Fig. 4b.

[0045] IL-13 zetakine-expressing CD8^{+} CTL clones are activated and proliferate when stimulated by glioma cells in culture. Figs. 5-7. MJ-IL13-pMG Cl. F2 responder cells expressing the IL13 zetakine were evaluated for receptor-mediated triggering of IFNγ, GM-CSF, and TNFα production *in vitro*. $2x10^{6}$ responder cells were co-cultured in 24-well tissue culture plates with $2x10^{5}$ irradiated stimulator cells (Daudi, Fibroblasts, Neuroblastoma 10HTB, and glioblastoma U251) in 2 ml total. Blocking rat anti-human-IL13 monoclonal antibody (Pharmingen, San Diego, CA), recombinant human IL13 (R&D Systems, Minneapolis, MN), and IL13Rα2-specific goat IgG (R&D Systems, Minneapolis, MN) were added to aliquots of U251 stimulator cells ($2x10^{5}$/ml) at concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 μg/ml, 30 minutes prior to the addition of responder cells. Plates were incubated for 72 hours at 37° C, after which time culture supernatants were harvested, aliquoted, and stored at -70°C. ELISA assays for IFNγ, GM-CSF, and TNFα were carried out using the R&D Systems (Minneapolis, MN) kit per manufacturer's instructions. Samples were tested in duplicate wells undiluted or diluted at 1:5 or 1:10. The developed ELISA plate was evaluated on a microplate reader and cytokine concentrations determined by extrapolation from a standard curve. Results are reported as picograms/ml, and show strong activation for cytokine production by glioma stimulator cells. Fig. 5, Fig. 6.

[0046] Lastly, IL-2 independent proliferation of IL13zetakine^{+} CD8^{+} CTL was observed upon co-cultivation with glioma stimulators (Fig. 7a), but not with IL13

Rα2 stimulators. Proliferation was inhibited by the addition of rhIL-13 antibody (Fig. 7b), showing that the observed proliferation was dependant on binding of zetakine to the IL-13Rα2 glioma cell-sepcific receptor.

EXAMPLE 5: Preparation of IL-13 zetakine$^+$ T cells suitable for therapeutic use

[0047] The mononuclear cells are separated from heparinized whole blood by centrifugation over clinical grade Ficoll (Pharmacia, Uppsula, Sweden). PBMC are washed twice in sterile phosphate buffered saline (Irvine Scientific) and suspended in culture media consisting of RPMI 1640 HEPES, 10% heat inactivated FCS, and 4 mM L-glutamine. T cells present in patient PBMC are polyclonally activated by addition to culture of Orthoclone OKT3 (30ng/ml). Cell cultures are then incubated in vented T75 tissue culture flasks in the study subject's designated incubator. Twenty-four hours after initiation of culture rhIL-2 is added at 25 U/ml.

[0048] Three days after the initiation of culture PBMC are harvested, centrifuged, and resuspended in hypotonic electroporation buffer (Eppendorf) at 20×10^6 cells/ml. 25 μg of the plasmid IL13zetakine/HyTK-pMG of Example 3, together with 400 μl of cell suspension, are added to a sterile 0.2 cm electroporation cuvette. Each cuvette is subjected to a single electrical pulse of 250V/40μs and again incubated for ten minutes at RT. Surviving cells are harvested from cuvettes, pooled, and resuspended in culture media containing 25 U/ml rhIL-2. Flasks are placed in the patient's designated tissue culture incubator. Three days following electroporation hygromycin is added to cells at a final concentration of 0.2 mg/ml. Electroporated PBMC are cultured for a total of 14 days with media and IL-2 supplementation every 48-hours.

[0049] The cloning of hygromycin-resistant CD8+ CTL from electroporated OKT3-activated patient PBMC is initiated on day 14 of culture. Briefly, viable patient PBMC are added to a mixture of 100×10^6 cyropreserved irradiated feeder PBMC and 20×10^6 irradiated TM-LCL in a volume of 200ml of culture media containing 30 ng/ml OKT3 and 50 U/ml rhIL-2. This mastermix is plated into ten 96-well cloning plates with each well receiving 0.2 ml. Plates are wrapped in aluminum foil to decrease evaporative loss and placed in the patient's designated

tissue culture incubator. On day 19 of culture each well receives hygromycin for a final concentration of 0.2 mg/ml. Wells are inspected for cellular outgrowth by visualization on an inverted microscope at Day 30 and positive wells are marked for restimulation.

[0050] The contents of each cloning well with cell growth are individually transferred to T25 flasks containing $50x10^6$ irradiated PBMC, $10x10^6$ irradiated LCL, and 30ng/mlOKT3 in 25mls of tissue culture media. On days 1,3,5,7,9,11, and 13 after restimulation flasks receive 50U/ml rhIL-2 and 15mls of fresh media. On day 5 of the stimulation cycle flasks are also supplemented with hygromycin 0.2 mg/ml. Fourteen days after seeding cells are harvested, counted, and restimulated in T75 flasks containing 150 x 10^6 irradiated PBMC, 30 x 10^6 irradiated TM-LCL and 30 ng/ml OKT3 in 50 mls of tissue culture media. Flasks receive additions to culture of rhIL-2 and hygromycin as outlined above.

[0051] CTL selected for expansion for possible use in therapy are analyzed by immunofluorescence on a FACSCalibur housed in CRB-3006 using FITC-conjugated monoclonal antibodies WT/31 (aßTCR), Leu 2a (CD8), and OKT4 (CD4) to confirm the requisite phenotype of clones (αβTCR+, CD4-, CD8+, and IL13+). Criteria for selection of clones for clinical use include uniform TCR αβ+, CD4-, CD8+ and IL13+ as compared to isotype control FITC/PE-conjugated antibody. A single site of plasmid vector chromosomal integration is confirmed by Southern blot analysis. DNA from genetically modified T cell clones will be screened with a DNA probe specific for the plasmid vector. Probe DNA specific for the HyTK in the plasmid vector is synthesized by random priming with florescein-conjugated dUTP per the manufacture's instructions (Amersham, Arlington Hts, IL). T cell genomic DNA is isolated per standard technique. Ten micrograms of genomic DNA from T cell clones is digested overnight at 37°C then electrophoretically separated on a 0.85% agarose gel. DNA is then transferred to nylon filters (BioRad, Hercules, CA) using an alkaline capillary transfer method. Filters are hybridized overnight with probe in 0.5 M Na_2PO_4, pH 7.2, 7% SDS, containing 10 μg/ml salmon sperm DNA (Sigma) at 65°C. Filters are then washed four times in 40 mM Na_2PO_4, pH 7.2, 1% SDS at 65°C and then visualized using a

chemiluminescence AP-conjugated anti-florescein antibody (Amersham, Arlington Hts, IL). Criteria for clone selection is a single band unique vector band.

[0052] Expression of the IL-13 zetakine is determined by Western blot procedure in which chimeric receptor protein is detected with an anti-zeta antibody. Whole cell lysates of transfected T cell clones are generated by lysis of 2 x 10^7 washed cells in 1 ml of RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 tablet/10ml Complete Protease Inhibitor Cocktail (Boehringer Mannheim). After an eighty minute incubation on ice, aliquots of centrifuged whole cell lysate supernatant are harvested and boiled in an equal volume of loading buffer under reducing conditions then subjected to SDS-PAGE electrophoresis on a precast 12% acrylamide gel (BioRad). Following transfer to nitrocellulose, membranes are blocked in blotto solution containing .07 gm/ml non-fat dried milk for 2 hours. Membranes are washed in T-TBS (.05% Tween 20 in Tris buffered saline pH 8.0) then incubated with primary mouse anti-human CD3ζ monoclonal antibody 8D3 (Pharmingen, San Diego, CA) at a concentration of 1 μg/ml for 2 hours. Following an additional four washes in T-TBS, membranes are incubated with a 1:500 dilution of goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody for 1 hour. Prior to developing, membranes are rinsed in T-TBS then developed with 30 ml of "AKP" solution (Promega, Madison, WI) per the manufacturer's instructions. Criteria for clone selection is the presence of a chimeric zeta band.

[0053] CD8+ cytotoxic T cell clones expressing the IL-13 zetakine chimeric immunoreceptor recognize and lyse human glioblastoma target cells following interaction of the chimeric receptor with the cell surface target epitope in a HLA-unrestricted fashion. The requirements for target IL-13Rα2 epitope expression and class I MHC independent recognition will be confirmed by assaying each aßTCR+, CD8+, CD4-, IL-13 zetakine+ CTL clones against IL-13Rα2+ Daudi cell transfectants and IL-13Rα2- Daudi cells. T cell effectors are assayed 12-14 days following stimulation with OKT3. Effectors are harvested, washed, and resuspended in assay media; and Daudi cell transfectants expressing IL-13Rα2. 2.5x10^5, 1.25x10^5, 0.25x10^5, and 0.05x10^5 effectors are plated in triplicate at 37°C

for 4 hours with $5x10^3$ target cells in V-bottom microtiter plates (Costar, Cambridge, MA). After centrifugation and incubation, 100 μL aliquots of cell-free supernatant is harvested and counted. Percent specific cytolysis is calculated as follows:

$$\frac{(\text{Experimental } ^{51}\text{Cr release}) - (\text{control } ^{51}\text{Cr release})}{(\text{Maximum } ^{51}\text{Cr release}) - (\text{control } ^{51}\text{Cr release})} \times 100$$

Control wells contain target cells incubated in assay media. Maximum ^{51}Cr release is determined by measuring the ^{51}Cr content of target cells lysed with 2% SDS. Criteria for clone selection is >25% specific lysis of IL-13Rα2+ Daudi transfectants at an E:T ratio of 5:1 and a <10% lysis of parental Daudi at the same E:T ratio.

EXAMPLE 6: Treatment of human glioma using IL-13 zetakine-expressing T cells.

[0054] T cell clones genetically modified according to Example 5 to express the IL-13R zetakine chimeric immunoreceptor and HyTK are selected for:

a. TCRα/β$^+$, CD4$^-$, CD8$^+$, IL-13$^+$ cell surface phenotype as determined by flow cytometry.

b. Presence of a single copy of chromosomally integrated plasmid vector DNA as evidenced by Southern blot.

c. Expression of the IL-13 zetakine protein as detected by Western blot.

d. Specific lysis of human IL-13Rα2$^+$ targets in 4-hr chromium release assays.

e. Dependence on exogenous IL-2 for *in vitro* growth.

f. Mycoplasma, fungal, bacterial sterility and endotoxin levels <5 EU/ml.

g. *In vitro* sensitivity of clones to ganciclovir.

[0055] Peripheral blood mononuclear cells are obtained from the patient by leukapheresis, preferably following recovery from initial resection surgery and at a time at least three weeks from tapering off steroids and/or their most recent

systemic chemotherapy. The target leukapheresis mononuclear cell yield is $5x10^9$ and the target number of hygromycin-resistant cytolytic T cell clones is 25 with the expectation that at least five clones will be identified that meet all quality control parameters for ex-vivo expansion. Clones are cryopreserved and patients monitored by serial radiographic and clinical examinations. When recurrence of progression of disease is documented, patients undergo a re-resection and/or placement of a reservoir-access device (Omaya reservoir) for delivering T cells to the tumor resection cavity. Following recovery from surgery and tapering of steroids, if applicable, the patient commences with T cell therapy.

[0056] The patient receives a target of at least four one-week cycles of therapy. During the first cycle, cell dose escalation proceeds from an initial dose on Day 0 of 10^7 cells, followed by $5x10^7$ cells on Day 3 to the target dose of 10^8 cells on Day 5. Cycle 2 commences as early as one week from commencement of cycle 1. Those patients demonstrating tumor regression with residual disease on MRI may have additional courses of therapy beginning no earlier than Week 7 consisting of repetition of Cycles 3 and 4 followed by one week of rest/restaging provided these treatments are well tolerated (max. toxicities <grade 3) until such time that disease progression or a CR is achieved based on radiographic evaluation.

[0057] Cell doses are at least a log less than doses given in studies employing intracavitary LAK cells (individual cell doses of up to 10^9 and cumulative cell numbers as high as $2.75x10^{10}$ have been safety administered), ex vivo expanded TILs (up to 10^9 cells/dose reported with minimal toxicity) and allo-reactive lymphocyte (starting cell dose 10^8 with cumulative cell doses up to $51.5x10^8$) delivered to a similar patient population[75-85]. The rationale for the lower cell doses as proposed in this protocol is based on the increased *in vitro* reactivity/anti-tumor potency of IL-13 zetakine+ CTL clones compared to the modest reactivity profile of previously utilized effector cell populations. Low-dose repetitive dosing is favored to avoid potentially dangerous inflammatory responses that might occur with single large cell number instillations. Each infusion will consist of a single T cell clone. The same clone will be administered throughout a patient's treatment course. On the days of T cell administration, expanded clones are aseptically

processed by washing twice in 50cc of PBS then resuspended in pharmaceutical preservative-free normal saline in a volume that results in the cell dose for patient delivery in 2mls. T cells are instilled over 5-10 minutes. A 2ml PFNS flush will be administered over 5 minutes following T cells. Response to therapy is assessed by brain MRI +/- gandolinium, with spectroscopy.

[0058] Expected side-effects of administration of T cells into glioma resection cavities typically consist of self-limited nausea and vomiting, fever, and transient worsening of existing neurological deficits. These toxicities can be attributed to both the local inflammation/edema in the tumor bed mediated by T cells in combination with the action of secreted cytokines. These side-effects typically are transient and less than grade II in severity. Should patients experience more severe toxicities it is expected that decadron alone or in combination with ganciclovir will attenuate the inflammatory process and ablate the infused cells. The inadvertent infusion of a cell product that is contaminated with bacteria or fungus has the potential of mediating serious or life-threatening toxicities. Extensive pre-infusion culturing of the cell product is conducted to identify contaminated tissue culture flasks and minimize this possibility. On the day of re-infusion, gram stains of culture fluids, as well as, endotoxin levels are performed.

[0059] Extensive molecular analysis for expression of IL-13Rα2 has demonstrated that this molecule is tumor-specific in the context of the CNS[44; 46; 48; 54]. Furthermore, the only human tissue with demonstrable IL-13Rα2 expression appears to be the testis[42]. This tumor-testis restrictive pattern of expression is reminiscent of the growing number of tumor antigens (i.e. MAGE, BAGE, GAGE) expressed by a variety of human cancers, most notably melanoma and renal cell carcinoma[109-111]. Clinical experience with vaccine and adoptive T cell therapy has demonstrated that this class of antigens can be exploited for systemic tumor immunotherapy without concurrent autoimmune attack of the testis[112-114]. Presumably this selectively reflects the effect of an intact blood-testis barrier and an immunologically privileged environment within the testis. Despite the exquisite specificity of the mutant IL-13 targeting moiety, toxicities are theoretically possible if cells egress into the systemic circulation in sufficient numbers and recognize

tissues expressing the IL-13Rα1/IL-4β receptor. In light of this remote risk, as well as the possibility that instilled T cells in some patients may mediate an overly exuberant inflammatory response in the tumor bed, clones are equipped with the HyTK gene which renders T cells susceptible to *in vivo* ablation with ganciclovir[115-118]. Ganciclovir-suicide, in combination with an intra-patient T cell dose escalation strategy, helps minimize the potential risk to research participants.

[0060] Side effects associated with therapy (headache, fever, chills, nausea, etc.) are managed using established treatments appropriate for the condition. The patient receives ganciclovir if any new grade 3 or any grade 4 treatment-related toxicity is observed that, in the opinion of the treating physician, puts that patient at significant medical danger. Parentally administered ganciclovir is dosed at 10 mg/kg/day divided every 12 hours. A 14-day course will be prescribed but may be extended should symptomatic resolution not be achieved in that time interval. Treatment with ganciclovir leads to the ablation of IL-13 zetakine$^+$ HyTK$^+$ CD8$^+$ CTL clones. Patients should be hospitalized for the first 72 hours of ganciclovir therapy for monitoring purposes. If symptoms do not respond to ganciclovir within 48 hours additional immunosuppressive agents including but not limited to corticosteroids and cyclosporin may be added at the discretion of the treating physician. If toxicities are severe, decadron and/or other immunosuppressive drugs along with ganciclovir are used earlier at the discretion of the treating physician.

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I claim:

1. A chimeric immunoreceptor comprising the following linked elements, arranged in the stated order:
   a) an extracellular domain comprising a soluble receptor ligand,
   b) a support region capable of tethering the extracellular domain to a cell surface,
   c) a transmembrane region, and
   d) an intracellular signalling domain.

2. The chimeric immunoreceptor of claim 1, wherein the extracellular domain comprises a soluble ligand selected from the group consisting of autocrine growth factors, paracrine growth factors, chemokines, cytokines, hormones, and engineered artificial small molecule ligands.

3. The chimeric immunoreceptor of claim 1, wherein the support region is selected from the group consisting of the constant regions of immunoglobins, CD8, and artificial linkers.

4. The chimeric immunoreceptor of claim 1, wherein the transmembrane region is a transmembrane domain of a leukocyte CD marker.

5. The chimeric immunoreceptor of claim 1, wherein the intracellular signalling domain is selected from the group consisting of the intracellular receptor signalling domain of the T cell antigen complex, Fcγ RIII costimulatory domains, CD28, DAP10 and CD2.

6. The chimeric immunoreceptor of claim 1, comprising the following linked elements, in the stated order:
   a) IL13(E13Y),
   b) an IgG4 constant region,
   c) a CD4 transmembrane domain, and

d) an intracellular T cell antigen receptor CD3 complex zeta chain.

7. The chimeric immunoreceptor of claim 1, wherein the immunoreceptor is expressed by a T lymphocyte cell line transformed with a DNA sequence encoding the immunoreceptor.

8. A method for treating human cancer, comprising administering to a human suffering from cancer a plurality of cells expressing an immunoreceptor of any of claims 1-7, wherein the soluble receptor ligand of the immunoreceptor is specific for a cancer-specific cell surface receptor.

9. The method of claim 8, wherein the the cancer-specific cell surface receptor is a cytokine receptor.

10. The method of claim 9, wherein the soluble receptor ligand is IL-13(E13Y).

Figure 1

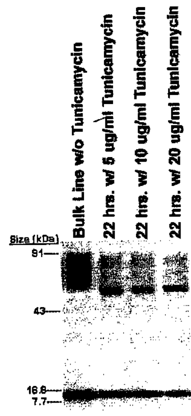

Figure 2

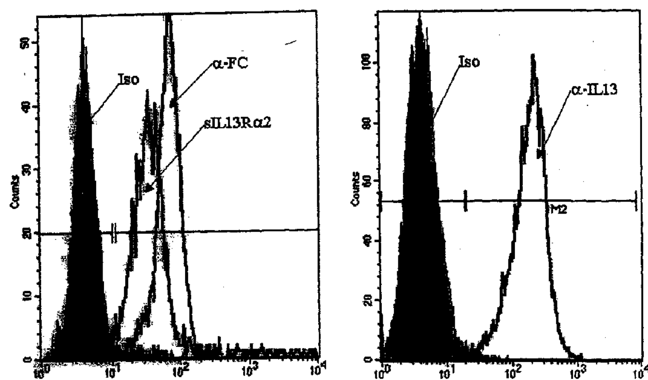

Figure 3

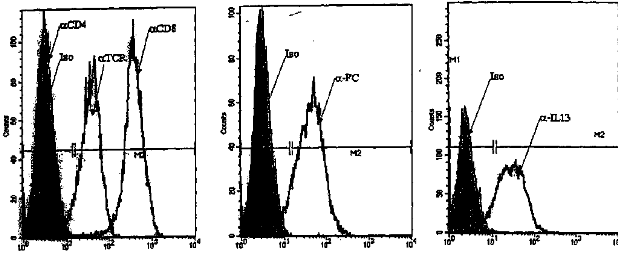

Figure 4a

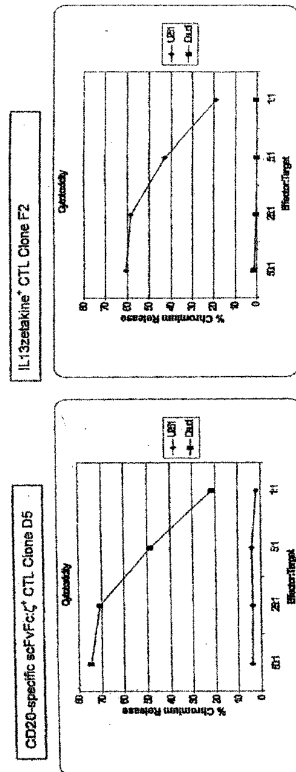

Figure 4b

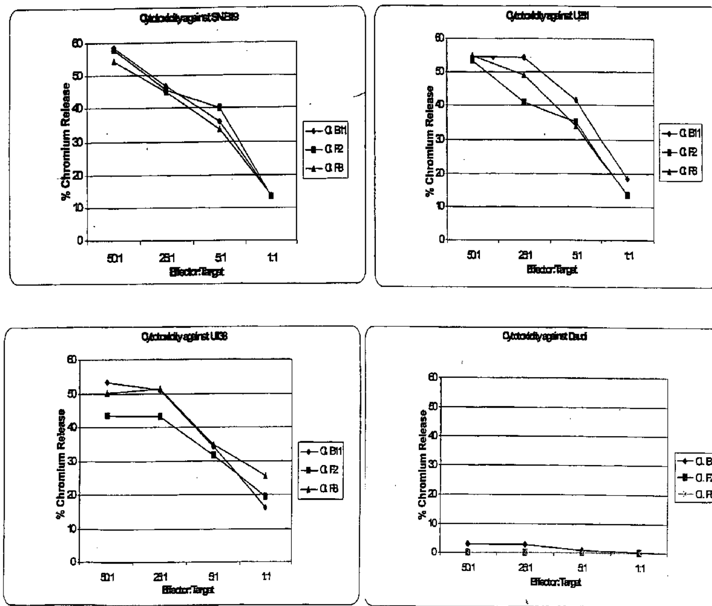

Figure 5

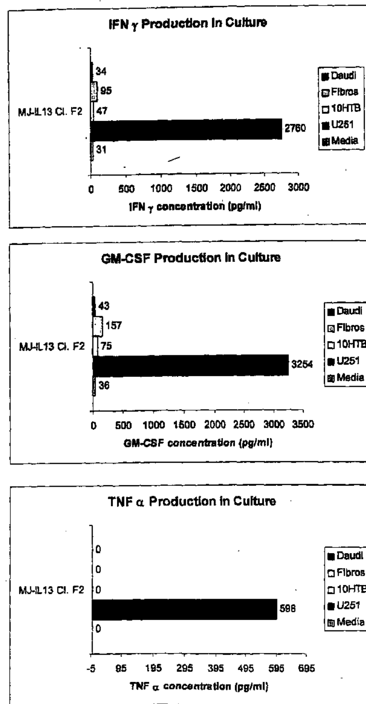

Figure 6

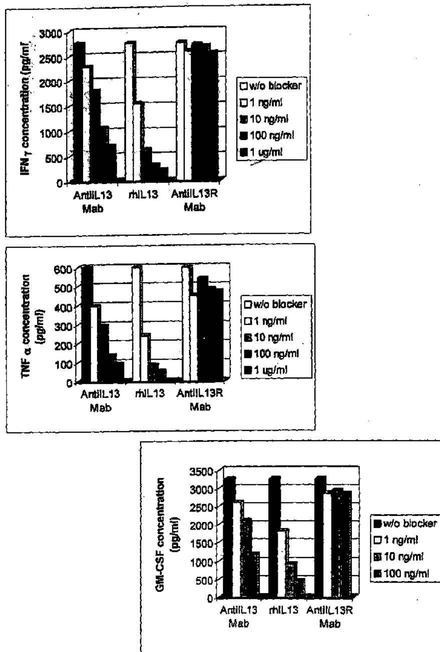

Figure 7a

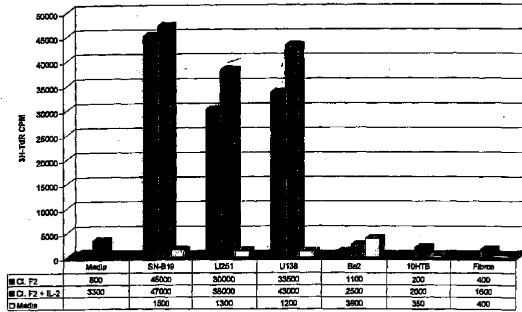

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Figure 7b
Inhibition of Glioma-Stimulated Proliferation of IL13zetakine CD8+ CTL by rhIL-13

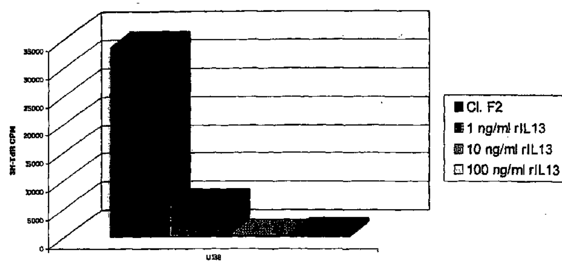


Figure 8

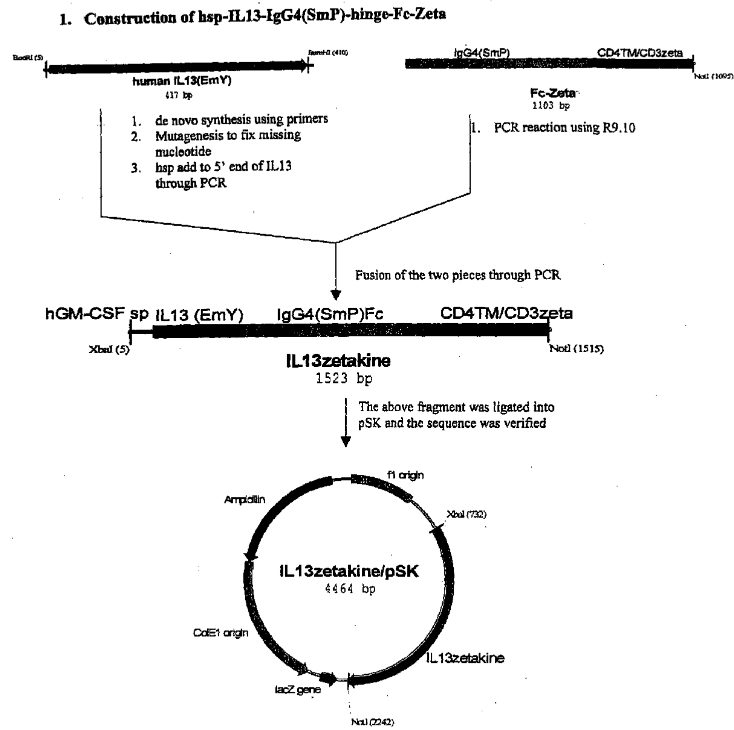
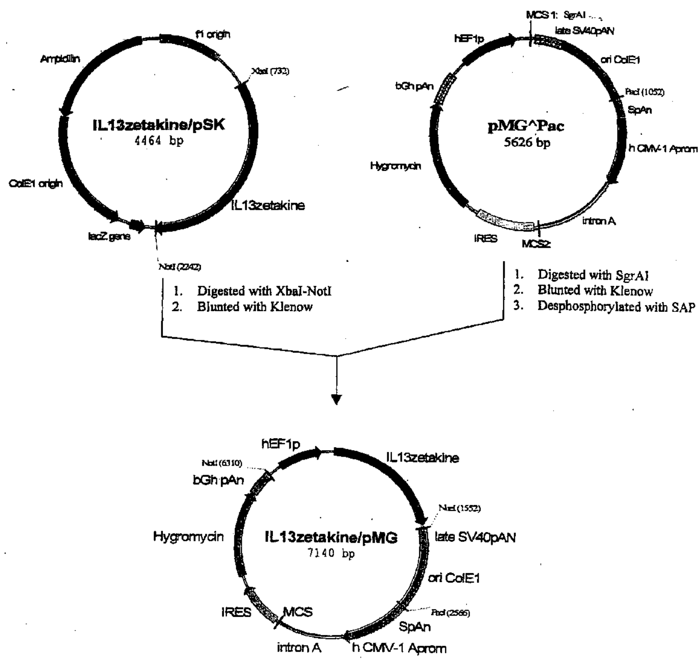


Fig. 8 (cont'd)

2. Construction of IL13 Fc:ζ/pMG^Pac



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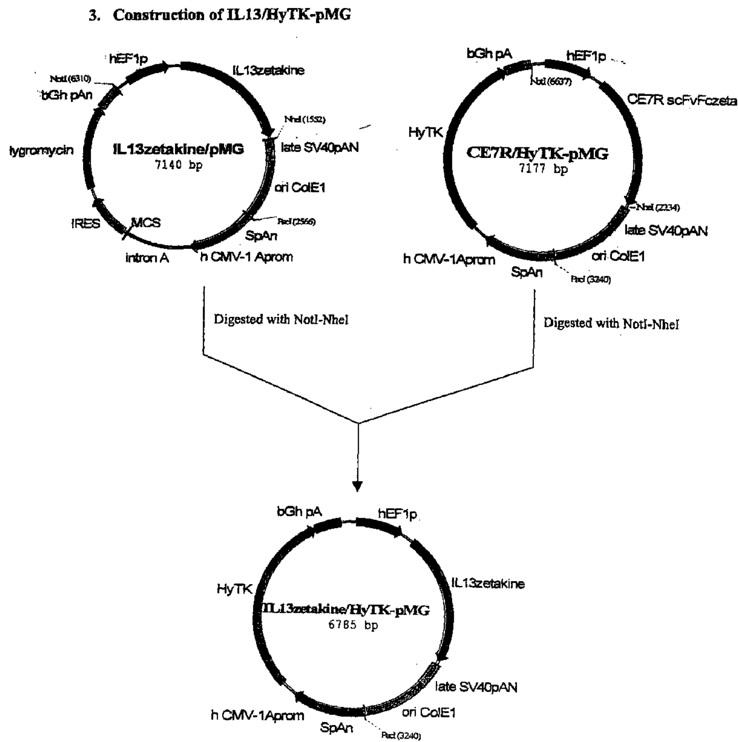
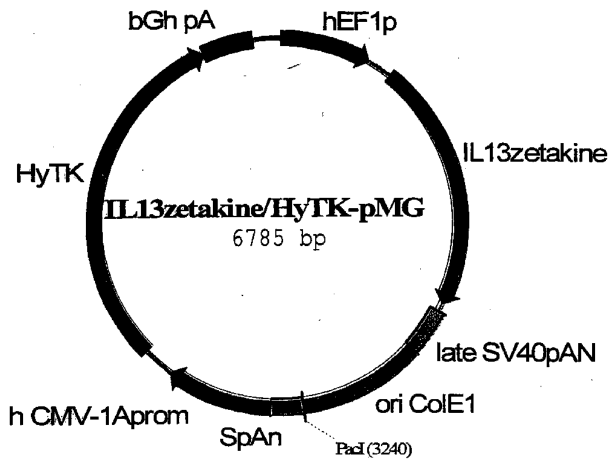


Figure 9



SEQUENCE LISTING

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Jensen, Michael

<120> Chimeric Immunoreceptor Useful in Treating Human Cancers

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<150> US 60/286,981
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【国際公開パンフレット（コレクトバージョン）】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 November 2002 (07.11.2002)

PCT

(10) International Publication Number
WO 02/088334 A1

(51) International Patent Classification[7]: C12N 5/06, 15/00, 5/00, 5/08, C07H 21/04, A01N 65/00

(21) International Application Number: PCT/US02/13500

(22) International Filing Date: 30 April 2002 (30.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/286,981 30 April 2001 (30.04.2001) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(48) Date of publication of this corrected version: 7 August 2003

(15) Information about Correction:
see PCT Gazette No. 32/2003 of 7 August 2003, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/088334 A1

(54) Title: CHIMERIC IMMUNORECEPTOR USEFUL IN TREATING HUMAN CANCERS

(57) Abstract: The present invention relates to chimeric transmembrane immunoreceptors, named "zetakines", comprised of an extracellular domain comprising a soluble receptor ligand linked to a support region capable of tethering the extracellular domain to a cell surface, a transmembrane region and an intracellular signalling domain. Zetakines, when expressed on the surface of T lymphocytes, direct T cell activity to those specific cells expressing a receptor for which the soluble receptor ligand is specific. Zetakine chimeric immunoreceptors represent a novel extension of antibody-based immunoreceptors for redirecting the antigen specificity of T cells, with application to treatment of a variety of cancers, particularly via the autocrin/paracrine cytokine systems utilized by human maligancy. In a preferred embodiment is a glioma-specific immunoreceptor comprising the extracellular targetting domain of the IL-13Rα2-specific IL-13 mutant IL-13(E13Y) linked to the Fc region of IgG, the transmembrane domain of human CD4, and the human CD3 zeta chain.

CHIMERIC IMMUNORECEPTOR USEFUL IN TREATING HUMAN CANCERS

TECHNICAL FIELD

[0001] This invention relates to cancer therapy, and the use of genetically-modified T-lymphocytes expressing chimeric immunoreceptors in the treatment of human brain tumors and other cancers.

BACKGROUND OF THE INVENTION

[0002] Primary brain tumors are the third leading contributor to cancer-related mortality in young adults, are the second leading contributor in children, and appear to be increasing in incidence both in the pediatric and geriatric population[1-4]. Gliomas are the most common type of primary brain tumors; 20,000 cases are diagnosed and 14,000 glioma-related deaths occur annually in the United States[5-8]. Gliomas are heterogeneous with respect to their malignant behavior and, in their most common and aggressive forms, anaplastic astrocytoma (AA-grade III) and glioblastoma multiforme (GBM-grade IV), are rapidly progressive and nearly uniformly lethal[9; 10]. Currently available therapeutic modalities have minimal curative potential for these high-grade tumors and often exacerbate the already severe morbidities imposed by their location in the central nervous system. Thus patients with malignant glioma are often struck in the most productive period of their lives; frequent deterioration of mental faculties and a high case:fatality ratio contribute to the unique personal and social impact of these tumors.

[0003] The cornerstones of oncologic management of malignant glioma are resection and radiation therapy[11-16]. With modern surgical and radiotherapeutic techniques the mean duration of survival has increased to 82 weeks for glioblastoma multiforme and 275 weeks for anaplastic astrocytoma, although 5-year survival rates have only increased from 3 to 6% for glioblastoma multiforme and 12.1% for anaplastic astrocytoma[6-8]. The major prognostic indicators for prolonged survival are younger age (<40yrs) and performance status (KPS score >70)[17]. Resections of >90% of bulky tumors are usually attempted provided that vital functional anatomy is spared. When used in conjunction with post-operative radiation therapy, the impact of extent of resection on duration of survival is less clear[18; 19]. The addition of chemotherapy to resection and radiation provides only marginal survival advantage to patients with anaplastic astrocytoma or glioblastoma multiforme[20-23]. Nitrosureas alone or in combination with procarbazine and vincristine are the conventional drugs used in the community and appear to improve the 1-year and 2-year survival rates by 15% without impacting on the overall median survival[24; 25]. More aggressive regimens incorporating platinum-based drugs and topoisomerase inhibitors are under investigation[26]. The role of high-dose chemotherapy with stem cell rescue has not been substantiated to date[27-29].

[0004] Approximately 80% of recurrent tumors arise from radiographically enhancing remnants of the original incompletely resected tumor[10; 30; 31]. Provided recurrences are unifocal and amenable in their location to aggressive re-resection, this approach can extend survival duration, particularly for patients with anaplastic astrocytoma and those glioblastoma multiforme patients with a KPS >70.[10] The median survival of recurrent glioblastoma multiforme patients treated with re-resection is 36 weeks[10; 30; 31]. Radiation therapy in the form of either brachytherapy or stereotactic radiosurgery may extend the duration of survival in re-resected recurrent glioblastoma multiforme patients by only 10-12 weeks[32]. The use of chemotherapy in the setting of recurrent disease should be in the context of available clinical trials, as its efficacy in this patient population is unsubstantiated.

[0005] The continued dismal prognosis of malignant glioma has prompted the clinical investigation of novel therapeutic entities, including, but not limited to: gene therapy (TK-suicide, antisense inhibition of tumor growth factor receptors, conditionally lethal viral vectors), immunotherapy (antibody, tumor cell vaccines, immunotoxins, adoptive transfer of activated lymphocytes), and anti-angiogenesis approaches[33-40]. The multiplicity of challenges faced in the development of effective adjuvant therapies for malignant glioma include the extensive infiltrative growth of tumor cells into normal brain parenchyma, the capacity of soluble factors elaborated from these tumors to attenuate the development of immune responses, and the difficulty of establishing clinically meaningful therapeutic ratios when administering therapeutics into the central nervous system (CNS). Early clinical evaluation of novel therapeutics is clearly indicated in this patient population.

[0006] Recently, receptors for transferrin and growth factors have been the subject of experimental glioma therapeutics utilizing ligands for these receptors conjugated to toxins or radionucleotides as a delivery system[41]. The specificity of this approach relies on the unique expression or over-expression of targeted receptors on glioma cells compared to normal brain. Interestingly, some receptor complexes for interleukins utilized by the immune system are expressed by gliomas, in particular high-affinity IL-13 receptors[42-48]. Unlike the IL-13 receptor trimolecular complex utilized by the immune system, which consists of the IL-13Rα1, the IL-4Rβ, and γc, glioma cells overexpress a unique IL-13Rα2 chain capable of binding IL-13 independently of the requirement for IL-4Rβ or γc[44; 49; 50]. Like its homologue IL-4, IL-13 has pleotrophic immunoregulatory activity outside the CNS[51-53]. Both cytokines stimulate IgE production by B lymphocytes and suppress pro-inflammatory cytokine production by macrophages. The immunobiology of IL-13 within the CNS is largely unknown.

[0007] Detailed studies by Debinski et al. using autoradiography with radiolabeled IL-13 have demonstrated abundant IL-13 binding on nearly all malignant glioma tissues studied[42; 45; 46; 48]. Moreover, the binding is highly homogeneous within tumor sections and from single cell analysis[46; 48]. Scatchard analyses of IL-13 binding to human glioma cell lines reveals on average 17,000-

28,000 binding sites/cell[45]. Molecular analysis using probes specific for IL-13Rα2 mRNA fail to demonstrate expression of the glioma-specific receptor by normal brain elements in all CNS anatomic locations[42; 43]. Furthermore, autoradiography with radiolabeled IL-13 failed to demonstrate detectable specific IL-13 binding in the CNS, suggesting that the shared IL13Rα1/IL-4β/γc receptor is also not expressed at detectable levels in the CNS[46]. These findings were independently verified using immunohistochemical techniques on non-pathologic brain sections with antibodies specific for IL-13Rα1 and IL-4β[54]. Thus IL-13Rα2 stands as the most specific and ubiquitously expressed cell-surface target for glioma described to date.

[0008] As a strategy to exploit the glioma-specific expression of IL-13Rα2 in the CNS, molecular constructs of the IL-13 cytokine have been described that fuse various cytotoxins (*Pseudomonas* exotoxin and *Diptheria* toxin) to its carboxyl terminal[55-58]. Internalization of these toxins upon binding to IL-13 receptors is the basis of the selective toxicity of these fusion proteins. These toxins display potent cytotoxicity towards glioma cells *in vitro* at picomolar concentrations[55]. Human intracranial glioma xenografts in immunodeficient mice can be eliminated by intratumor injection of the IL-13-toxin fusion protein without observed toxicities[55]. These studies support the initiation of clinical investigation utilizing IL-13-directed immunotoxins loco-regionally for malignant glioma.

[0009] However, the binding of IL-13-based cytotoxins to the broadly expressed IL-13Rα1/IL-4β/γc receptor complex has the potential of mediating untoward toxicities to normal tissues outside the CNS, and thus limits the systemic administration of these agents. IL-13 has been extensively dissected at the molecular level: structural domains of this cytokine that are important for associating with individual receptor subunits have been mapped[55; 58]. Consequently, selected amino acid substitutions in IL-13 have predictable effects on the association of this cytokine with its receptor subunits. Amino acid substitutions in IL-13's alpha helix A, in particular at amino acid 13, disrupt its ability to associate with IL-4β, thereby selectively reducing the affinity of IL-13 to the IL-13Rα1/IL-4β/γc receptor by a factor of five[55; 57; 58]. Surprisingly, binding of

mutant IL-13(E13Y) to IL-13Rα2 was not only preserved but increased relative to wild-type IL-13 by 50-fold. Thus, minimally altered IL-13 analogs can simultaneously increase IL-13's specificity and affinity for glioma cells via selective binding to IL-13Rα2 relative to normal tissues bearing IL-13Rα1/IL-4β/γc receptors.

[0010] Malignant gliomas represent a clinical entity that is highly attractive for immunotherapeutic intervention since 1) most patients with resection and radiation therapy achieve a state of minimal disease burden and 2) the anatomic location of these tumors within the confines of the CNS make direct loco-regional administration of effector cells possible. At least two pathologic studies have demonstrated that the extent of perivascular lymphocytic infiltration in malignant gliomas correlates with an improved prognosis[59-61]. Animal model systems have established that glioma-specific T cells, but not lymphokine-activated killer (LAK) cells, can mediate the regression of intracerebrally implanted gliomas[62-71]. T cells, unlike LAK cells, have the capacity to infiltrate into brain parenchyma and thus can target infiltrating tumor cells that may be distant from the primary tumor. Despite these findings, there is a substantial body of evidence that gliomas actively subvert immune destruction, primarily by the elaboration of immunosuppressive cytokines (TGF-β2) and prostaglandins, which, inhibit the induction/amplification of glioma-reactive T cell responses[72-74]. These findings have prompted the evaluation of *ex vivo* expanded anti-glioma effector cells for adoptive therapy as a strategy to overcome tumor-mediated limitations of generating responses *in vivo*.

[0011] At least ten pilot studies involving the administration of *ex vivo* activated lymphocytes to malignant glioma resection cavities have been reported to date[75-85]. Despite the variety of effector cell types (LAK, TILs, alloreactive CTLs), their heterogeneous composition/variability of composition from patient to patient, and the often modest *in vitro* reactivity of these effector cells towards glioma targets, these studies, in aggregate, report an approximate 50% response rate in patients with recurrent/refractory disease with anecdotal long-term survivors. These studies support the premise that a superior clinical effect of cellular immumotherapy for glioma might be expected with homogenous highly potent effector cells.

[0012] These pilot studies also report on the safety and tolerability of direct administration of *ex vivo* activated lymphocytes and interleukin-2 (IL-2), a T cell growth factor, into the resection cavity of patients with malignant glioma[75; 76; 78; 82; 86-92]. Even at large individual cell doses (>10^9 cells/dose), as well as high cumulative cell doses (>$27x10^9$ cells), toxicities are modest, and typically consist of grade II or less transient headache, nausea, vomiting and fever. As noted above, these studies also employed the co-administration of rhIL-2 to support the *in vivo* survival of transferred lymphocytes. Multiple doses given either concurrently with lymphocytes or sequentially after lymphocyte administration were tolerated at doses as high as $1.2x10^6$ IU/dose for 12-dose courses of IL-2 delivered every 48-hours.

[0013] Based on the findings outlined above, strategies to improve the anti-tumor potency of lymphocyte effector cells used in glioma immunotherapy are under development. One approach utilizes bi-specific antibodies capable of co-localizing and activating T lymphocytes via an anti-CD3 domain with glioma targets utilizing an epidermal growth factor receptor (EGFR) binding domain[93-96]. Preliminary clinical experience with this bi-specific antibody in combination with autologous lymphocytes suggests that T cells are activated *in situ* in the resection cavity. Targeting infiltrating tumor cells within the brain parenchyma, however, is a potentially significant limitation of this approach. T cells might have significantly increased anti-glioma activity if they are specific for target antigens expressed by gliomas. A growing number of human genes encoding tumor antigens to which T lymphocytes are reactive have been cloned, including the SART-1 gene, which appears to be expressed by nearly 75% of high-grade gliomas[97]. Both dendritic cell-based *in vitro* cell culture techniques, as well as tetramer-based T cell selection technologies are making feasible the isolation of antigen-specific T cells for adoptive therapy. Since antigens like SART-1 are recognized by T cells in the context of restricting HLA alleles, antigen-specific approaches will require substantial expansion in the number of antigens and restricting HLA alleles capable of presenting these antigens to be broadly applicable to the general population of glioma patients.

[0014] Chimeric antigen receptors engineered to consist of an extracellular single chain antibody (scFvFc) fused to the intracellular signaling domain of the T cell antigen receptor complex zeta chain (scFvFc:ζ) have the ability, when expressed in T cells, to redirect antigen recognition based on the monoclonal antibody's specificity[98]. The design of scFvFc:ζ receptors with target specificities for tumor cell-surface epitopes is a conceptually attractive strategy to generate antitumor immune effector cells for adoptive therapy as it does not rely on pre-existing anti-tumor immunity. These receptors are "universal" in that they bind antigen in a MHC independent fashion, thus, one receptor construct can be used to treat a population of patients with antigen-positive tumors. Several constructs for targeting human tumors have been described in the literature including receptors with specificities for Her2/Neu, CEA, ERRB-2, CD44v6, and epitopes selectively expressed on renal cell carcinoma[98-104]. These epitopes all share the common characteristic of being cell-surface moieties accessible to scFv binding by the chimeric T cell receptor. *In vitro* studies have demonstrated that both CD4+ and CD8+ T cell effector functions can be triggered via these receptors. Moreover, animal models have demonstrated the capacity of adoptively transferred scFvFc:ζ expressing T cells to eradicate established tumors[105]. The function of primary human T cells expressing tumor-specific scFvFc:ζ receptors have been evaluated *in vitro*; these cells specifically lyse tumor targets and secrete an array of pro-inflammatory cytokines including IL-2, TNF, IFN-γ, and GM-CSF[104]. Phase I pilot adoptive therapy studies are underway utilizing autologous scFvFc:ζ-expressing T cells specific for HIV gp120 in HIV infected individuals and autologous scFvFc:ζ-expressing T cells with specificity for TAG-72 expressed on a variety of adenocarcinomas, including breast and colorectal adenocarcinoma.

[0015] Investigators at City of Hope have engineered a CD20-specific scFvFc:ζ receptor construct for the purpose of targeting CD20+ B-cell malignancy and an L1-CAM-specific chimeric immunoreceptor for targeting neuroblastoma[106]. Preclinical laboratory studies have demonstrated the feasibility of isolating and expanding from healthy individuals and lymphoma patients CD8+ CTL clones that contain a single copy of unrearranged chromosomally integrated vector DNA and

express the CD20-specific scFvFc:ζ receptor[107]. To accomplish this, purified linear plasmid DNA containing the chimeric receptor sequence under the transcriptional control of the CMV immediate/early promoter and the NeoR gene under the transcriptional control of the SV40 early promoter was introduced into activated human peripheral blood mononuclear cells by exposure of cells and DNA to a brief electrical current, a procedure called electroporation. Utilizing selection, cloning, and expansion methods currently employed in FDA-approved clinical trials at the Fred Hutchinson Cancer Research Center, Seattle, Washington, gene modified CD8+ CTL clones with CD20-specific cytolytic activity have been generated from each of six healthy volunteers in 15 separate electroporation procedures. These clones when co-cultured with a panel of human CD20+ lymphoma cell lines proliferate, specifically lyse target cells, and are stimulated to produce cytokines.

SUMMARY OF THE INVENTION

[0016] The present invention relates to chimeric transmembrane immunoreceptors, named "zetakines," comprised of an extracellular domain comprising a soluble receptor ligand linked to a support region capable of tethering the extracellular domain to a cell surface, a transmembrane region and an intracellular signaling domain. Zetakines, when expressed on the surface of T lymphocytes, direct T cell activity to those cells expressing a receptor for which the soluble receptor ligand is specific. Zetakine chimeric immunoreceptors represent a novel extension of antibody-based immunoreceptors for redirecting the antigen specificity of T cells, with application to treatment of a variety of cancers, particularly via the autocrine/paracrine cytokine systems utilized by human malignancy.

[0017] In one preferred embodiment exploiting the tumor-restricted expression of IL-13Rα2 by malignant glioma and renal cell carcinoma as a target for cellular immunotherapy, a mutant of the IL-13 cytokine, IL-13(E13Y), having selective high-affinity binding to IL-13Rα2 has been converted into a type I transmembrane chimeric immunoreceptor capable of redirecting T cell antigen specificity to IL-13Rα2-expressing tumor cells. This embodiment of the zetakine consists of

extracellular IL-13(E13Y) fused to human IgG4 Fc, transmembrane CD4, and intracellular T cell antigen receptor CD3 complex zeta chain. Analogous immunoreceptors can be created that are specific to any of a variety of cancer cell types that selectively express receptors on their cell surfaces, for which selective ligands are known or can be engineered.

[0018] Bulk lines and clones of human T cells stably transformed to express such an immunoreceptor display redirected cytolysis of the cancer cell type to which they are specific, while showing negligible toxicity towards non-target cells. Such engineered T cells are a potent and selective therapy for malignancies, including difficult to treat cancers such as glioma.

BRIEF DESCRIPTION OF THE FIGURES

[0019] Figure 1: Results of a Western Blot showing that the IL13zetakine Chimeric Immunoreceptor is expressed as an intact glycosylated protein in Jurkat T cells.

[0020] Figure 2: Results of flow cytometric analysis showing that expressed IL13zetakine chimeric immunoreceptor trafficks to the cell-surface as a type I transmembrane protein.

[0021] Figure 3: Results of flow cytometric analysis showing the cell surface phenotype of a representative primary human IL13zetakine$^+$ CTL clone.

[0022] Figure 4: Results of a chromium release assays showing (a) that the IL13zetakine$^+$ CTL clone acquired glioma-specific re-directed cytolytic activity, and (b) the profile of anti-glioma cytolytic activity by primary human IL13zetakine$^+$ CD8$^+$ CTL clones was observed in glioma cells generally.

[0023] Figure 5: Results of *in vitro* stimulation of cytokine production, showing that IL13zetakine$^+$ CTL clones are activated for cytokine production by glioma stimulator cells.

[00024] Figure 6: Results of *in vitro* stimulation of cytokine production, showing the specific inhibition of IL13zetakine$^+$ CTL activation for cytokine production by anti-IL13R Mab and rhIL13.

[0025] Figure 7: Results of growth studies showing (a) that IL13zetakine^{+} CD8^{+} CTL cells proliferate upon co-culture with glioma stimulators, and (b) the inhibition of glioma-stimulated proliferation of IL13zetakine^{+} CD8^{+} CTL cells by rhIL-13.

[0026] Figure 8: Flow chart of the construction of IL13zetakine/HyTK-pMG.

[0027] Figure 9: Plasmid map of IL13zetakine/HyTK-pMG.

DETAILED DESCRIPTION

[0028] An ideal cell-surface epitope for tumor targeting with genetically-engineered re-directed T cells would be expressed solely on tumor cells in a homogeneous fashion and on all tumors within a population of patients with the same diagnosis. Modulation and/or shedding of the target molecule from the tumor cell membrane may also impact on the utility of a particular target epitope for re-directed T cell recognition. To date few "ideal" tumor-specific epitopes have been defined and secondary epitopes have been targeted based on either lack of expression on critical normal tissues or relative over-expression on tumors. In the case of malignant glioma, the intracavitary administration of T cells for the treatment of this cancer permits the expansion of target epitopes to those expressed on tumor cells but not normal CNS with less stringency on expression by other tissues outside the CNS. The concern regarding toxicity from cross-reactivity of tissues outside the CNS is mitigated by a) the sequestration of cells in the CNS based on the intracavitary route of administration and b) the low cell numbers administered in comparison to cell doses typically administered systemically.

[0029] The IL-13Rα2 receptor stands out as the most ubiquitous and specific cell-surface target for malignant glioma[47]. Sensitive autoradiographic and immunohistochemical studies fail to detect IL-13 receptors in the CNS[46; 48]. Moreover, mutation of the IL-13 cytokine to selectively bind the glioma-restricted IL-13Rα2 receptor is a further safeguard against untoward reactivity of IL-13-directed therapeutics against IL-13Rα1/IL-4β+ normal tissues outside the CNS[55; 57]. The potential utility of targeting glioma IL-13Rα2 the design and testing of a novel engineered chimeric immunoreceptor for re-directing the specificity of T cells that

consists of an extracellular IL-13 mutant cytokine (E13Y) tethered to the plasma membrane by human IgG4 Fc which, in turn, is fused to CD4TM and the cytoplasmic tail of CD3 zeta. This chimeric immunoreceptor has been given the designation of "IL-13 zetakine". The IL-13Rα2 receptor/IL-13(E13Y) receptor-ligand pair is an excellent guide for understanding and assessing the suitability of receptor-ligand pairs generally for use in zetakines. An ideal zetakine comprises an extracellular soluble receptor ligand having the properties of IL-13(E13Y) (specificity for a unique cancer cell surface receptor, *in vivo* stability due to it being derived from a naturally-occurring soluble cell signal molecule, low immunogenicity for the same reason). The use of soluble receptor ligands as distinct advantages over the prior art use of antibody fragments (such as the scFvFc immunoreceptors) or cell adhesion molecules, in that soluble receptor ligands are more likely to be stable in the extracellular environment, non-antigenic, and more selective.

[0030] Chimeric immunoreceptors according to the present invention comprise an extracellular domain comprised of a soluble receptor ligand linked to an extracellular support region that tethers the ligand to the cell surface via a transmembrane domain, in turn linked to an intracellular receptor signaling domain. Examples of suitable soluble receptor ligands include autocrine and paracrine growth factors, chemokines, cytokines, hormones, and engineered artificial small molecule ligands that exhibit the required specificity. Natural ligand sequences can also be engineered to increase their specificity for a particular target cell. Selection of a soluble receptor ligand for use in a particular zetakine is governed by the nature of the target cell, and the qualities discussed above with regard to the IL-13(E13Y) molecule, a preferred ligand for use against glioma. Examples of suitable support regions include the constant (Fc) regions of immunoglobins, human CD8∝, and artificial linkers that serve to move the targeting moiety away from the cell surface for improved access to receptor binding on target cells. A preferred support region is the Fc region of an IgG (such as IgG4). Examples of suitable transmembrane domains include the transmembrane domains of the leukocyte CD markers, preferably that of CD8.

Examples of intracellular receptor signaling domains are those of the T cell antigen receptor complex, preferably the zeta chain of CD3 also Fcγ RIII costimulatory signaling domains, CD28, DAP10, CD2, alone or in a series with CD3zeta.

[0031] In the IL-13 zetakine embodiment, the human IL-13 cDNA having the E13Y amino acid substitution was synthesized by PCR splice overlap extension. A full length IL-13 zetakine construct was assembled by PCR splice overlap extension and consists of the human GM-CSF receptor alpha chain leader peptide, IL-13(E13Y)-Gly-Gly-Gly, human IgG4 Fc, human CD4TM, and human cytoplasmic zeta chain. This cDNA construct was ligated into the multiple cloning site of a modified pMG plasmid under the transcriptional control of the human Elongation Factor-1alpha promoter (Invivogen, San Diego). This expression vector co-expresses the HyTK cDNA encoding the fusion protein HyTK that combines in a single molecule hygromycin phosphotransferase activity for *in vitro* selection of transfectants and HSV thymidine kinase activity for *in vivo* ablation of cells with ganciclovir from the CMV immediate/early promoter. Western blot of whole cell Jurkat lysates pre-incubated with tunicamycin, an inhibitor of glycosylation, with an anti-zeta antibody probe demonstrated that the expected intact 56-kDa chimeric receptor protein is expressed. This receptor is heavily glycosylated consistent with post-translational modification of the native IL-13 cytokine[108]. Flow cytometric analysis of IL-13 zetakine+ Jurkat cells with anti-human IL-13 and anti-human Fc specific antibodies confirmed the cell-surface expression of the IL-13 zetakine as a type I transmembrane protein.

[0032] Using established human T cell genetic modification methods developed at City of Hope[107], primary human T cell clones expressing the IL-13 zetakine chimeric immunoreceptor have been generated for pre-clinical functional characterization. IL-13 zetakine+ CD8+ CTL clones display robust proliferative activity in *ex vivo* expansion cultures. Expanded clones display re-directed cytolytic activity in 4-hr chromium release assays against human IL-13Rα2+ glioblastoma cell lines. The level of cytolytic activity correlates with levels of zetakine expression on T cells and IL-13Rα2 receptor density on glioma target cells. In addition to killing, IL-13 zetakine+ clones are activated for cytokine

secretion (IFN-γ, TNF-α, GM-CSF). Activation was specifically mediated by the interaction of the IL-13 zetakine with the IL-13Rα2 receptor on glioma cells since CTL clones expressing an irrelevant chimeric immunoreceptor do not respond to glioma cells, and, since activation can be inhibited in a dose-dependent manner by the addition to culture of soluble IL-13 or blocking antibodies against IL-13 on T cell transfectants and IL-13Rα2 on glioma target cells. Lastly, IL-13 zetakine-expressing CD8+ CTL clones proliferate when stimulated by glioma cells in culture. IL-13 zetakine+ CTL clones having potent anti-glioma effector activity will have significant clinical activity against malignant gliomas with limited collateral damage to normal CNS.

[0033] An immunoreceptor according to the present invention can be produced by any means known in the art, though preferably it is produced using recombinant DNA techniques. A nucleic acid sequence encoding the several regions of the chimeric receptor can prepared and assembled into a complete coding sequence by standard techniques of molecular cloning (genomic library screening, PCR, primer-assisted ligation, site-directed mutagenesis, etc.) . The resulting coding region is preferably inserted into an expression vector and used to transform a suitable expression host cell line, preferably a T lymphocyte cell line, and most preferably an autologous T lymphocyte cell line. A third party derived T cell line/clone, a transformed humor or xerogenic immunologic effector cell line, for expression of the immunoreceptor. NK cells, macrophages, neutrophils, LAK cells, LIK cells, and stem cells that differentiate into these cells, can also be used. In a preferred embodiment, lymphocytes are obtained from a patient by leukopharesis, and the autologous T cells are transduced to express the zetakine and administered back to the patient by any clinically acceptable means, to achieve anti-cancer therapy.

[0034] Suitable doses for a therapeutic effect would be between about 10^6 and about 10^9 cells per dose, preferably in a series of dosing cycles. A preferred dosing regimen consists of four one-week dosing cycles of escalating doses, starting at about 10^7 cells on Day 0, increasing incrementally up to a target dose of about 10^8 cells by Day 5. Suitable modes of administration include intravenous,

subcutaneous, intracavitary (for example by reservoir-access device), intraperitoneal, and direct injection into a tumor mass.

[0035] The following examples are solely for the purpose of illustrating one embodiment of the invention.

EXAMPLE 1: Construction of an immunoreceptor coding sequence

[0036] The coding sequence for an immunoreceptor according to the present invention was constructed by de novo synthesis of the IL13(E13Y) coding sequence using the following primers (see Fig. 8 for a flow chart showing the construction of the immunoreceptor coding sequence and expression vector):

IL13P1:

EcoRI
TAT**GAATTC**ATGGCGCTTTTGTTGACCACGGTCATTGCTCTCACTTGCC
TTGGCGGCTTTGCCTCCCCAGGCCCTGTGCCTCCCTCTACAGCCCTCAG
GTAC [SEQ ID NO. 1]

IL13P2:
GTTGATGCTCCATACCATGCTGCCATTGCAGAGCGGAGCCTTCTGGTTC
TGGGTGATGTTGACCAGCTCCTCAATGAGGTACCTGAGGGCTGTAGAG
GGAG [SEQ ID NO. 2]

IL13P3:
CTCTGGGTCTTCTCGATGGCACTGCAGCCTGACACGTTGATCAGGGATT
CCAGGGCTGCACAGTACATGCCAGCTGTCAGGTTGATGCTCCATACCAT
GC [SEQ ID NO. 3]

IL13P4:
CCTCGATTTTGGTGTCTCGGACATGCAAGCTGGAAAACTGCCCAGCTGA
GACCTTGTGCGGGCAGAATCCGCTCAGCATCCTCTGGGTCTTCTCGATG
GC [SEQ ID NO. 4]

IL13P5:

BamHI
TC**GGATCC**TCAGTTGAACCGTCCCTCGCGAAAAAGTTTCTTTAAATGTA
AGAGCAGGTCCTTTACAAACTGGGCCACCTCGATTTTGGTGTCTCGG
[SEQ ID NO. 5]

[0037] The final sequence (417bp) was end-digested with EcoRI-BamHI, and ligated into the plasmid pSK (stratagene, LaJolla, CA) as ligation 312#3. Ligation 312#3 was mutagenized (stratagene kit, per manufacturer's instructions) to fix a

deleted nucleotide using the primers 5': IL13 312#3 mut5-3 (CAACCTGACAGCTGGCATGTACTGTGCAGCCCTGGAATC [SEQ ID NO. 6]) and 3':IL13 312#3 mut3-5 (GATTCCAGGGCTGCACAGTACATGCCAGCTGTCAGGTTG [SEQ ID NO. 7]), and ligation 312#3 as a template, to form ligation 348#1 (IL13zetakine/pSK).

[0038] The coding Human GM-CSFR alpha chain Signal Peptide (hsp) coding sequence was fused to the 5' end of IL13(E13Y) by standard PCR splice overlap extension. The hsp sequence (101 bp) was obtained from the template ligation 301#10 (hsp/pSK) (human GCSF receptor α-chain leader sequence from human T cell cDNA), using the primers 5':19hsp5' (ATC**TCTAGA**GCCGCCACCATGCTTCTCCTGGTGACAAGCCTTC [SEQ ID NO. 8]) (XbaI site highlighted in bold), and 3': hsp-IL13FR (GAGGGAGGCACAGGGCCTGGGATCAGGAGGAATG [SEQ ID NO. 9]). The IL-13 sequence (371 bp) was obtained using the primers 5': hsp-IL13FF (CATTCCTCCTGATCCCAGGCCCTGTGCCTCCCTC [SEQ ID NO. 10]) and 3': IL13-IgG4FR (GGGACCATATTTGGACTCGTTGAACCGTCCCTCGC [SEQ ID NO. 11]), and ligation 312#3 as template. Fusion was achieved using the 101 bp hsp sequence and 371 bp IL13 sequence thus obtained, and the primers 5': 19hsp5' and 3': IL13-IgG4FR, to yeild a 438 bp fusion hsp-IL13 sequence.

[0039] A sequence encoding the IgG4 Fc region IgG4m:zeta was fused to the 3' end of the hsp-IL13 fusion sequence using the same methods. The IgG4m:zeta sequence (1119 bp) was obtained using the primers 5': IL13-IgG4FF (GCGAGGGACGGTTCAACGAGTCCAAATATGGTCCC [SEQ ID NO. 12]) and 3': ZetaN3' (AT**GCGGCCGC**TCAGCGAGGGGGCAGG [SEQ ID NO. 13]) (NotI site highlighted in bold), using the sequence R9.10 (IgG4mZeta/pSK) as template. The 1119 bp IgG4m:zeta sequence was fused to the hsp-IL13 fusion sequence using the respective sequences as templates, and the primers 5': 19hsp5' and 3': ZetaN3', to yeild a 1522 bp hsp-IL13-IgG4m:zeta fusion sequence. The ends were digested with XbaI-NotI, and ligated into pSK as ligation 351#7, to create the plasmid IL13zetakine/pSK (4464 bp).

EXAMPLE 2: Construction of expression vector

[0040] An expression vector containing the IL13 zetakine coding sequence was created by digesting the IL13zetakine/pSK of Example 1 with XbaI-NotI, and creating blunt ends with Klenow, and ligating the resulting fragment into the plasmid pMG^Pac (Invirogen) (first prepared by opening with SgrAI, blunting with Klenow, and dephosphorylation with SAP), to yield the plasmid IL13zetakine/pMG. See Fig. 8. The hygromycin resistance region of IL13zetakine/pMG was removed by digestion with NotI-NheI, and replaced by the selection/suicide fusion HyTK, obtained from plasmid CE7R/HyTK-pMG (Jensen, City of Hope) by digestion with NotI-NheI, to create the expression vector IL13zetakine/HyTK-pMG (6785 bp). This plasmid comprises the Human Elongation Factor-1α promoter (hEF1p) at bases 6-549, the IL13zetakine coding sequence at bases 692-2185, the Simian Virus 40 Late polyadenylation signal (Late SV40pAN) at bases 2232-2500, a minimal *E. coli* origin of replication (Ori ColE1) at bases 2501-3247, a synthetic poly A and Pause site (SpAN) at bases 3248-3434, the Immeate-early CMV enhancer/promoter (h CMV-1Aprom) at bases 3455-4077, the Hygromycin resistance-Thymidine kinase coding region fusion (HyTK) at bases 4259-6334, and the bovine growth hormone polyadenylation signal and a transcription pause (BGh pAn) at bases 6335-6633. The plasmid has a PacI linearization site at bases 3235-3242. The hEF1p and IL13zetakine elements derived from IL13zetakine/pMG, and the remaining elements derived from CE7R/HyTk-pMG (and with the exception of the HyTK element, ultimately from the parent plasmid pMG^Pac). In sum, IL13zetakine/HyTK-pMG is a modified pMG backbone, expressing the IL13zetakine gene from the hEF1 promoter, and the HyTK fusion from the h CMV-1A promoter. A map of the plasmid IL13zetakine/HyTK-pMG appears in Fig. 9.

EXAMPLE 3: Expression of the immunoreceptor

[0041] Assessment of the integrity of the expressed construct was first delineated by Wester blot probed with an anit-zeta antibody of whole cell lysates derived from Jurkat T cell stable transfectants[107] cocultured in the presence or absence of

tunicamycin, an inhibitor of glycosylation. Fig. 1. Jurkat T cell stable transfectants (Jurkat-IL13-pMG bulk line) were obtained by electroporating Jurkat T cells with the IL13zetakine/HyTK-pMG expression vector, followed by selection and expansion of positive transfectants. $2x10^6$ cells from the Jurkat-IL13-pMG bulk line were plated per well in a 24-well plate with or without 5 μg/ml, 10 μg/ml, or 20 μg/ml Tunicamycin. The plate was incubated at 37°C for 22 hrs. Cells were harvested from each well, and each sample was washed with PBS and resuspended in 50 μl RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 tablet/10ml Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN). Samples were incubated on ice for 30 minutes then disrupted by aspiration with syringe with 21 gauge needle then incubated on ice for an additional 30 minutes before being centrifuged at 4°C for 20 minutes at 14,000 rpm. Samples of centrifuged lysate supernatant were harvested and boiled in an equal volume of sample buffer under reducing conditions, then subjected to SDS-PAGE electrophoresis on a 12% acrylamide gel. Following transfer to nitrocellulose, membrane was allowed to dry O/N at 4°C. Next morning, membrane was blocked in a Blotto solution containing 0.04 gm/ml non-fat dried milk in T-TBS (0.02% Tween 20 in Tris buffered saline pH 8.0) for 1 hour. Membrane was then incubated with primary mouse anti-human CD3ζ monoclonal antibody (Pharmingen, San Diego, CA) at a concentration of 1 μg/ml for 2 hours, washed, and then incubated with a 1:3000 dilution (in Blotto solution) of goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Bio-Rad ImmunoStar Kit, Hercules, CA) for 1 hour. Prior to developing, membrane was washed 4 additional times in T-TBS, and then incubated with 3 ml of phosphatase substrate solution (Biorad ImmunoStar Kit, Hercules, CA) for 5 minutes at room temperature. Membrane was then covered with plastic, and exposed to x-ray film. Consistant with the known glycosylation pattern of wild-type human IL-13, the electrophoretic mobility of expressed IL-13(E13Y) zetakine is demonstrative of a heavily glycosylated protein which, when expressed in the presence of tunicamycin, is reduced to an amino acid backbone of approximately 54 kDa.

[0042] The IL-13(E13Y) zetakine traffics to the cell surface as a homodimeric type I transmembrane protein, as evidenced by flow cytometric analysis of transfectants with a phycoerythrin (PE)-conjugated anti human-IL13 monoclonal antibody and a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Fc (gamma) fragment-specific $F(ab')_2$ antibody. Fig. 2. Jurkat IL13zetakine-pMG transfectants were stained with anti-human Fc(FITC) antibody (Jackson ImmunoResearch, West Grove, PA), recombinant human IL13Rα2/human IgG1 chimera (R&D Systems, Minneapolis, MN) followed by FITC-conjugated anti human-IgG1 monoclonal antibody (Sigma, St. Louis, MO), and an anti-IL13(PE) antibody (Becton Dickinson, San Jose, CA) for analysis of cell surface chimeric receptor expression. Healthy donor primary cells were also stained with FITC-conjugated anti-CD4, anti-CD8, anti-TCR, and isotype control monoclonal antibodies (Becton Dickinson, San Jose, CA) to assess cell surface phenotype. For each stain, 10^6 cells were washed and resuspended in 100μl of PBS containing 2% FCS, 0.2 mg/ml NaN_3, and 5 μl of stock antibody. Following a 30 minute incubation at 4°C, cells were washed twice and either stained with a secondary antibody, or resuspended in PBS containing 1% paraformaldehyde and analyzed on a FACSCaliber cytometer.

EXAMPLE 4: Binding of IL13(E13Y) zetakine to IL13Rα2 receptor

[0043] IL-13(E13Y), tethered to the cell membrane by human IgG4 Fc (i.e., IL13(E13Y) zetakine), is capable of binding to its target IL13Rα2 receptor as assessed by flow cytometric analysis using soluble IL13Rα2-Fc fusion protein. Fig. 3. Cloned human PBMC IL13zetakine-pMG transfectants were obtained by electroporating PBMC with the IL13zetakine/HyTK-pMG expression vector, followed by selection and expansion of positive transfectants[107]. IL13zetakine$^+$ CTL clonal cells were stained with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Fc (gamma) fragment-specific $F(ab')_2$ (Jackson ImmunoResearch, West Grove, PA), recombinant human IL13Rα2/human IgG1 chimera (R&D Systems, Minneapolis, MN) followed by FITC-conjugated anti human-IgG1 monoclonal antibody (Sigma, St. Louis, MO), and a phycoerythrin

(PE)-conjugated anti human-IL13 monoclonal antibody (Becton Dickinson, San Jose, CA) for analysis of cell surface chimeric receptor expression. Healthy donor primary cells were also stained with FITC-conjugated anti-CD4, anti-CD8, anti-TCR, and isotype control monoclonal antibodies (Becton Dickinson, San Jose, CA) to assess cell surface phenotype. For each stain, 10^6 cells were washed and resuspended in 100μl of PBS containing 2% FCS, 0.2 mg/ml NaN_3, and 5 μl of antibody. Following a 30 minute incubation at 4°C, cells were washed twice and either stained with a secondary antibody, or resuspended in PBS containing 1% paraformaldehyde and analyzed on a FACSCaliber cytometer.

[0044] Next, the immunobiology of the IL-13(E13Y) zetakine as a surrogate antigen receptor for primary human T cells was evaluated. Primary human T cells were electroporated with the plasmid expression vector. Positive transformants were selected with hygromycin, cloned in limiting dilution, then expanded by recursive stimulation cyles with OKT3, IL-2 and irradiated feeder cells. Clones demonstrating IL 13zetakine expression by Western blot and FACS were then subjected to functional evaluation in 4-hr chromium release assays against a variety of IL-13$\alpha2^+$/CD20$^-$ glioma cell lines (U251, SN-B19, U138), and the IL-13α^-/CD20$^+$ B cell lyphocyte line Daudi). These tests showed that IL13zetakine conferred cytolytic activity that was specific for glioma cells (Fig. 4a), and that this specific cytolytic activity is present for glioma cells as a class (Fig. 4b). The cytolytic activity of MJ-IL13-pMG clones was assayed by employing ^{51}Cr-labeled SN-B19, U251, and U138 glioma cell lines (IL13α2+/CD20-) and Daudi (CD20+/IL13α2-) as targets. MJ-IL13 effectors were assayed 8-12 days following stimulation. Effectors were harvested, washed, and resuspeded in assay media: $2.5\text{x}10^5$, $1.25\text{x}10^5$, $2.5\text{x}10^4$, and $5\text{x}10^3$ effectors were cultured in triplicate at 37°C for 4 hours with $5\text{x}10^3$ target cells in 96-well V-bottom microtiter plates. After incubation, 100μl aliquots of cell-free supernatant were harvested and ^{51}Cr in the supernatants was assayed with a γ-counter. Percent specific cytolysis was calculated as follows:

$$\frac{(\text{Experimental } ^{51}\text{Cr release}) - (\text{control } ^{51}\text{Cr release})}{(\text{Maximum } ^{51}\text{Cr release}) - (\text{control } ^{51}\text{Cr release})} \times 100$$

Control wells contained target cells incubated in the presence of target cells alone. Maximum ^{51}Cr release was determined by measuring the ^{51}Cr released by labeled target cells in the presence of 2% SDS. Bulk lines of stabley transfected human T cells consisting of approximately 40% IL-13(E13Y) zetakine^{+} TCRα/β^{+} lymphocytes displayed re-directed cytolysis specific for 13R$\alpha2^{+}$ glioma targets in 4-hr chromium release assays (>50% specific lysis at E:T ratios of 25:1), with negligable acitivity against IL-13R$\alpha2^{-}$ targets (<8% specific lysis at E:T ratios of 25:1). IL-13(E13Y) zetakine^{+}CD8^{+}TCRα/β^{+} CTL clones selected on the basis of high-level binding to anti-IL-13 antibody also display redirected IL13Rα2-specific glioma cell killing. Fig. 4b.

[0045] IL-13 zetakine-expressing CD8^{+} CTL clones are activated and proliferate when stimulated by glioma cells in culture. Figs. 5-7. MJ-IL13-pMG Cl. F2 responder cells expressing the IL13 zetakine were evaluated for receptor-mediated triggering of IFNγ, GM-CSF, and TNFα production *in vitro*. 2×10^{6} responder cells were co-cultured in 24-well tissue culture plates with 2×10^{5} irradiated stimulator cells (Daudi, Fibroblasts, Neuroblastoma 10HTB, and glioblastoma U251) in 2 ml total. Blocking rat anti-human-IL13 monoclonal antibody (Pharmingen, San Diego, CA), recombinant human IL13 (R&D Systems, Minneapolis, MN), and IL13Rα2-specific goat IgG (R&D Systems, Minneapolis, MN) were added to aliquots of U251 stimulator cells (2×10^{5}/ml) at concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 μg/ml, 30 minutes prior to the addition of responder cells. Plates were incubated for 72 hours at 37° C, after which time culture supernatants were harvested, aliquoted, and stored at -70°C. ELISA assays for IFNγ, GM-CSF, and TNFα were carried out using the R&D Systems (Minneapolis, MN) kit per manufacturer's instructions. Samples were tested in duplicate wells undiluted or diluted at 1:5 or 1:10. The developed ELISA plate was evaluated on a microplate reader and cytokine concentrations determined by extrapolation from a standard curve. Results are reported as picograms/ml, and show strong activation for cytokine production by glioma stimulator cells. Fig. 5, Fig. 6.

[0046] Lastly, IL-2 independent proliferation of IL13zetakine^{+} CD8^{+} CTL was observed upon co-cultivation with glioma stimulators (Fig. 7a), but not with IL13

Rα2 stimulators. Proliferation was inhibited by the addition of rhIL-13 antibody (Fig. 7b), showing that the observed proliferation was dependant on binding of zetakine to the IL-13Rα2 glioma cell-sepcific receptor.

EXAMPLE 5: Preparation of IL-13 zetakine$^+$ T cells suitable for therapeutic use

[0047] The mononuclear cells are separated from heparinized whole blood by centrifugation over clinical grade Ficoll (Pharmacia, Uppsula, Sweden). PBMC are washed twice in sterile phosphate buffered saline (Irvine Scientific) and suspended in culture media consisting of RPMI 1640 HEPES, 10% heat inactivated FCS, and 4 mM L-glutamine. T cells present in patient PBMC are polyclonally activated by addition to culture of Orthoclone OKT3 (30ng/ml). Cell cultures are then incubated in vented T75 tissue culture flasks in the study subject's designated incubator. Twenty-four hours after initiation of culture rhIL-2 is added at 25 U/ml.

[0048] Three days after the initiation of culture PBMC are harvested, centrifuged, and resuspended in hypotonic electroporation buffer (Eppendorf) at $20x10^6$ cells/ml. 25 μg of the plasmid IL13zetakine/HyTK-pMG of Example 3, together with 400 μl of cell suspension, are added to a sterile 0.2 cm electroporation cuvette. Each cuvette is subjected to a single electrical pulse of 250V/40μs and again incubated for ten minutes at RT. Surviving cells are harvested from cuvettes, pooled, and resuspended in culture media containing 25 U/ml rhIL-2. Flasks are placed in the patient's designated tissue culture incubator. Three days following electroporation hygromycin is added to cells at a final concentration of 0.2 mg/ml. Electroporated PBMC are cultured for a total of 14 days with media and IL-2 supplementation every 48-hours.

[0049] The cloning of hygromycin-resistant CD8+ CTL from electroporated OKT3-activated patient PBMC is initiated on day 14 of culture. Briefly, viable patient PBMC are added to a mixture of $100x10^6$ cyropreserved irradiated feeder PBMC and $20x10^6$ irradiated TM-LCL in a volume of 200ml of culture media containing 30 ng/ml OKT3 and 50 U/ml rhIL-2. This mastermix is plated into ten 96-well cloning plates with each well receiving 0.2 ml. Plates are wrapped in aluminum foil to decrease evaporative loss and placed in the patient's designated

tissue culture incubator. On day 19 of culture each well receives hygromycin for a final concentration of 0.2 mg/ml. Wells are inspected for cellular outgrowth by visualization on an inverted microscope at Day 30 and positive wells are marked for restimulation.

[0050] The contents of each cloning well with cell growth are individually transferred to T25 flasks containing $50x10^6$ irradiated PBMC, $10x10^6$ irradiated LCL, and 30ng/mlOKT3 in 25mls of tissue culture media. On days 1,3,5,7,9,11, and 13 after restimulation flasks receive 50U/ml rhIL-2 and 15mls of fresh media. On day 5 of the stimulation cycle flasks are also supplemented with hygromycin 0.2 mg/ml. Fourteen days after seeding cells are harvested, counted, and restimulated in T75 flasks containing 150 x 10^6 irradiated PBMC, 30 x 10^6 irradiated TM-LCL and 30 ng/ml OKT3 in 50 mls of tissue culture media. Flasks receive additions to culture of rhIL-2 and hygromycin as outlined above.

[0051] CTL selected for expansion for possible use in therapy are analyzed by immunofluorescence on a FACSCalibur housed in CRB-3006 using FITC-conjugated monoclonal antibodies WT/31 (aßTCR), Leu 2a (CD8), and OKT4 (CD4) to confirm the requisite phenotype of clones (αβTCR+, CD4-, CD8+, and IL13+). Criteria for selection of clones for clinical use include uniform TCR αβ+, CD4-, CD8+ and IL13+ as compared to isotype control FITC/PE-conjugated antibody. A single site of plasmid vector chromosomal integration is confirmed by Southern blot analysis. DNA from genetically modified T cell clones will be screened with a DNA probe specific for the plasmid vector. Probe DNA specific for the HyTK in the plasmid vector is synthesized by random priming with florescein-conjugated dUTP per the manufacture's instructions (Amersham, Arlington Hts, IL). T cell genomic DNA is isolated per standard technique. Ten micrograms of genomic DNA from T cell clones is digested overnight at 37°C then electrophoretically separated on a 0.85% agarose gel. DNA is then transferred to nylon filters (BioRad, Hercules, CA) using an alkaline capillary transfer method. Filters are hybridized overnight with probe in 0.5 M Na_2PO_4, pH 7.2, 7% SDS, containing 10 μg/ml salmon sperm DNA (Sigma) at 65°C. Filters are then washed four times in 40 mM Na_2PO_4, pH 7.2, 1% SDS at 65°C and then visualized using a

chemiluminescence AP-conjugated anti-florescein antibody (Amersham, Arlington Hts, IL). Criteria for clone selection is a single band unique vector band.

[0052] Expression of the IL-13 zetakine is determined by Western blot procedure in which chimeric receptor protein is detected with an anti-zeta antibody. Whole cell lysates of transfected T cell clones are generated by lysis of 2 x 10^7 washed cells in 1 ml of RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 tablet/10ml Complete Protease Inhibitor Cocktail (Boehringer Mannheim). After an eighty minute incubation on ice, aliquots of centrifuged whole cell lysate supernatant are harvested and boiled in an equal volume of loading buffer under reducing conditions then subjected to SDS-PAGE electrophoresis on a precast 12% acrylamide gel (BioRad). Following transfer to nitrocellulose, membranes are blocked in blotto solution containing .07 gm/ml non-fat dried milk for 2 hours. Membranes are washed in T-TBS (.05% Tween 20 in Tris buffered saline pH 8.0) then incubated with primary mouse anti-human CD3ζ monoclonal antibody 8D3 (Pharmingen, San Diego, CA) at a concentration of 1 µg/ml for 2 hours. Following an additional four washes in T-TBS, membranes are incubated with a 1:500 dilution of goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody for 1 hour. Prior to developing, membranes are rinsed in T-TBS then developed with 30 ml of "AKP" solution (Promega, Madison, WI) per the manufacturer's instructions. Criteria for clone selection is the presence of a chimeric zeta band.

[0053] CD8+ cytotoxic T cell clones expressing the IL-13 zetakine chimeric immunoreceptor recognize and lyse human glioblastoma target cells following interaction of the chimeric receptor with the cell surface target epitope in a HLA-unrestricted fashion. The requirements for target IL-13Rα2 epitope expression and class I MHC independent recognition will be confirmed by assaying each aßTCR+, CD8+, CD4-, IL-13 zetakine+ CTL clones against IL-13Rα2+ Daudi cell transfectants and IL-13Rα2- Daudi cells. T cell effectors are assayed 12-14 days following stimulation with OKT3. Effectors are harvested, washed, and resuspended in assay media; and Daudi cell transfectants expressing IL-13Rα2. 2.5x10^5, 1.25x10^5, 0.25x10^5, and 0.05x10^5 effectors are plated in triplicate at 37°C

for 4 hours with $5x10^3$ target cells in V-bottom microtiter plates (Costar, Cambridge, MA). After centrifugation and incubation, 100 μL aliquots of cell-free supernatant is harvested and counted. Percent specific cytolysis is calculated as follows:

$$\frac{(\text{Experimental } ^{51}\text{Cr release}) - (\text{control } ^{51}\text{Cr release})}{(\text{Maximum } ^{51}\text{Cr release}) - (\text{control } ^{51}\text{Cr release})} \times 100$$

Control wells contain target cells incubated in assay media. Maximum ^{51}Cr release is determined by measuring the ^{51}Cr content of target cells lysed with 2% SDS. Criteria for clone selection is >25% specific lysis of IL-13Rα2+ Daudi transfectants at an E:T ratio of 5:1 and a <10% lysis of parental Daudi at the same E:T ratio.

EXAMPLE 6: Treatment of human glioma using IL-13 zetakine-expressing T cells.

[0054] T cell clones genetically modified according to Example 5 to express the IL-13R zetakine chimeric immunoreceptor and HyTK are selected for:

a. TCRα/β$^+$, CD4$^-$, CD8$^+$, IL-13$^+$ cell surface phenotype as determined by flow cytometry.
b. Presence of a single copy of chromosomally integrated plasmid vector DNA as evidenced by Southern blot.
c. Expression of the IL-13 zetakine protein as detected by Western blot.
d. Specific lysis of human IL-13Rα2$^+$ targets in 4-hr chromium release assays.
e. Dependence on exogenous IL-2 for *in vitro* growth.
f. Mycoplasma, fungal, bacterial sterility and endotoxin levels <5 EU/ml.
g. *In vitro* sensitivity of clones to ganciclovir.

[0055] Peripheral blood mononuclear cells are obtained from the patient by leukapheresis, preferably following recovery from initial resection surgery and at a time at least three weeks from tapering off steroids and/or their most recent

systemic chemotherapy. The target leukapheresis mononuclear cell yield is $5x10^9$ and the target number of hygromycin-resistant cytolytic T cell clones is 25 with the expectation that at least five clones will be identified that meet all quality control parameters for ex-vivo expansion. Clones are cryopreserved and patients monitored by serial radiographic and clinical examinations. When recurrence of progression of disease is documented, patients undergo a re-resection and/or placement of a reservoir-access device (Omaya reservoir) for delivering T cells to the tumor resection cavity. Following recovery from surgery and tapering of steroids, if applicable, the patient commences with T cell therapy.

[0056] The patient receives a target of at least four one-week cycles of therapy. During the first cycle, cell dose escalation proceeds from an initial dose on Day 0 of 10^7 cells, followed by $5x10^7$ cells on Day 3 to the target dose of 10^8 cells on Day 5. Cycle 2 commences as early as one week from commencement of cycle 1. Those patients demonstrating tumor regression with residual disease on MRI may have additional courses of therapy beginning no earlier than Week 7 consisting of repetition of Cycles 3 and 4 followed by one week of rest/restaging provided these treatments are well tolerated (max. toxicities $<$grade 3) until such time that disease progression or a CR is achieved based on radiographic evaluation.

[0057] Cell doses are at least a log less than doses given in studies employing intracavitary LAK cells (individual cell doses of up to 10^9 and cumulative cell numbers as high as $2.75x10^{10}$ have been safety administered), ex vivo expanded TILs (up to 10^9 cells/dose reported with minimal toxicity) and allo-reactive lymphocyte (starting cell dose 10^8 with cumulative cell doses up to $51.5x10^8$) delivered to a similar patient population[75-85]. The rationale for the lower cell doses as proposed in this protocol is based on the increased *in vitro* reactivity/anti-tumor potency of IL-13 zetakine+ CTL clones compared to the modest reactivity profile of previously utilized effector cell populations. Low-dose repetitive dosing is favored to avoid potentially dangerous inflammatory responses that might occur with single large cell number instillations. Each infusion will consist of a single T cell clone. The same clone will be administered throughout a patient's treatment course. On the days of T cell administration, expanded clones are aseptically

processed by washing twice in 50cc of PBS then resuspended in pharmaceutical preservative-free normal saline in a volume that results in the cell dose for patient delivery in 2mls. T cells are instilled over 5-10 minutes. A 2ml PFNS flush will be administered over 5 minutes following T cells. Response to therapy is assessed by brain MRI +/- gandolinium, with spectroscopy.

[0058] Expected side-effects of administration of T cells into glioma resection cavities typically consist of self-limited nausea and vomiting, fever, and transient worsening of existing neurological deficits. These toxicities can be attributed to both the local inflammation/edema in the tumor bed mediated by T cells in combination with the action of secreted cytokines. These side-effects typically are transient and less than grade II in severity. Should patients experience more severe toxicities it is expected that decadron alone or in combination with ganciclovir will attenuate the inflammatory process and ablate the infused cells. The inadvertent infusion of a cell product that is contaminated with bacteria or fungus has the potential of mediating serious or life-threatening toxicities. Extensive pre-infusion culturing of the cell product is conducted to identify contaminated tissue culture flasks and minimize this possibility. On the day of re-infusion, gram stains of culture fluids, as well as, endotoxin levels are performed.

[0059] Extensive molecular analysis for expression of IL-13Rα2 has demonstrated that this molecule is tumor-specific in the context of the CNS[44; 46; 48; 54]. Furthermore, the only human tissue with demonstrable IL-13Rα2 expression appears to be the testis[42]. This tumor-testis restrictive pattern of expression is reminiscent of the growing number of tumor antigens (i.e. MAGE, BAGE, GAGE) expressed by a variety of human cancers, most notably melanoma and renal cell carcinoma[109-111]. Clinical experience with vaccine and adoptive T cell therapy has demonstrated that this class of antigens can be exploited for systemic tumor immunotherapy without concurrent autoimmune attack of the testis[112-114]. Presumably this selectively reflects the effect of an intact blood-testis barrier and an immunologically privileged environment within the testis. Despite the exquisite specificity of the mutant IL-13 targeting moiety, toxicities are theoretically possible if cells egress into the systemic circulation in sufficient numbers and recognize

tissues expressing the IL-13Rα1/IL-4β receptor. In light of this remote risk, as well as the possibility that instilled T cells in some patients may mediate an overly exuberant inflammatory response in the tumor bed, clones are equipped with the HyTK gene which renders T cells susceptible to *in vivo* ablation with ganciclovir[115-118]. Ganciclovir-suicide, in combination with an intra-patient T cell dose escalation strategy, helps minimize the potential risk to research participants.

[0060] Side effects associated with therapy (headache, fever, chills, nausea, etc.) are managed using established treatments appropriate for the condition. The patient receives ganciclovir if any new grade 3 or any grade 4 treatment-related toxicity is observed that, in the opinion of the treating physician, puts that patient at significant medical danger. Parentally administered ganciclovir is dosed at 10 mg/kg/day divided every 12 hours. A 14-day course will be prescribed but may be extended should symptomatic resolution not be achieved in that time interval. Treatment with ganciclovir leads to the ablation of IL-13 zetakine$^+$ HyTK$^+$ CD8$^+$ CTL clones. Patients should be hospitalized for the first 72 hours of ganciclovir therapy for monitoring purposes. If symptoms do not respond to ganciclovir within 48 hours additional immunosuppressive agents including but not limited to corticosteroids and cyclosporin may be added at the discretion of the treating physician. If toxicities are severe, decadron and/or other immunosuppressive drugs along with ganciclovir are used earlier at the discretion of the treating physician.

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I claim:

1. A chimeric immunoreceptor comprising the following linked elements, arranged in the stated order:
   a) an extracellular domain comprising a soluble receptor ligand,
   b) a support region capable of tethering the extracellular domain to a cell surface,
   c) a transmembrane region, and
   d) an intracellular signalling domain.

2. The chimeric immunoreceptor of claim 1, wherein the extracellular domain comprises a soluble ligand selected from the group consisting of autocrine growth factors, paracrine growth factors, chemokines, cytokines, hormones, and engineered artificial small molecule ligands.

3. The chimeric immunoreceptor of claim 1, wherein the support region is selected from the group consisting of the constant regions of immunoglobins, CD8, and artificial linkers.

4. The chimeric immunoreceptor of claim 1, wherein the transmembrane region is a transmembrane domain of a leukocyte CD marker.

5. The chimeric immunoreceptor of claim 1, wherein the intracellular signalling domain is selected from the group consisting of the intracellular receptor signalling domain of the T cell antigen complex, Fcγ RIII costimulatory domains, CD28, DAP10 and CD2.

6. The chimeric immunoreceptor of claim 1, comprising the following linked elements, in the stated order:
   a) IL13(E13Y),
   b) an IgG4 constant region,
   c) a CD4 transmembrane domain, and

d) an intracellular T cell antigen receptor CD3 complex zeta chain.

7. The chimeric immunoreceptor of claim 1, wherein the immunoreceptor is expressed by a T lymphocyte cell line transformed with a DNA sequence encoding the immunoreceptor.

8. A method for treating human cancer, comprising administering to a human suffering from cancer a plurality of cells expressing an immunoreceptor of any of claims 1-7, wherein the soluble receptor ligand of the immunoreceptor is specific for a cancer-specific cell surface receptor.

9. The method of claim 8, wherein the the cancer-specific cell surface receptor is a cytokine receptor.

10. The method of claim 9, wherein the soluble receptor ligand is IL-13(E13Y).

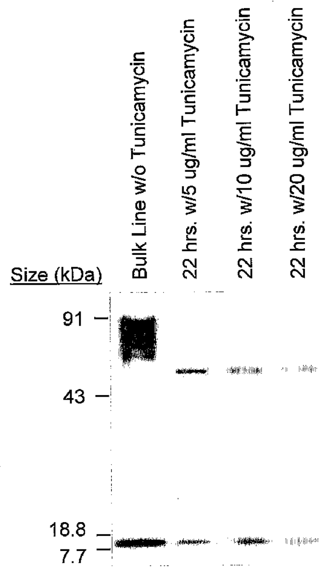


FIG.1

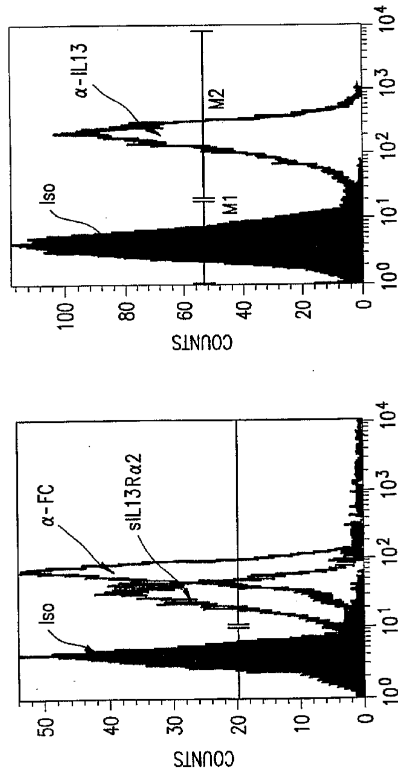
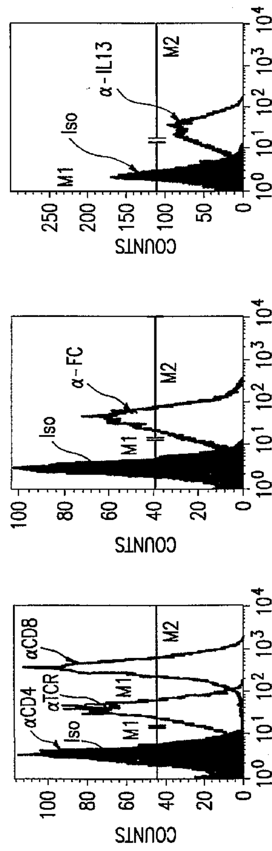


FIG. 2



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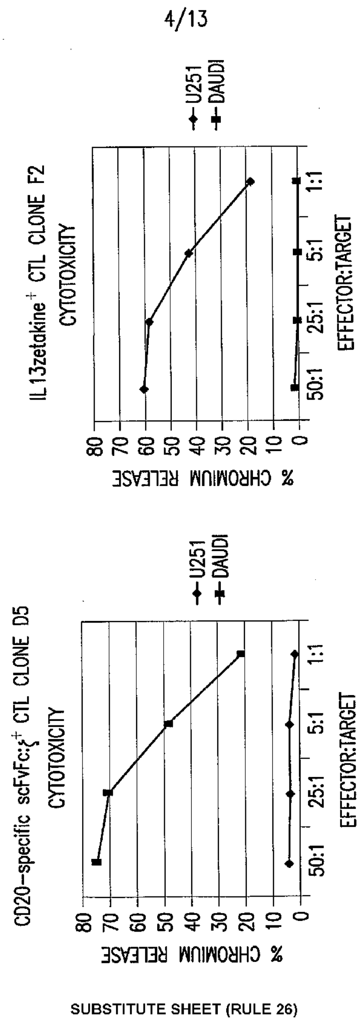

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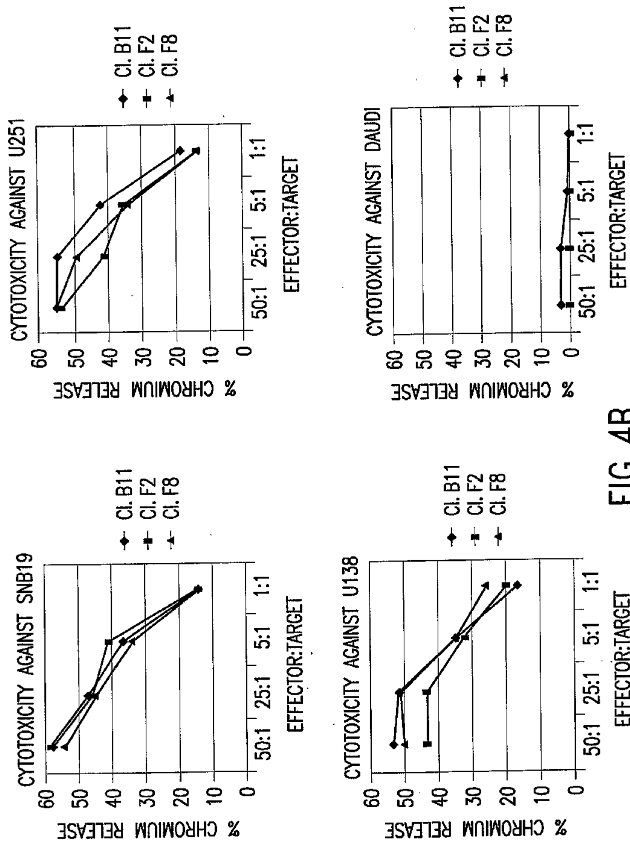


FIG.4B

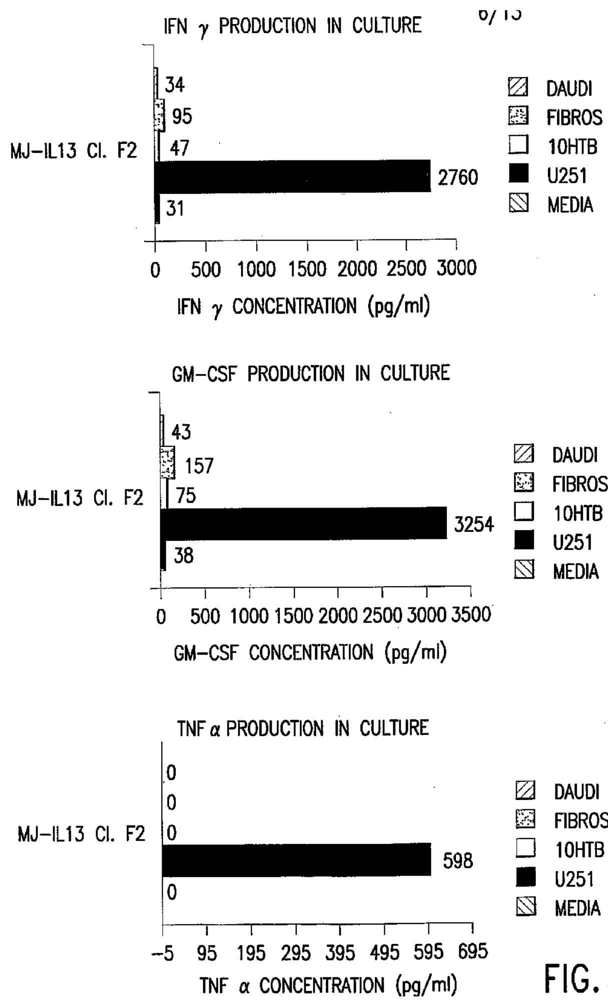


FIG.5

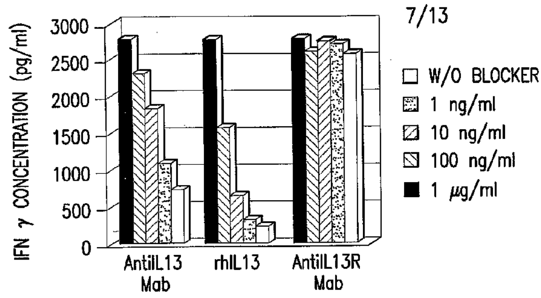


FIG. 6A

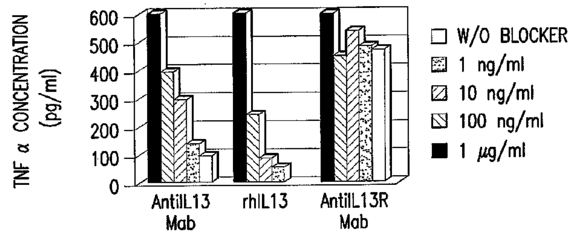


FIG. 6B

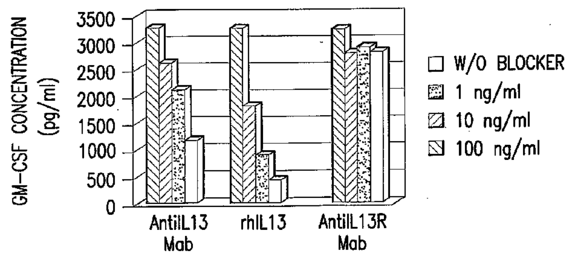
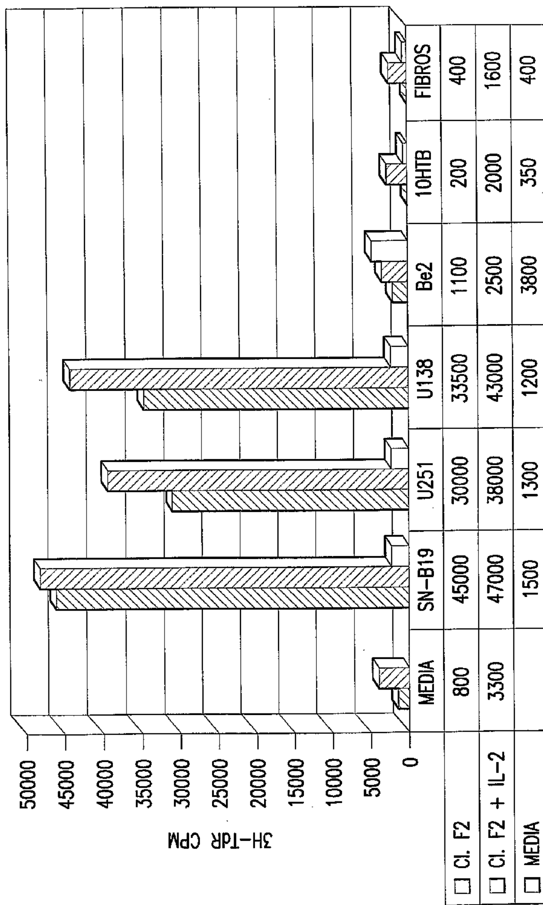


FIG. 6C



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□ Cl. F2 + IL-2	3300	47000	38000	43000	2500	2000	1600
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FIG. 7A

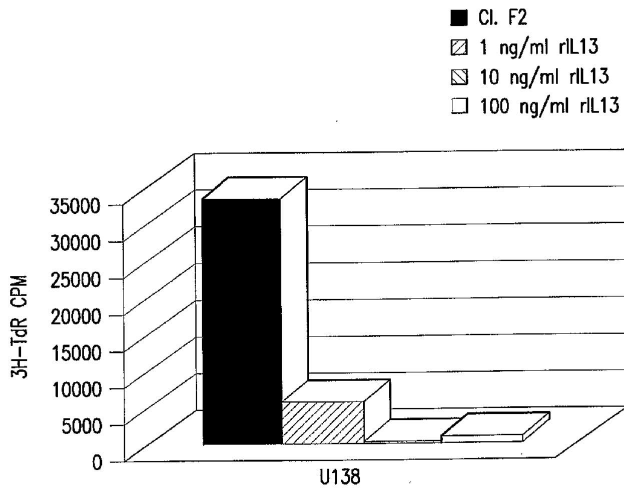

FIG.7B

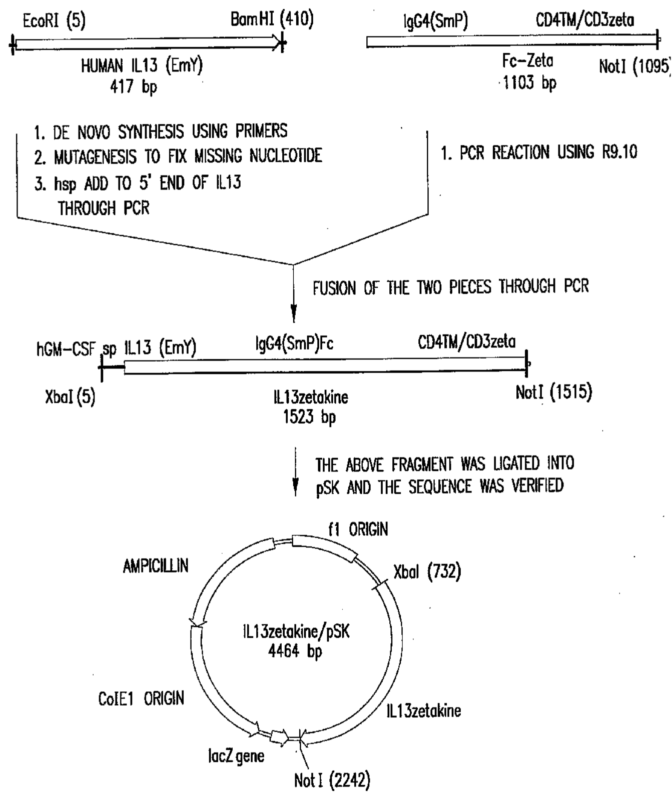

FIG.8A

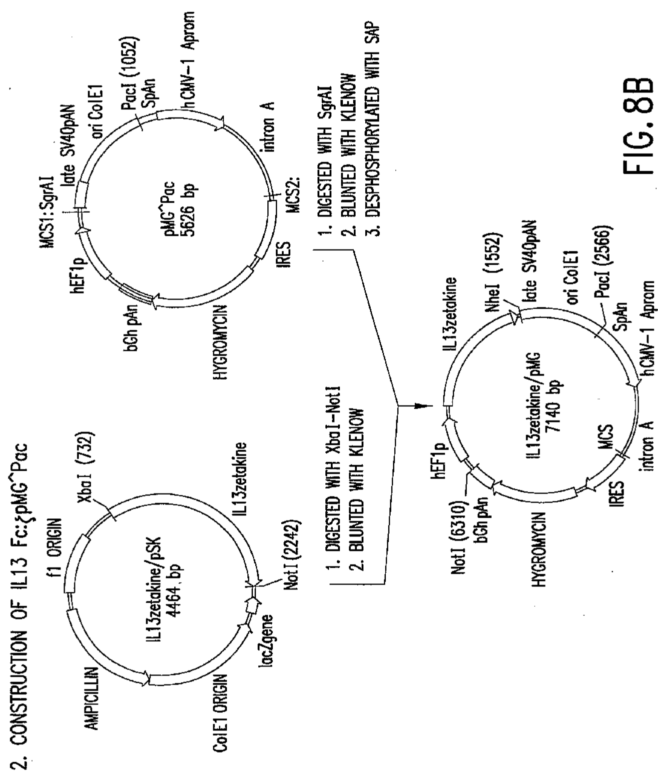

FIG. 8B

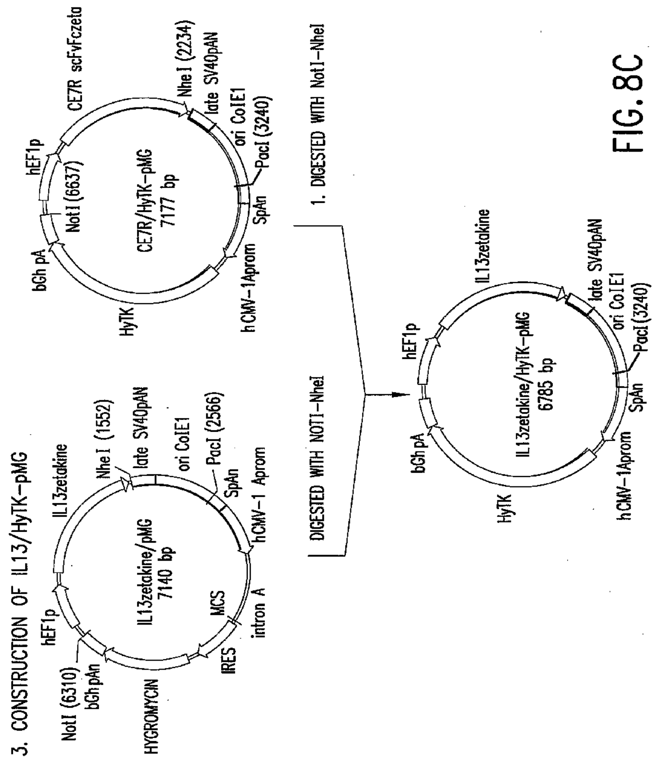

FIG. 8C

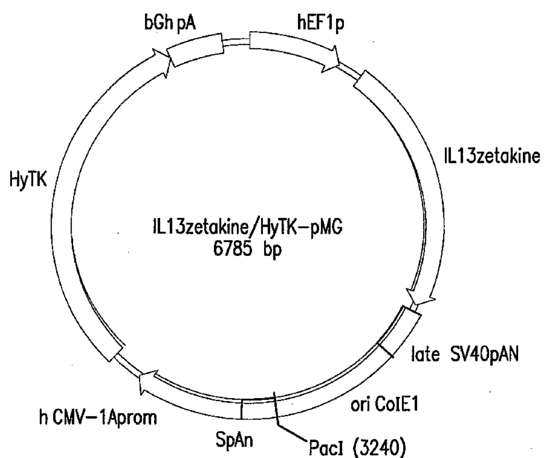

FIG. 9

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<120> Chimeric Immunoreceptor Useful in Treating Human Cancers

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<151> 2001-04-30

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【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/13500

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12N 5/06, 15/00, 5/00, 5/08; C07H 21/04; A01N 65/00
US CL : 435/343.1, 320.1, 325, 326, 328, 332, 343, 344, 372, 372.2, 372.3 455; 536/23.53; 424/93.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/343.1, 320.1, 325, 326, 328, 332, 343, 344, 372, 372.2, 372.3 455; 536/23.53; 424/93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, STN, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US 6410,319 B1 (RAUBITSCHEK et al) 25 Jun 2002 (25.06.2002), columns 31-32.	1-5
Y	DEBINSKI et al. Retargeting Interleukin 13 for Radioimmunodetection and Radioimmunotherapy of Human High-grade Gliomas. Clin Cancer Res. October 1999, Vol. 5, pages 3143s-3147s, entire document.	6-10
X	JENSEN, M. et al., CD20 is a molecular target for scFvFc:zeta receptorredirected T cells: implications for cellular immunotherapy of CD20+ malignancy Biol. Blood Marrow Transplant. 1998, Vol.4 pages 75-83, entire document.	1-5
X	XU, X et al. Targeting an d therapy of Carcinoembryonic Antigen-expressing Tumors in Transgenic Mice with an Antibody-Interleukin 2 Fusion protein. Cancer Research. August 2000, Vol. 60, pages 4475-4484, especially page 4475.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search
11 July 2002 (11.07.2002)

Date of mailing of the international search report
12 SEP 2002

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Authorized officer
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Telephone No. 703 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

フロントページの続き

(51)Int.Cl.[7] ＦＩ テーマコード（参考）
Ｃ０７Ｋ 14/705

(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OM,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZM,ZW

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アメリカ合衆国カリフォルニア州９１１０４，パサディーナ，ウッドリン・ロード ２３０５

Ｆターム(参考) 4B024 AA01 BA63 CA04 DA02 EA04 GA11 HA12
4C085 AA16 DD62
4C087 AA01 AA02 BB43 CA05 NA14 ZB26
4H045 AA10 AA30 BA41 CA40 DA50 EA28 FA74