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最終頁に続く

(54)【発明の名称】移植における免疫応答の予防および処置に適した間葉幹細胞から獲得した上澄み

(57)【要約】
受容者における移植片に対する免疫応答を減少させる一つの方法であって、移植片の宿主拒絶を減少または阻害するのに有効な間葉幹細胞の量で前記受容者を処置する方法。間葉幹細胞は移植の前、移植と同時、または移植後に投与することができる。更に、間葉幹細胞を用いる処置で外来組織、すなわち対宿主性移植片による宿主に対する減少免疫応答を誘導する方法が開示される。

【特許請求の範囲】
【請求項１】
同種異系抗原に対する免疫応答を減少させる一つの方法であって、免疫応答を減少させるのに有効な量での間葉幹細胞培養物から獲得した上澄みに、免疫エフェクター細胞を接触させることを含むことを特徴とする方法。
【請求項２】
請求項１記載の方法であって、ここでエフェクター細胞がＴ細胞であることを特徴とする方法。
【請求項３】
活性化されたＴ細胞の再活性化を予防しまたは減少させる一つの方法であって、前記活性化Ｔ細胞の再刺激を抑制するのに有効な量での間葉幹細胞から獲得した上澄みに、同種異系抗原でこれまでに活性化されたＴ細胞を接触させることを含むことを特徴とする方法。
【請求項４】
提供者移植片に対する免疫応答を減少させる一つの方法であって、移植片に対する受容者の免疫応答を減少させるのに有効な量での間葉幹細胞から獲得した上澄みで受容者を処置することを含むことを特徴とする方法。
【請求項５】
請求項４記載の方法であって、ここで間葉幹細胞が受容者にとって自己由来であることを特徴とする方法。
【請求項６】
請求項４記載の方法であって、ここで間葉幹細胞が受容者にとって同種異系であることを特徴とする方法。
【請求項７】
請求項６記載の方法であって、ここで間葉幹細胞が移植片の提供者から得られることを特徴とする方法。
【請求項８】
請求項４記載の方法であって、ここで間葉幹細胞が移植片の提供者および受容者の双方にとって同種異系であることを特徴とする方法。
【請求項９】
請求項４記載の方法であって、ここで間葉幹細胞が移植片の提供者および受容者の双方にとって異種であることを特徴とする方法。
【請求項１０】
請求項４記載の方法であって、ここで移植片が皮膚であることを特徴とする方法。
【請求項１１】
請求項４記載の方法であって、ここで上澄みが移植片の投与に先立ち受容者に投与されることを特徴とする方法。
【請求項１２】
請求項４記載の方法であって、ここで上澄みが移植片の投与と同時に投与されることを特徴とする方法。
【請求項１３】
請求項４記載の方法であって、ここで上澄みが移植片の一部として投与されることを特徴とする方法。
【請求項１４】
請求項４記載の方法であって、ここで上澄みが移植片の後で投与されることを特徴とする方法。
【請求項１５】
請求項４記載の方法であって、ここで上澄みが受容者による移植片拒絶を処置するために移植片受容者に投与されることを特徴とする方法。
【請求項１６】
請求項４記載の方法であって、更に免疫抑制薬を受容者に投与することを含むことを特徴

とする方法。
【請求項１７】
請求項４記載の方法であって、ここで移植片が固体器官であることを特徴とする方法。
【請求項１８】
請求項１７記載の方法であって、ここで固体器官が心臓、膵臓、腎臓、肺または肝臓から選択されることを特徴とする方法。
【請求項１９】
対宿主性移植片病のために移植片受容者を処置する一つの方法であって、移植片による受容者に対する免疫応答を減少させるのに有効な量での間葉幹細胞培養物から獲得した上澄みで、提供者移植片の受容者を処置することを含むことを特徴とする方法。
【請求項２０】
請求項１９記載の方法であって、ここで間葉幹細胞が受容者にとって自己由来であることを特徴とする方法。
【請求項２１】
請求項１９記載の方法であって、ここで間葉幹細胞が提供者移植片にとって自己由来であることを特徴とする方法。
【請求項２２】
請求項１９記載の方法であって、ここで間葉幹細胞が提供者および受容者の双方にとって同種異系であることを特徴とする方法。
【請求項２３】
請求項１９記載の方法であって、更に受容者に免疫抑制薬を投与することを含むことを特徴とする方法。
【請求項２４】
提供者移植片に対して不都合な免疫応答を減少させる一つの組成物であって、提供者移植片に対して不都合な免疫応答を阻害しまたは減少させるのに有効な量での間葉幹細胞培養物から獲得した上澄みと、薬理担体とを含むことを特徴とする組成物。
【請求項２５】
請求項２４記載の組成物であって、ここで間葉幹細胞が受容者にとって自己由来であることを特徴とする組成物。
【請求項２６】
請求項２４記載の組成物であって、ここで間葉幹細胞が提供者にとって自己由来であることを特徴とする組成物。
【請求項２７】
請求項２４記載の組成物であって、ここで間葉幹細胞が受容者および提供者双方にとって同種異系であることを特徴とする組成物。
【請求項２８】
請求項２４記載の組成物であって、ここで間葉幹細胞が受容者および提供者双方にとって異種であることを特徴とする組成物。
【請求項２９】
移植片により起こる移植片受容者に対して不都合な免疫応答を減少させる一つの組成物であって、移植片により起こる移植片受容者に対して不都合な免疫応答を減少させるのに有効な量での間葉幹細胞培養物から獲得した上澄みと、薬理担体とを含むことを特徴とする組成物。
【請求項３０】
請求項２９記載の組成物であって、ここで間葉幹細胞が受容者にとって自己由来であることを特徴とする組成物。
【請求項３１】
請求項２９記載の組成物であって、ここで間葉幹細胞が提供者にとって自己由来であることを特徴とする組成物。
【請求項３２】

請求項２９記載の組成物であって、ここで間葉幹細胞が受容者と提供者の双方にとって同種異系であることを特徴とする組成物。
【請求項３３】
請求項２９記載の組成物であって、ここで間葉幹細胞が受容者と提供者の双方にとって異種であることを特徴とする組成物。
【請求項３４】
請求項１記載の方法であって、ここで上澄みが混合リンパ球反応を受けるＴ細胞と共存培養された間葉幹細胞から獲得されることを特徴とする方法。
【請求項３５】
同種異系抗原に対する免疫応答を減少させる一つの方法であって、免疫応答を減少させるために有効な量での異種間葉幹細胞に免疫エフェクター細胞を接触させることを特徴とする方法。
【請求項３６】
同種異系抗原に対する免疫応答を減少させる一つの方法であって、同種異系抗原に対する免疫応答を減少させるのに有効な量での間葉幹細胞から獲得した上澄みにエフェクター細胞を接触させ、これにより同種異系抗原と接触した前記エフェクター細胞が前記同種異系抗原に対する減少した免疫応答を持つことを含むことを特徴とする方法。
【請求項３７】
請求項３６記載の方法であって、ここで前記エフェクター細胞が前もって活性化されたＴ細胞であり、また前記免疫応答が前記Ｔ細胞の再活性化であることを特徴とする方法。
【請求項３８】
請求項３６記載の方法であって、ここで減少される前記免疫応答が試験管内免疫応答であることを特徴とする方法。
【請求項３９】
請求項３６記載の方法であって、ここで減少される前記免疫応答が生体内免疫応答であることを特徴とする方法。
【請求項４０】
請求項３６記載の方法であって、ここで減少される前記免疫応答が提供者移植片に対する免疫応答であることを特徴とする方法。
【請求項４１】
請求項４０記載の方法であって、ここで前記提供者移植片が異種提供者移植片であることを特徴とする方法。
【発明の詳細な説明】
【０００１】
【発明の属する技術分野】
本発明は、１９９８年３月１８日に出願された合衆国暫定特許出願番号６０／０７８，４６３と１９９８年６月１９日に出願された合衆国暫定特許出願番号６０／０８９，９６４の優先権に基づきまたこれを主張する１９９９年３月１２日に出願された合衆国特許出願番号０９／２６７，５３６の一部継続出願である１９９９年１０月２６日に出願された合衆国特許出願番号０９／４２７，３３３の優先権を主張する。
【０００２】
本発明は同種異系抗原に対するＴ細胞応答を阻害することに関し、また更に前活性化Ｔ細胞の再活性化を阻害およびまたは予防することに関する。より詳細には、本発明は免疫エフェクター細胞により引き起こされる外来組織およびまたは細胞およびまたは器官に対する免疫応答を予防し、減少または処置する分野に関する。本発明は更に移植片拒絶およびまたは対宿主性移植片反応を予防し、減少または処置することに関する。
【０００３】
【発明の背景技術】
寛容は通常免疫応答の起こる抗原に対する特異的反応の獲得性欠損である。典型的には、寛容を誘導するには寛容化しようとする抗原に対する露出が必要であり、その結果として

若干のリンパ球の死または機能的不活性化が起こる。完全な寛容は、二次抗原攻撃に対する検出可能な免疫応答の欠損が特徴である。部分的寛容は免疫応答の量的減少が特徴である。

【０００４】

免疫機構の機能は病原体を含む外来物質を除去し、自己抗原に対する非応答性または寛容を維持することである。Ｔ細胞寛容は、１）自己ペプチドに反応する胸腺細胞がクローン消失により除去される胸腺において（中心寛容）、および２）免疫寛容を生じる条件下で自己抗原に露出される末梢において（末梢寛容）獲得される。クローン消失は抗原提示細胞に対する細胞死分子の発現からも起こり得る。細胞死分子の代表的な例はＦａｓリガンド（ＦａｓＬ）とＴＲＡＩＬリガンドであり、これらは活性化Ｔ細胞上のそれぞれのレセプターであるＦａｓとＤＲ４に結合しＴ細胞のアポトーシスを誘導する。ＴＮＦＲ上科の構成員であるＣＤ２７およびＣＤ２７リガンド（ＣＤ７０）の相互作用もまたＴ細胞アポトーシスを誘導する。

【０００５】

不都合なことに、免疫機能は移植片組織などの有益な侵入物と有害な侵入物とを区別せず、そのため免疫機構は移植された組織または器官を拒絶する。移植された器官に対する拒絶には、宿主内に存在し提供者同種異系抗原または異種抗原を認識する同種異系反応性Ｔ細胞が大きく介在する。

【０００６】

現在は、移植片に対する免疫応答を予防または減少させるために、患者は強力な免疫抑制剤で処置される。Ｔ細胞免疫応答を予防または抑制する薬剤の個々人への注入は移植片拒絶を阻害するが、全面的な免疫抑制、毒性、ましてや日和見感染による死すらも起こし得る。従来の提供者組織拒絶への処置に対する毒性と不完全な応答速度のために、現在の薬剤治療の方式に耐性を持てないまたは良い応答を示さない患者を処置するための代替アプローチが必要である。

【０００７】

従って、免疫エフェクター細胞による移植片に対する望ましくない宿主免疫応答の予防およびまたは減少のために、提供者組織に対する宿主拒絶が起こるのを防ぐ方法が必要である。また受容者組織に対する提供者組織による望ましくない免疫応答を除去または減少させる方法も有益であろう。このような免疫応答は対宿主性移植片病として知られている。

【０００８】

【発明の概要】

間葉幹細胞が移植において免疫機構による応答を改善するために用いることができ、それにより抗原に対する免疫応答は減少または除去されることは知られている。

【０００９】

本発明の一見地に従って、同種異系抗原、特に同種異系組織、器官または細胞に応答するＴ細胞により引き起こされる免疫応答を減少または抑制するための方法が提供され、免疫応答は間葉幹細胞を用いることで減少または抑制される。間葉幹細胞はＴ細胞に対して、自己由来である（同一宿主から獲得した）か、または同種異系あるいは異種のものである。Ｔ細胞に対して同種異系の間葉幹細胞の場合には、間葉幹細胞はＴ細胞が応答する細胞または組織に対して自己由来（同一宿主から獲得）であるかまたはＴ細胞の提供源とＴ細胞が応答する細胞または組織の提供源の両方に同種異系の宿主から獲得したものである。選択肢として、間葉幹細胞はＴ細胞の提供源およびＴ細胞が応答する細胞または組織の提供源のどちらかまたは両方に異種の提供源から獲得することができる。

【００１０】

本発明の別の見地に従って、（同種異系抗原、特に同種異系器官、組織または細胞に対して活性化した）活性化Ｔ細胞を続いて起こる外来抗原に対するＴ細胞応答を予防およびまたは減少させるのに有効な量で間葉幹細胞と接触させることにより、活性化Ｔ細胞の再刺激を予防するためのプロセスが提供される。用いられる間葉幹細胞はＴ細胞に対して自己由来およびまたは同種異系である。同種異系間葉幹細胞を用いる場合、間葉幹細胞はＴ細

胞を活性化する組織または細胞と同一の宿主、またはＴ細胞およびＴ細胞を活性化する細胞または組織を提供する宿主の両方に同種異系である宿主から獲得することができる。

【００１１】

本発明のもう一つの見地に従って、間葉幹細胞は、移植片受容者に対して間葉幹細胞を投与することにより移植片（組織、器官、細胞その他）に対する免疫応答を抑制または改善するために用いられる。間葉幹細胞は移植片に対する免疫応答を抑制または改善するのに有効な量で投与される。間葉幹細胞は移植片受容者に対して自己由来または同種異系あるいは異種である。

【００１２】

従って、本発明の一つの方法は提供者組織の受容者の間葉幹細胞への接触を提供する。この見地の一つの実施例において、この方法は間葉幹細胞を提供者移植片の受容者に投与することを含む。間葉幹細胞は移植片の前にまたは同時に、あるいは移植片に続いて受容者に投与することができる。間葉幹細胞は受容者に対して自己由来または同種異系であり、提供者から獲得することができる。本発明の別の見地において、同種異系間葉幹細胞は提供者以外の提供源からも獲得することができ、その提供源は提供者型または受容者型に適合する必要はない。

【００１３】

この方法の更なる見地において、移植方法の一部として間葉幹細胞は細胞死を誘導する分子を発現するように修飾される。間葉幹細胞は、分子に対するレセプターを保持する活性化Ｔ細胞のアポトーシスを誘導する分子を免疫機構に運搬するために用いることができる。この結果活性化Ｔリンパ球は削除され、移植片に対する望まれない免疫応答は抑制される。本発明のこの見地に従って、同種異系間葉幹細胞は細胞死分子を発現するように修飾される。分子は間葉幹細胞に対して外因性または内因性であり得る。ここに開示された本方法の望ましい実施例において、間葉幹細胞は細胞死分子ＦａｓリガンドまたはＴＲＡＩＬリガンドを発現する。

【００１４】

間葉幹細胞は移植片の一部として受容者に投与することもできる。この目的のために、本発明は間葉幹細胞で灌流しまたは間葉幹細胞を含む提供者組織または器官を受容者に提供することにより免疫応答を減少または改善する方法を提供する。間葉幹細胞は器官または組織の提供者または第三者から獲得したものか、あるいはＴ細胞に対して自己由来のものである。間葉幹細胞は、外来組織が受容者に移植された時に起こる受容者Ｔ細胞による外来組織に対する免疫応答を改善する。

【００１５】

本発明の更なる実施例において、器官または組織に灌流された間葉幹細胞は活性化Ｔ細胞死を誘導する分子を含有することができる。

【００１６】

もう一つの実施例において、本発明の方法は、移植片に対する拒絶症状の発病度を減少または除去するために移植片を受容した患者を処置することを提供する。この処置は提供者組織が受容者に移植された後、提供者移植片の受容者に対して間葉幹細胞を投与することからなる。間葉幹細胞は受容者に対して自己由来または同種異系であり得る。同種異系間葉幹細胞は提供者または第三者提供源から獲得することができる。移植片に対する不都合な免疫応答を受ける受容者への間葉幹細胞の提示は、更なる抗原刺激に対するＴ細胞の無応答を誘導し、それにより活性化Ｔ細胞による提供者組織または器官に対する不都合な応答を減少または除去する。

【００１７】

本発明の更なる一見地において、提供者組織、器官または細胞による受容者に対する免疫応答すなわち対宿主性移植片応答を減少させる方法が提供され、この方法は提供者組織、器官または細胞を生体外で、受容者体内への組織、器官または細胞の移植に先立って、同種異系（提供者に対して同種異系）間葉幹細胞で処置することから成る。間葉幹細胞は、続いて受容者抗原提示細胞に対して活性化するであろう移植片中のＴ細胞の反応性を減少

させる。そして移植片は、宿主に対する移植片の不都合な応答を起こすことなくあるいは減少されて受容者（宿主）体内に導入される。このようにして、対宿主性移植片病が起こることを防ぐことができる。

【００１８】

望ましい実施例において、提供者移植片は、提供者移植片中のＴ細胞を活性化するために、最初に受容者、または第三者組織、あるいは細胞に対して生体外で露出される。提供者移植片はその後提供者に対して自己由来または同種異系の間葉幹細胞に接触させられる。間葉幹細胞は受容者または第三者の間葉幹細胞でもあり得る。提供者移植片が続いて受容者体内に置かれたときに、間葉幹細胞は、提供者移植片中のＴ細胞による受容者の抗原刺激に対する不都合な二次免疫応答を減少または阻害するであろう。

【００１９】

従って、間葉幹細胞は例えば移植片に先立って受容者から獲得することができる。間葉幹細胞は単離し、必要時まで凍結保存することができる。また間葉幹細胞は必要量まで培養して増殖させ、必要時まで保存される。間葉幹細胞は、提供者移植片によって引き起こされる、受容者（宿主）に対する進行中の不都合な免疫応答を減少させるまたは除去するのに有効な量で受容者に投与される。移植片によって引き起こされる不都合な免疫応答を受ける受容者に対する間葉幹細胞の提示は、進行中の応答を阻害しＴ細胞の再刺激を予防する。それにより活性化Ｔ細胞による受容者組織に対する不都合な応答を減少または除去する。

【００２０】

更なる実施例は、受容者間葉幹細胞を活性化Ｔ細胞死を誘導する分子で修飾することを含む。

【００２１】

このように、本発明の望ましい実施例に従って、ヒト間葉幹細胞は、移植の結果起こる移植片拒絶およびまたは対宿主性移植片病を処置するために、およびまたは移植片拒絶およびまたは対宿主性移植片病を予防または減少させるために用いられる。ヒト間葉幹細胞はまた異種移植片の使用を容易にするために用いられる。前に記載した目的のために非ヒト霊長類細胞などの異種細胞を用いることも本発明に含まれる。

【００２２】

更に、ＭＳＣ培養系およびＭＳＣ／混合リンパ球反応培養系から得た上澄みは同種異系抗原に対するＴ細胞応答に対して抑制効果を持つことが知られている。従って、本発明は更に上澄みを用いる方法を提供する。

【００２３】

【発明の説明】

ここで定義したように、同種異系間葉幹細胞は受容者と同種の異なる固体から獲得される。提供者抗原は、受容者に移植された提供者組織が発現する抗原を指す。同種異系抗原は受容者が発現する抗原とは異なる抗原である。移植される提供者組織、器官または細胞が移植片である。移植片の例としては皮膚、骨髄および心臓、膵臓、腎臓、肺および肝臓などの固体組織が含まれる。従って、同種異系抗原は受容者に対して外来の抗原である。

【００２４】

発明者は、間葉幹細胞が試験管内で同種異系Ｔリンパ球と接触した時は同種異系Ｔ細胞は増殖しないことを発見した。通常、異なる個体から獲得した細胞の共存培養はＴ細胞の活性化と増殖により発現するＴ細胞応答を引き起こし、これは混合リンパ球反応（ＭＬＲ）として知られている。

【００２５】

この予期しない結果は、Ｔ細胞が不適合間葉幹細胞に応答しないことを示す。同種異系Ｔ細胞によるヒト間葉幹細胞に対する増殖応答の欠如は予期しないものであった。それはヒト間葉幹細胞が免疫原性を与える表面分子を発現する、すなわち同種異系クラスＩ　ＭＨＣ分子を発現するからである。この発見は間葉幹細胞が免疫機構に対して免疫原性が無いことを示している。

【００２６】
発明者は更に間葉幹細胞が同種異系細胞間のＭＬＲを抑制できることを発見した。間葉幹細胞は投与量依存方法において混合リンパ球反応における同種異系Ｔ細胞応答を積極的に減少させる。加えて、異なる提供者から獲得した間葉幹細胞はＭＨＣ型に関して減少応答の特異性を示さない。従って、間葉幹細胞は、間葉幹細胞に対する同種異系Ｔ細胞の増殖応答を減少させるためには、混合リンパ球反応における標的細胞集団に対してＭＨＣが適合する必要はない。間葉幹細胞は応答または刺激細胞あるいはその両方に対して異種でもあり得る。
【００２７】
発明者はまた、間葉幹細胞培養系から得た上澄みが同種異系細胞間のＭＬＲを抑制することができることも発見した。ここで用いられたように、間葉幹細胞培養系から得た上澄みは、ここでは「ＭＳＣ上澄み」とも示されるが、単独培養した間葉幹細胞または免疫応答を受けている細胞、すなわち混合リンパ球反応を受けているＴ細胞と共存培養した間葉幹細胞から獲得できる。
【００２８】
間葉幹細胞上澄みは、投与量依存方法において混合リンパ球反応における同種異系Ｔ細胞応答を積極的に減少させる。間葉幹細胞の場合と同様に、異なる提供者から獲得した間葉幹細胞培養系から得た上澄みはＭＨＣ型に関して減少応答の特異性を示さなかった。
【００２９】
加えて、間葉幹細胞と接触させた混合リンパ球反応から得た上澄みもまた同種異系細胞間のＭＬＲを抑制することができる。このＭＬＲ／間葉幹細胞上澄みは投与量依存方法において混合リンパ球反応における同種異系Ｔ細胞応答を積極的に減少させ、ＭＨＣ型に関して減少応答の特異性を示さなかった。
【００３０】
可溶性因子または化合物が混合リンパ球反応に対する抑制効果を持つ間葉幹細胞培養培地中に分泌されると考えられる。混合リンパ球反応に露出されたＭＳＣｓから獲得した上澄みの使用では強力な抑制効果が見られる。
【００３１】
従って、本発明は、提供者組織、器官または細胞の受容者に同種異系間葉幹細胞を投与することにより、免疫応答を減少、阻害または除去する方法を提供する。一つの実施例において、間葉幹細胞は移植片と同時に受容者に投与される。選択肢として、間葉幹細胞を移植片の導入に先だって投与することも可能である。例えば、間葉幹細胞を提供者組織移植の約３日乃至７日前に受容者に投与することも可能である。代替的に、間葉幹細胞を移植片に続いて投与することも可能である。
【００３２】
このように間葉幹細胞は、提供者組織の移植に先だって、または同時に受容者に投与することにより、提供者または外来の組織に対する受容者の免疫機構を調整するために用いることが可能である。間葉幹細胞は例えば受容者Ｔ細胞による移植片に対する免疫応答を減少または除去するのに有効な量で投与される。間葉幹細胞は受容者Ｔ細胞に影響を与え、それにより提供者または外来の組織が提示されたときのＴ細胞の応答は減少または除去される。このようにして、移植片に対する宿主拒絶は回避されまたはその発症度は減少する。
【００３３】
発明者は更に、すでに抗原刺激に露出された、すなわち活性化されたＴリンパ球が続いて間葉幹細胞に露出されると、Ｔ細胞は続く同種異系細胞による抗原刺激に対して免疫応答を生じさせないまたは減少した免疫応答を生じさせることを発見した。このように間葉幹細胞はＴ細胞の低反応性状態を誘導する。
【００３４】
これらの予期しない結果は、活性化Ｔ細胞が前活性化Ｔ細胞の間葉幹細胞への露出により、更なる同種異系刺激に対して無応答になることを示す。間葉幹細胞はＴ細胞に対して自

己由来または同種異系であり得る。

【００３５】

従って、本発明は、免疫応答を減少または抑制するのに有効な量の間葉幹細胞を移植片に対する不都合な免疫応答を受ける患者に投与することにより、患者を処置する方法を提供する。間葉幹細胞は組織提供者、移植片受容者または第三者から獲得される。更に代わって、ＭＳＣｓは提供者、受容者またはその両方に対して異種であり得る。

【００３６】

間葉幹細胞は更に、活性化Ｔ細胞の除去を促進するための細胞死分子を発現するように修飾することができる。例えば細胞死分子は、外因性の細胞死分子を発現するように遺伝子操作された間葉幹細胞によって発現される。

【００３７】

もう一つの見地において、本発明は、提供者移植片（対宿主性移植片）による移植片受容者に対する免疫応答を減少、または阻害、あるいは除去する方法を提供する。

【００３８】

従って、本発明は、移植に先だって提供者器官または組織を間葉幹細胞と接触させることを提供する。間葉幹細胞は、提供者移植片による受容者に対する不都合な応答を改善、阻害または減少させる。

【００３９】

望ましい実施例において、移植に先立って提供者移植片は、提供者移植片中のＴ細胞を活性化する同種異系（受容者）組織または細胞で処置される。提供者移植片はその後、移植の前に自己由来または同種異系の間葉幹細胞で処置される。間葉幹細胞は、続いて起こる抗原刺激に対するＴ細胞の再刺激を予防し、または低反応性を誘導する。

【００４０】

提供者移植片の前調整のために、間葉幹細胞は更に細胞死分子を発現するように修飾され、それにより間葉幹細胞と接触した活性化Ｔ細胞は除去される。

【００４１】

従って、例えば骨髄およびまたは末梢血から獲得した造血幹細胞移植の場合においても、移植片による宿主への攻撃は減少または除去できる。骨髄または末梢血幹細胞の受容者への移植に先だって、提供者骨髄を受容者間葉幹細胞で前処置することができる。望ましい実施例において、提供者骨髄は最初に受容者組織／細胞に露出され、その後間葉幹細胞で処置される。これに限定されないが、最初に受容者組織または細胞に接触させることが骨髄中のＴ細胞を活性化する役割を果たすと考えられている。続く間葉幹細胞での処置が骨髄中でのＴ細胞の更なる活性化を阻害または除去し、それにより提供者組織による不都合な影響を減少または除去する。すなわちこの治療が対宿主性移植片応答を減少または除去する。

【００４２】

更なる実施例において、対宿主性移植片病を患う移植片受容者は、そのような受容者に間葉幹細胞を投与することにより、その発症を減少または除去する処置を受けることができる。間葉幹細胞は提供者に対して自己由来または同種異系であり、同種異系細胞は受容者に対して自己由来の細胞間葉幹細胞または第三者間葉幹細胞であり得る。間葉幹細胞は宿主の移植片拒絶を減少または除去するのに有効な量で投与される。間葉幹細胞は、提供者組織中の活性化Ｔ細胞が受容者に対する免疫応答を持つことを阻害または抑制し、それにより対宿主性移植片応答を減少または除去する。

【００４３】

受容者の間葉幹細胞は移植に先立って受容者から獲得し保存し、およびまたは進行中の宿主に対する移植片の攻撃を処置するのに十分な量の間葉幹細胞を得るために培養して増殖させる。

【００４４】

本発明のもう一つの方法において、提供者組織は間葉幹細胞に露出され、それにより、移植に先立って間葉幹細胞は器官移植片そのものに結合する。この状況では、同種異系受容

者細胞により引き起こされる移植片に対する免疫応答は、移植片中に存在する間葉幹細胞によって抑制されるであろう。この免疫応答は移植片拒絶を予防する通常の処置、例えば薬剤を介した免疫抑制を免れたものである。間葉幹細胞は受容者に対して同種異系のものが望ましく、提供者間葉幹細胞、または提供者または受容者以外から獲得した間葉幹細胞である。受容者に対して自己由来の間葉幹細胞を移植片に対する免疫応答を抑制するために用いることができる場合もある。

【００４５】

本方法の更なる実施例において、間葉幹細胞は細胞死分子を発現するように遺伝子操作され、それにより同種異系宿主Ｔ細胞はこの間葉幹細胞と接触させられて除去されるであろう。

【００４６】

更に、初期免疫応答の予防または改善に加えて、局在する間葉幹細胞は起こり得る、続くいずれのＴ細胞応答をも抑制すると考えられる。

【００４７】

ここで用いられるように、「細胞死分子」は、刺激されたＴ細胞上のこれが認識するレセプターと相互作用しまたは結合して、Ｔ細胞死またはアポトーシスを誘導する分子である。Ｆａｓは刺激物質に再び露出され活性化されたＴ細胞のアポトーシスを仲介する（ヴァン　パレイス他、Ｉｍｍｕｎｉｔｙ４巻、３２１－３２８ページ（１９９６年））。ＦａｓはＩ型膜レセプターであり、Ｆａｓがコグネイトするリガンドにより架橋されると多様な細胞にアポトーシスを誘導する。標的Ｔ細胞上のＦａｓ分子（ＣＤ９５）と間葉幹細胞上のＦａｓ分子のリガンドＦａｓ　Ｌとの相互作用はレセプター凝集を引き起こし、標的細胞のアポトーシスを引き起こす信号を導入する。Ｆａｓシステムは、胸腺細胞の陰性選択、体内の免疫細胞侵入阻止部位の維持および細胞障害性Ｔリンパ球（ＣＴＬ）介在性細胞障害を含む生体内での一連の細胞機能に含まれると見なされている（グリーンとウエア、Ｐｒｏｃ　Ｎａｔｌ　Ａｃａｄ　Ｓｃｉ、９４（１２）巻、５９８６－９０ページ、（１９９７年））。

【００４８】

腫瘍壊死因子レセプター（ＴＮＦＲ）科のその他はプログラムされた細胞死に役割を持つ。ＴＲＡＩＬリガンドはそのレセプターＤＲ４と相互作用し、種々の形質転換細胞系にアポトーシスを誘導することができる（Ｇ．パン他、Ｓｃｉｅｎｃｅ、２７７巻、８１５－８１８ページ（１９９７年））。また、ＣＤ２７とそのリガンドＣＤ７０（プラサッド他、Ｐｒｏｃ　Ｎａｔｌ　Ａｃａｄ　Ｓｃｉ、９４巻、６３４６－６３５１ページ（１９９７年））の発現もアポトーシスを誘導する。Ｆａｓの発現は刺激されたＴ細胞および免疫細胞侵入阻止部位に限られる。ＴＲＡＩＬは多くの通常組織に検出される。

【００４９】

ＴｒａｉｌリガンドおよびＣＤ２７の両方は、Ｆａｓリガンドとは異なり、操作されていないヒト間葉幹細胞上にも発現する。活性化されしかし休止していないＴ細胞はＴｒａｉｌレセプターとＣＤ７０を発現する。体内に見られるＴ細胞のほとんどは休止状態にある。Ｔ細胞は、ＭＨＣおよびＢ７－１またはＢ７－２などの適切な補助的刺激分子の両方の場合において細胞と遭遇したとき活性化される。

【００５０】

このように、間葉幹細胞上に発現するリガンドが活性化Ｔ細胞上の細胞死レセプターへ嵌入することにより、アポトーシスによるＴ細胞死が起こる。リガンドおよびそのレセプターで特に前に記載したもの以外は、間葉幹細胞中に存在するかまたは間葉幹細胞に導入されるかのどちらかでこの機能を果たすことができる。従って、個人に投与される間葉幹細胞は活性化Ｔ細胞を削除し、移植片拒絶病の発症度を減少させる。

【００５１】

ここに開示された本発明の方法に従って、本発明の間葉幹細胞は、提供者組織拒絶または対宿主性移植片病を処置する現行の方法と連係して用いることができる、と考えられる。このような使用法の利点は、移植片受容者体内での免疫応答の発病度を改善することによ

り、治療に用いる薬剤の量およびまたは薬剤治療の適用頻度を減らすことができ、その結果一般免疫の抑制と望まれない副作用が軽減する、ということになる。

【００５２】

更に考えられるのは、本発明での間葉幹細胞単独の処置だけが必要であり、長期的な免疫抑制剤治療の必要性は除去される、ということである。代わって、多角的な間葉幹細胞の投与が行われる。

【００５３】

従って、ここに開示された本発明は、間葉幹細胞の投与により移植片拒絶を予防または処置することを提供する。間葉幹細胞は、同一種から獲得した器官、組織または細胞、あるいは異種移植器官または組織移植片の移植片拒絶、およびまたは対宿主性移植片病の予防、または処置、あるいは改善のために有効な量で投与される。

【００５４】

間葉幹細胞の単一投与量の投与は、Ｔ細胞に対して同種異系の組織または「非自己」組織に対するＴ細胞応答の減少または除去に有効である。Ｔリンパ球が、間葉幹細胞から分離した後も同種異系細胞に対する非応答性（すなわち、寛容またはアネルギー）を保持する場合には、特に有効である。

【００５５】

間葉幹細胞の投与量は広範囲の制限域内で変更され、もちろんそれぞれ特定の場合における個々人の要求量に合わせられる。一般に非経口投与の場合、通常は受容者体重１ｋｇ当たり約０．０１乃至５百万個の細胞が投与される。用いられる細胞数は受容者の体重と体調、投与量または頻度、そして通常の技術に習熟した人には既知のその他の変数に依存する。間葉幹細胞は移植される組織、器官、または細胞に適した継代を通して、投与することが可能となる。間葉幹細胞は全身に、すなわち非経口的に、静脈内注射によって投与することができ、または、骨髄などの特定組織または器官を標的とすることができる。間葉幹細胞は、細胞の皮下埋め込み、または結合組織、例えば筋肉〔原文通り〕内への幹細胞の注入によって投与することができる。

【００５６】

細胞は適切な希釈液で、１ｍｌ当たり約０．０１乃至約５ｘ１０^{6}個の細胞濃度で懸濁され得る。注入溶液に適切な希釈液は、細胞および受容者に生物学的、生理学的に適合するもので、緩衝生理食塩水またはその他の適切な希釈液などである。投与する組成物は、適切な無菌状態および安定性を満たす通常の方法に従って処方、産生、および保存しなくてはならない。

【００５７】

しかしながら本発明はそれらに限定されることはなく、間葉幹細胞は望ましくは骨髄から単離し、精製して、培養すなわち試験管内で増殖させることができる。間葉幹細胞はここに開示された方法に用いるのに十分な細胞数を獲得するために培養して増殖させる。間葉幹細胞は骨中で発見された形成多能性芽細胞であり、通常骨髄およびその他間葉組織中に非常に低頻度（骨髄では１：１００，０００）で存在する。キャプランとヘインズワース、合衆国特許番号５，４８６，３５９を参照されたい。間葉幹細胞への遺伝子導入は、ガーソン他、合衆国特許番号５，５９１，６２５に開示されている。

【００５８】

別途言及されていない限り、遺伝子操作は、サムブルックとマニエイティス、ＭＯＬＥＣＵＬＡＲ　ＣＬＯＮＩＮＧ：Ａ　ＬＡＢＯＲＡＴＯＲＹ　ＭＡＮＵＡＬ、第２版、コールド　スプリング　ハーバー　ラボラトリー　プレス、コールド　スプリング　ハーバー、ニューヨーク（１９８９年）に開示されたように行われる。

【００５９】

考慮すべきは、ここに開示された方法は多方面で、また通常の技術で良く知られる様々な修正、変更と共に実施されるということである。また十分に留意すべきは、細胞型間の作用または相互作用の形態について説明する理論が本発明をある方法に限定すると解釈されるべきでなく、この理論があることにより本発明の方法をより十分に理解できるというこ

とである。

【００６０】

次に記載する実施例は本発明の見地を更に説明する。しかしながら、ここで説明するように、それらは決して本発明の教義または開示を限定するものではない。

【００６１】

【発明の実施の形態】

（実施例１）

間葉幹細胞の同種異系反応性欠損

混合リンパ球反応は提供者の表面抗原の適合性を測定し、提供者組織の拒絶の可能性を示唆するものである。移植片拒絶を引き出す原因となる細胞表面抗原はクラスＩ、クラスＩＩ　ＭＨＣ抗原である。Ｔ細胞は外来ＭＨＣ抗原に対して同種異系反応性である。クラスＩ、クラスＩＩ　ＭＨＣ分子は混合リンパ球反応を刺激する。

【００６２】

正常ヒト有志者はＣＯＢＥ　ＳＰＥＣＴＲＡ^{TM}アフェレーシスシステム（コーブ、レークウッド、コロラド）で白血球瀉血された。個人Ａから獲得したＴ細胞（$Ｔ_{A}$）１ｘ１０^{5}個は個人Ｂから獲得された同種異系ＰＢＭＣｓ（$ｍＰＢＭＣ_{B}$）を（Ｔ細胞に対するＰＢＭＣｓの増殖を予防するために）処置するマイトマイシンＣと共に平底マイクロタイタウェル内で７日間培養された。$ｍＰＢＭＣ_{B}$ｓは２０Ｋおよび１００Ｋで接種された。培養基は、Ｔ細胞増殖を測定するための培養期間の最終１８時間の間^{3}Ｈ－チミジンで標識された。結果は図１に示され、$Ｔ_{A}$細胞が$ＰＢＭＣ_{B}$を外来のものと認識したことを示唆している（「$Ｔ_{A}$＋$ｍＰＢＭＣ_{B}$」部参照）。$ＰＢＭＣ_{B}$ｓの量が多いほど、Ｔ細胞増殖も多かった。

【００６３】

ＰＢＭＣｓと同一の提供者から獲得したヒト間葉幹細胞（ｈＭＳＣｓ）２ｘ１０^{4}個は個人Ａから獲得したＴ細胞（$Ｔ_{A}$）１ｘ１０^{5}個と共培養された。細胞は平底マイクロタイタウェル内で合計７日間培養された。培養基はＴ細胞増殖を測定するための培養期間の最終１８時間の間^{3}Ｈ－チミジンで標識された。Ｔ細胞との培養前の２日間、ヒト間葉幹細胞は前に記載された細胞数（密集）でマイクロタイタウェル内に接種され、ＭＳＣｓ上の表面抗原発現を刺激するためにＩＦＮ－γ（５０ｕｎｉｔｓ／ｍｌ）で処置された。非形質導入ｈＭＳＣｓ、またはヒトＢ７－１あるいはヒトＢ７－２補助的刺激分子を導入されたｈＭＳＣｓはＴ細胞と共にインキュベートされた。対照細胞はＮｅｏを導入された。

【００６４】

結果は図１に示され（図１「$Ｔ_{A}$＋形質導入ｈＭＳＣｓ」参照）、Ｔリンパ球がヒト間葉幹細胞に対して非応答性（増殖しない）であった、すなわち間葉幹細胞が外来のものとして認識されなかったことを示している。

【００６５】

この結果は、間葉幹細胞に対する応答の欠如が個体間の遺伝的適合性に依らなかったことを示している。これはＴ細胞がｈＭＳＣ提供者から獲得した末梢血単核細胞（ＰＢＭＣｓ）を外来のものとして認識したためである。

【００６６】

（実施例２）

混合リンパ球反応の抑制

間葉幹細胞が同種異系応答を積極的に抑制するかどうかを確認するために、混合リンパ球反応は、組織培養プレート中に準備された。組織培養プレートは異なる二提供者から獲得した粘着性間葉幹細胞を伴うものと伴わないものである。異なる二提供者は、一方はＭＬＲ中の刺激細胞に適合する提供者であり他方は刺激または応答細胞に無関係の提供者である。

【００６７】

個人Ａから獲得したＰＢＭＣｓ（$ＰＢＭＣ_{A}$）１０^{5}個は個人Ｂの標的ＰＢＭＣｓ（$ＰＢＭＣ_{B}$）１０^{5}個と混合された。$ＰＢＭＣ_{B}$ｓは$ＰＢＭＣ_{A}$ｓによる活性化に依る増殖を

予防するために３０００ラドでＸ線を照射された。従って、ＰＢＭＣ$_A$ｓのみが増殖する。ＰＢＭＣ$_A$ｓとＰＢＭＣ$_B$ｓが混合されると混合リンパ球反応が起こり、その中でＰＢＭＣ$_A$ｓ細胞（応答細胞）はＰＢＭＣ$_B$ｓ（刺激細胞）上の表面抗原により活性化された。培養基は７日間インキュベートされ^{3}Ｈ－チミジンで最終１８時間標識された。ＰＢＭＣ$_B$ｓの存在下ではＰＢＭＣ$_A$ｓは４０，０００カウントまで増殖した。図２、第一棒グラフ（「ＮＯＮＥ」は間葉幹細胞不在を示す）参照。

【００６８】

しかし間葉幹細胞の存在下でＰＢＭＣ$_A$ｓとＰＢＭＣ$_B$ｓが混合されると、混合リンパ球反応は抑制された。ＰＢＭＣ$_A$ｓ１０^{5}個は、ヒト間葉幹細胞の粘着性単層で覆われたマイクロタイタプレート内でＰＢＭＣ$_B$ｓ１０^{5}個と混合された。間葉幹細胞は、ウェル当たり７５００個乃至２２，５００個の範囲の量でウェル内で平板培養された。二つの間葉幹細胞集団が試験された：ヒト間葉幹細胞が個人Ｂから獲得したものおよび個人Ａまたは個人ＢのＭＨＣ型に適合しない個人（第三者）から獲得したものであった。培養基は７日間培養され^{3}Ｈ－チミジンで最終１８時間標識された。ヒト間葉幹細胞の存在下ではＭＬＲは抑制された。図２を参照。従って、間葉幹細胞のＭＨＣ系統に関係なく間葉幹細胞は混合リンパ球反応を抑制した。

【００６９】

結果は図２に示され、ヒト間葉幹細胞が投与依存法において混合リンパ球反応を減少させたことを示唆している。どの提供者からの間葉幹細胞も増殖を同等に十分に抑制し、これはＭＨＣ型に関して抑制の特異性が無かったことを示唆している。これらの結果は、細胞が共存培養された場合、間葉幹細胞が積極的に混合リンパ球反応を抑制したことを示す。

【００７０】

（実施例３）

二次混合リンパ球反応における無応答性

これらの実験は、ＭＳＣｓによる前活性化Ｔ細胞の抑制により二次刺激時に特異的な無応答性が生じるかどうかを確認するために行われた。

【００７１】

提供者２４８（ｄ２４８）から獲得したＴ細胞は提供者２７３（ｄ２７３）から獲得した同種異系ＰＢＭＣｓにより７日間特発され、その後単独または同一提供者（ｄ２７３）から獲得したＩＦＮ－γ処置ＭＳＣｓの存在下で３日間培養された。細胞はその後同一提供者（ｄ２７３）、自己由来（ｄ２４８）または「第三者」（ｄ２４４）ＰＢＭＣｓにより再刺激された。

【００７２】

リンパ球調製

末梢血単核細胞（ＰＢＭＣ）はフィコールパック（ファルメイシア）による密度勾配遠心法により調製された。細胞の一部は１０％ＤＭＳＯ添加９０％ＦＣＳ中で凍結され、液体窒素内で保存された。解凍後、細胞はＭＳＣ培地（低グルコースおよび１０％ＦＣＳ添加ＤＭＥＭ）で２回洗浄され、分析培地（２５ｍＭヘペス、１ｍＭピルビン酸ナトリウム、１００μＭ可欠アミノ酸、１００Ｕ／ｍｌペニシリン、１００μｇ／ｍｌストレプトマイシン、０．２５μｇ／ｍｌアンホテリシンＢ、５．５ｘ１０$^{-5}$Ｍ２－メルカプトエタノール（全試薬はギブコＢＬＲ）および５％ヒトＡＢ血清（シグマ、ＭＬＲ試験済）添加イスコーブ）中に再懸濁された。

【００７３】

Ｔ細胞富化分画を調製するために、免疫磁気陰性選別法によりＰＢＭＣｓから単球とＢリンパ球を除去した。ＰＢＭＣｓはマウス抗ヒトＣＤ１９およびＣＤ１４モノクローナル抗体（非アジ化合物／低菌体内毒素（ＮＡ／ＬＥ）型）と共にインキュベートされ、続いてビオチン結合ヤギ抗マウスＩｇＧ（多部位吸着）抗体（全試薬はファルメイシア）とストレプトアビジンマイクロビーズ（ミルテニー　バイオテック）と共にインキュベートされた。細胞はその後磁気細胞選別機（ＭＡＣＳ、ミルテニー　バイオテック）で分離された。Ｔ細胞富化分画は約７０－約９０％のＣＤ３＋細胞を含む。

【００７４】
ＭＳＣ培養
ヒトＭＳＣｓは、合衆国特許番号５，４８６，３５９に記載された通りに骨髄から単離されて、ＭＳＣ培地を含む培養基中に保持され、３代乃至６代継代したものが使用された。細胞は０．０５％トリプシン／ＥＤＴＡ溶液を用いて取り出され、ＭＳＣ培地で１回洗浄され、７０－８０％の密集度ですなわち１０ｃｍ組織培養ディッシュに対してプレート当たり$1x10^6$個で平板培養された。平板培養後、５００Ｕ／ｍｌのＩＦＮ－γ（ベーリンガー　マンハイム）が添加され、細胞は更に３日間インキュベートされた。Ｔ細胞を移す前に、平板培養されたＭＳＣはＨＢＳＳで４回、イスコーブで１回洗浄され、分析培地が１０ｃｍ組織培養ディッシュ中にウェル当たり１０ｍｌで添加された。

【００７５】
初期（1^O）ＭＬＲ
Ｔ細胞（ｄ２４８）はＸ線照射ＰＢＭＣｓ（ｄ２７３）により活性化された。刺激に用いたＰＢＭＣｓはキャビネットＸ線システム（ファクシトロン・エックスレイ、バッファローグローブ、イリノイ）を用いて３，０００ラドでＸ線を照射された。初期刺激のために応答細胞$2x10^7$個は、１０ｃｍ組織培養ディッシュ中の分析培地２０ｍｌ中で刺激細胞$2x10^7$個と混合された。細胞は３７℃、５％二酸化炭素濃度で７日間インキュベートされた。

【００７６】
活性化Ｔ細胞／ＭＳＣ培養
1^OＭＬＲで活性化されたＴ細胞は回収されＭＳＣ培地で一回洗浄され、分析培地１０ｍｌ中に10^6個／ｍｌで再懸濁された。そして自己由来または同種異系ＭＳＣｓが含まれあるいは培地単独の１０ｃｍ組織培養ディッシュに添加され、更に３日間培養された。

【００７７】
再刺激分析
ＭＳＣｓまたは培地と共に培養されたＴ細胞は回収されＭＳＣ培地で一回洗浄され、始原提供者、無関係の提供者から獲得したＸ線照射ＰＢＭＣｓまたは自己由来ＰＢＭＣｓで再刺激された。分析のために、活性化応答細胞$5x10^4$個とＸ線照射刺激細胞$5x10^4$個は９６ウェルプレート内でインキュベートされた。分析は三回行われた。培養細胞は回収前に〔^{3}Ｈ〕チミヂンの１μＣｉ（エイマシャム）で１８時間標識された。培養基はハーベスター９６（トムテック）を用いて回収され、濾過器はマイクロベータトリラックス液体シンチレーションと発光計数器（Ｅ．Ｇ．アンドＧ．ウォーラック）を用いて解析された。データは３回の反復実験の平均ｃｐｍ±ＳＤとして表示されている。

【００７８】
単独培養されたＴ細胞（正の対照）は２日目に最大となる「同一提供者」再刺激に対する応答の促進を示す。「第三者」応答もまた促進されたが、実際には「同一提供者」と同じ動態であるが最大値は低くまたわずかに開始の遅れが見られた（図３）。同種異系ＭＳＣｓ上で培養されたＴ細胞は、続く培養６日目までの間は「同一提供者」または「第三者」ＰＢＭＣｓに対して無応答を示した（図４）。

【００７９】
（実施例４）
二次混合リンパ球反応における無応答性
提供者４１３から獲得されたＴ細胞は提供者２７３から獲得されたＸ線照射ＰＢＭＣｓで７日間再刺激された（容積２０ｍｌ培養基に$1.5x10^6$ｍｌ）。異なる提供者４１３、４１８および２７３から獲得したＭＳＣｓは１０ｃｍ組織培養ディッシュ中に$1x10^6$／ディッシュで平板培養された。ディッシュはＩＦＮ－γで３日間前処置され、前活性化Ｔ細胞の混合に先立って洗浄された。

【００８０】
ＭＬＲ中で７日間前活性化されたＴ細胞は単独でまたはＭＳＣｓと共に３日間インキュベートされた（$1.0x10^6$／ｍｌＴ細胞、１０ｍｌ／ディッシュ）。ＭＳＣｓとのイン

キュベートの３日後Ｔ細胞は回収され、自己由来（ｄ４１３）ＰＢＭＣの存在または不在下で、Ｘ線刺激ＰＢＭＣ２７３（始原提供者）、４１３（自己由来）、ＰＢＭＣ１０（第三者）またはＰＨＡ（５μｇ／ｍｌ）で再刺激された。細胞はウェル当たり5×10^4個で添加され、培養細胞は〔^{3}Ｈ〕チミヂンで表示時間で１８時間標識された。

【００８１】

結果は、自己由来（ｄ４１３）（図５Ｃ）、同一提供者（ｄ２７３）（図５Ｂ）および第三者（ｄ４１８）（図５Ｄ）ＭＳＣｓでの活性化Ｔ細胞の処置がＴ細胞中の抗原刺激に対する無応答性を誘導することを示唆している。ＭＳＣ処置を伴わない対照培養（図５Ａ）は同種異系ＰＢＭＣｓに対する露出による細胞の再刺激を示した。

【００８２】

（実施例５）

イヌＭＳＣｓによる初期ＭＬＲの抑制

イヌＰＢＭＣｓはフィコールパック勾配遠心法（１．０７７）により末梢血から精製された。刺激ＰＢＭＣｓは２２００ラド（７分、７０ｋＶ）でＸ線照射された。Ｘ線照射刺激細胞10^5個は、前平板培養イヌＭＳＣ（Ｅ６４７、2×10^4個／ウェル）の存在または不在下で応答ＰＢＭＣｓ10^5個と９６ウェルプレート中で混合された。培養細胞は６日間インキュベートされ〔^{3}Ｈ〕ＴｄＲ（５Ｃｉ／ｍｍｏｌ、１μＣｉ／ウェル）で１６時間標識された。結果は図６Ａ－６Ｄに示す。Ｅ６４７とＥ６４５は同腹子（ＤＬＡ同一）である。結果は同種異系と同様自己由来ＭＳＣｓも初期ＭＬＲを抑制することを示す。

【００８３】

（実施例６）

非粘着性ＭＳＣｓによる初期ＭＬＲの抑制

ｄ２７３から獲得したＴ細胞（2×10^5個／ウェル）はｄ２４４から獲得したＸ線照射ＰＢＭＣｓ（2×10^5個／ウェル）と混合された。ｄ２４４またはｄ２７３から獲得したＭＳＣｓはＩＦＮ－γ（９００Ｕ／ｍｌ、３日間）で前処置されまたは無処置のままで、試験日にトリプシン処理されＴ細胞およびＰＢＭＣｓと同時に添加された。培養細胞は７日間インキュベートされ、〔^{3}Ｈ〕ＴｄＲ（５Ｃｉ／ｍｍｏｌ、１μＣｉ／ウェル）が１６－１８時間添加された。結果は図７に示され、非粘着性ＭＳＣｓも初期ＭＬＲを抑制することを示している。

【００８４】

（実施例７）

同種ＭＳＣｓ支持皮膚異系移植片の生存

研究集団

若いヒヒ（パピオ　アヌビス）が研究された。雄と受胎していない雌ヒヒの体重は７－２０ｋｇで３－１６歳の年齢であった。これら動物は結核乳頭腫ウイルスの有無を検査され、サイトメガロウイルス（ＣＭＶ）で滴定され、糞便浮遊及び塗沫検査を含むシミアンウイルスの検査より成る霊長類ウイルススクリーンで試験された。提供者と受容者の対はＰＣＲ類別化を通じて主要組織適合遺伝子複合体（ＭＨＣ）不等性により決定された。研究期間中、ヒヒは付き添い動物のそばで個別の地域をあてがわれた。

【００８５】

ＭＳＣ単離と培養抗張のための提供者骨髄収穫

ＭＳＣｓの単離と培養抗張のために骨髄針吸引液が腸骨稜から得られた。骨髄吸引液は週１回連続４週で交互に脇腹から獲得された。吸引液量は動物の血液量の１０％の推定値で決められた。血液量は体重の７％と推定された。従って１０ｋｇのヒヒは推定血液量０．７リットルであった。血液量の１０％の吸引液は従って７０ミリリットルになるであろう。

【００８６】

手続きに先立ち、セファゾリン５００ｍｇが手術時抗菌予防のために筋肉内（ＩＭ）投与された。ヒヒは手術のためにケタミン１０ｍｇ／ｋｇ、ＩＭ、またキシラジン１ｍｇ／ｋｇ、ＩＭで手術のために鎮静化され麻酔された。針挿入部位はポビドンヨードでこすり洗

いされ次いでアルコールで洗浄された。吸引液は腸骨稜から１６ゲージ、２インチ骨髄針を使って獲得された。注射器が針に取付けられ、骨髄液を抜くための吸入装置が利用された。手術後の痛みを除くために鎮痛剤ブプレノルフィンが０．０３ｍｇ／ｋｇ、ＩＭ．Ｑ１２ｘ２用量で与えられた。

【００８７】

提供者骨髄吸引液の送り

骨髄吸引液は注射器からヘパリンナトリウムを含む無菌バキュテーナー（Ｒ）に移された。この管はスタイロフォームコンテナーに安置され、室温（ＲＴ）で細胞処理設備に送られた。

【００８８】

ＭＳＣｓの単離と培養物の樹立

骨髄の５乃至１０ｍｌアリコートがポリプロピレン培養管内でダルベッコ食塩加リン酸緩衝液（ＤＰＢＳ）５０ｍｌで希釈された。細胞懸濁液は２２００ＲＰＭで１０分室温（ＲＴ）で遠心された。全有核細胞数は酢酸４％液で確認された。細胞は次いで最終濃度で２０ｘ１０^{6}細胞／ｍｌになるようにＤＰＢＳで希釈された。１０ｍｌまたは２００ｘ１０^{6}細胞が５０ｍｌの円錐管でパーコール２０ｍｌ（比重１．０７３ｇｍ／ｍｌ）に負荷され１３００ＲＰＭで２０分遠心された。単核細胞を含む細胞中間期はＤＰＢＳで洗浄され、完全培地に再懸濁され、回収物を得るために計数された。パーコール中間期で得られた洗浄単核細胞は完全培地３０ｍｌと１５－２０ｘ１０^{6}細胞／フラスコ（８．１ｘ１０^{4}ＭＳＣ／ｃｍ^{2}）を含むＴ－１８５フラスコ内で確立され、炭酸ガス濃度５％で３７℃のインキュベーターに安置された。

【００８９】

ＭＳＣの収穫

三つ組フラスコ内の培地は傾瀉されフラスコはＤＰＢＳ５０ｍｌで洗浄された。ＤＰＢＳを傾瀉した後、０．０５％のトリプシン２３ｍｌが各三つ組みフラスコに加えられた。フラスコは３７℃インキュベーターに３分配置された。細胞剥離後に、完全培地２３ｍｌが各フラスコに加えられた。細胞懸濁液は５０ｍｌ円錐管に移されフラスコは３０ｍｌのＨＢＳＳで洗浄された。管は２２００ＲＰＭ、５分ＲＴで遠心された。

【００９０】

形成／パッケージング

収穫されたＭＳＣｓはプラズマ－ライトＡ（バクスターＩＶセラピー）８５％、ＤＭＳＯ、１０％およびＭＳＣ提供者血清５％よりなる不凍溶液内で約１０ｘ１０^{6}細胞／ｍｌに形成され、１５－２０ｍｌを含む袋で冷凍保存された。

【００９１】

標識／貯蔵／送り

細胞は１分１－２°で９０℃の制御速度フリーザー（クライオメド、フォーマ・サイエンティフィック）を用いて冷凍保存された。サンプルは次いで気相（－１２０°乃至－１５０℃）で液体窒素貯蔵フリーザーに移された。

【００９２】

用量

２０ｘ１０^{6}細胞／ｋｇのＭＳＣ用量を達成するために、最終産物は注入日必要とされる用量の１１５％で調製された。

【００９３】

皮膚の収穫

手術に先立ち、ヒヒは手術時抗菌予防として５００ｍｇ、ＩＭでセファゾリンを与えられた。ヒヒは１０ｍｇ／ｋｇ、ＩＭでのケタミンで鎮静化され、また静脈内チオペンタール誘導、１－２％イソフルラン吸入麻酔で麻酔された。皮膚が腹前壁から収穫され、前もって標識された食塩水湿潤ガーゼパットに置かれた。創傷欠損部は次いで閉じられた。ヒヒは麻酔から覚めた時にコロニーに戻された。手術後の痛みを除くために、鎮痛剤ブプレノルフィンがＱ１２ｘ２用量でまたアンセフは２日間毎日投与された。

【００９４】
受容者皮膚移植とＭＳＣ注入
手術に先立ち、ヒヒは手術時抗菌予防として５００ｍｇ、ＩＭでセファゾリンを与えられた。ヒヒは鎮静化され１０ｍｇ／ｋｇ、ＩＭと静脈内チオペンタール誘導、１－２％イソフルラン吸入麻酔で麻酔された。皮膚が腹前壁から収穫され前もって標識された食塩水湿潤ガーゼパッドに置かれた。この皮膚は２個の移植片に分割された。１個はも一つの受容者ヒヒの第三者パーティ対照として使用され、も一つはこの同じ動物の自己由来対照として使用された。動物は次いでうつぶせの位置に置かれた。３個の３ｘ２ｃｍの皮膚の切片が肩甲骨の棘に沿った背中から除去された。前にＭＳＣ提供者第三者パーティおよび自身に適合するよう手入れされ、適所に縫合された。

【００９５】
移植後ヒヒは２０ｘ１０^{6}提供者ＭＳＣ／ｋｇの用量でＭＳＣの静脈内注入を受けた。末梢血サンプルはＭＳＣ注入前、注入１時間後、およびＭＳＣ注入１－３日後に確保された。骨髄吸引液はＭＳＣ注入０日後、３日、１４日および３０日後に得られた。

【００９６】
手術後の痛みを除くために、鎮痛剤ブプレノルフィンがＱ１２ｘ２用量で、またアンセフが２日間毎日投与された。動物は毎日観察され、移植片は移植７日後に開始して１日おきに写真撮影された。

【００９７】
物理的試験と診断検査
各ヒヒは試験のためにケタミン１０ｍｇ／ｋｇ、ＩＭで鎮静化された。鎮静の間に、２－３ミリリットルの骨髄が腸骨稜から針吸引で確保され、４日、１３日および研究終了の３０日後にヘパリンナトリウムに収集された。皮膚生検が骨髄吸引の得られた同じ日に収穫された。

【００９８】
結果
皮膚同種異系移植片生存に対するＭＳＣ注入の作用
未処置対照動物（Ｎ＝２）は皮膚同種異系移植片平均生存時間が８日±０日であった。未処置ＭＳＣ提供者のＭＳＣ提供者（Ｎ＝２）への注入は皮膚移植片平均生存時間が１１．５±０．７１日への延長をもたらした（マン－ホイットニー　Ｕ　テスト、Ｐ＜０．０５）。未処置第三者提供者ＭＳＣの提供者同種異系移植片（Ｎ＝４）への注入は平均生存時間１２．３±０．９６日の著しい延長をもたらした（マン－ホイットニー　Ｕ　テスト、Ｐ＜０．００３）。

【００９９】
受容者６１４０と６２００はＭＳＣ提供者６２４３から、お互いから（第三者移植片）、また彼等自身から（自己移植片）同種移植片を受けた。ＭＳＣ提供者６２４３から皮膚移植片収穫の２４時間前に６２４３からのＭＳＣが移植のために輪郭を描かれた腹前部皮膚下部に注射された。移植後に受容者は２０ｘ１０^{6}ＭＳＣ／ｋｇ（６２４３）の静脈内注入を投与された。第三者同種移植片はいずれも１３日に拒絶された。ＭＳＣ提供者（６２４３）同種移植片は４日目に出血したことが発見され、この出血は技術的な失敗によるものとされた。病理学試験では、ケラチンは真皮の下でわだち状に徐々に入り込んでいることが認められた。これらのわだちの性質はそれが皮下ＭＳＣ注射の際に針で形成されたことを示唆している。これら細胞の存在は非常に大きい炎症性応答を引き出した。この炎症性応答は皮膚移植片が適切に粘着／取り込む能力を妨げ、これらの移植片は７日までに完全に壊死した。自己移植片に拒絶されなかった。

【０１００】
受容者６６５４と６６５９はＭＳＣ提供者６５９３から、お互いから（第三者移植片）、また彼等自身から（自己移植片）同種移植片を受けた。移植後に受容者は２０ｘ１０^{6}ＭＳＣ／ｋｇの静脈内注入を投与された。ＭＳＣ提供者同種移植片は１１日と１２日に拒絶され、また第三者提供者同種移植片は１１日と１２日に拒絶された。自己移植片は拒絶さ

れなかった。
【０１０１】
同様に受容者６６６３と６６５８はＭＳＣ提供者６６５６から、お互いから（第三者移植片）、また彼等自身から（自己移植片）同種移植片を受けた。移植後受容者は２０ｘ１０^{6}ＭＳＣ／ｋｇの静脈内注入を投与された。ＭＳＣ提供者同種移植片は１１日に拒絶され、また第三者同種移植片は１０日と１２日に拒絶された。自己移植片は拒絶されなかった。
【０１０２】
研究の対照部門にある受容者６５３２と６７２０はＭＳＣの投与なしで注入または注射により自己移植片と同種移植片を受けた。同種移植片は８日に拒絶された。自己移植片は拒絶されなかった。
【０１０３】
同種異系ＭＳＣ注入と関連する同定可能毒性は存在せず、また続く３０日の追跡期間に不都合な臨床的後遺症は見られなかった。血液サンプルはＭＳＣの注入前および移植とＭＳＣ注入後１時間、２時間、更に１日、２日、および３日後に獲得された。骨髄吸引液は移植とＭＳＣ注入後４日と１３日に確保された。
【０１０４】
これらの結果は、同種異系ヒヒＭＳＣｓの単一注入が同種異系皮膚移植片の拒絶を遅延できることを示している。他の免疫抑制治療法は行なわれなかった。同種異系または第三者ＭＳＣｓの１回の投薬量は拒絶に至る時間を５０％増加した（グッドマン他、Ａｍ　Ｓｕｒｇ　６２巻（６号）：４３５－４４２ページ（１９９６年））。
【０１０５】
（実施例８）
本研究の目的は、イヌにおける適度に高い投与量の提供者イヌ白血球抗原（ＤＬＡ）－同一同腹子イヌ間葉幹細胞（ｃＭＳＣ）の注入の実現可能性および安定性を証明することである。提供者イヌ白血球抗原（ＤＬＡ）－同一同腹子イヌ間葉幹細胞（ｃＭＳＣ）は同種異系骨髄移植片定植において細胞１０ｘ１０^{6}個／ｋｇで投与される。第二の目的は、移植後５０および１００日目での提供者ｎｅｏ－およびＧＦＰ－標識ｃＭＳＣの分布および機能を調べることである。
【０１０６】
材料と方法
実験動物
ビーグル犬が本研究のために用いられた。０日目で７または９カ月齢のＤＬＡ同一同腹子の雄２匹、雌２匹が用いられた。分類のために用いられた方法は、主要組織適合抗原複合体が同じイヌのイヌ白血球抗原（ＤＬＡ）中クラスＩＩ　ＤＲＢ領域の遺伝形質を追跡するための高い多型マイクロサテライト標識の使用を含む。マイクロサテライトは小さい２、３，または４ヌクレオチドの反復であり、対立遺伝子中で十分な長さの違いが見られるため、多世代交雑を経た染色体断片の遺伝形質の追跡に用いることができる。対立遺伝子の分離は、通常各反復を取り囲むＤＮＡの特有な配列から得たプライマーを用いた一段階ポリメラーゼ連鎖反応を用いて測定される。加えて、混合白血球反応はＰＣＲマイクロサテライト標識分析の結果を確定するために、研究用ＤＬＡ同一同腹子のつがいに対して行われた。
【０１０７】
研究計画
研究用イヌはＤＬＡ同一同腹子の同一提供者から獲得したｃＭＳＣおよび骨髄の移植を受けた。骨髄移植片は、全身性Ｘ線照射（ＴＢＩ）に先だって０日目に２匹のＤＬＡ同一同腹子提供者から回収され交換された。骨髄離解は、０日目にイヌに９２０センチグレイ（ｃＧｙ）（７ｃＧｙ（９．３Ｒ）／分の割合で送られる二つの阻害^{60}ＣＯ源からの正中大気露出）の単独ＴＢＩ照射により誘発された。移植４週前またはそれ以上前の週で提供者骨髄吸引液から単離された培養増殖ｃＭＳＣは、グリーン蛍光タンパク質（ＧＦＰ）お

よびネオマイシンホスホトランスフェラーゼ（ｎｅｏ）遺伝子を含むＰａｐｐ@ＯＴ－２４〔原文通り〕が導入された。ｃＭＳＣは継代１（Ｐ１）または継代２（Ｐ２）の後に低温保存された。ＴＢＩに続いて、ｃＭＳＣｓは解凍され携帯注入ポンプにより１５分間静脈内に輸送された。ｃＭＳＣ注入後の１時間乃至２時間の間、骨髄移植片は全有核細胞（ＴＮＣ）≧１ｘ１０^{8}個／ｋｇの投与量で静脈内に注入された。

【０１０８】

シクロスポリンは移植片対宿主性病（ＧＶＨＤ）の予防のために０日目乃至５日目にかけて１０ｍｇ／ｋｇ　ＢＩＤ（２０ｍｇ／ｋｇ／ｄａｙ）（サンジミューン（Ｒ）インジェクションソリューション、サンド　ファーマシューティカル　コーポレーション）の投与量で４頭のイヌ全ての静脈内に投与された。グループＩ．１．ａについては６日目乃至５０日目（研究の終了）にかけて、またはグループＩ．１．ｂについては６日目乃至１００日目にかけてシクロスポリンは１０ｍｇ／ｋｇ　ＢＩＤ　ＰＯ（２０ｍｇ／ｋｇ／ｄａｙ）（ネオラル（Ｒ）ソフトゼラチンカプセル、サンド　ファーマシューティカル　コーポレーション）で投与された。受容者への抗生物質の経口投与による通常の補助介護は５日前から、抗生物質の全身性投与は０日目から開始し、移植片が得られるまで続けられた。液体補助は必要に応じて行われた。血小板輸注は、回復中の４頭のイヌ全てに必要とされなかった。標準イヌ処置法では、血小板値が一定して１０，０００個／ｍｍ^{3}より下に下がりまたは処置スタッフが出血の徴候を確認した場合には全血輸血が必要である。必要であれば血小板輸注は、任意の提供者から獲得した全Ｘ線照射（２０００ｃＧｙ）血の５０ｍｌ量で実施される。移植片は最初の完全好中球細胞＞５００個／ｍｍ^{3}、＞１，０００個／ｍｍ^{3}および血小板＞１０，０００個／ｍｍ^{3}、５０，０００個／ｍｍ^{3}＞、および＞１００，０００個の３連続した測定値の時に定植された。

【０１０９】

続く造血回復のために、完全血球算定（ＣＢＣｓ）が０日目乃至５０日目にかけて、１００日の研究グループについてはその後各週で得られた。血清化学分析は０、２日目、それ以降は毎週一回行われた。末梢血サンプルはＤＮＡ単離のために全ＭＳＣ注入の０日目、５分および１５分、１時間および２時間そして１日、２日、３日および４日目に採取された。ＤＮＡは、産物中に取り込まれるジゴキシゲニンを用いた抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ法および第２段階として抗ジゴキシゲニン比色定量分析によりＧＦＰ標識細胞の存在について評価された。骨髄吸引液は血小板数値が一定して５０，０００個／ｍｍ^{3}に達した時に得られ、同一ＰＣＲ法を用いてＧＦＰ標識細胞の存在について調べられた。培養ｃＭＳＣはコロニー形成単位（ＣＦＵ）を調べるために、また更なる抗ＥＧＦＰ　ＰＣＲ分析用にｃＭＳＣを増殖させるために樹立された。剖検時に末梢血、骨髄吸引液および骨髄生検材料は抗ＥＧＦＰ　ＰＣＲ分析のために得られた。ＣＦＵ分析は骨髄吸引液に対して行われ、抗ＥＧＦＰ　ＰＣＲ分析は培養増殖ｃＭＳＣに対して行われた。組織学的解析は種々の組織におけるＧＦＰの存在について行われた。

【０１１０】

ｃＭＳＣの単離、培養増殖、形質導入および低温保存

両側の骨髄吸引液はｃＭＳＣの単離、およびイヌＣＡＮ－０７－０１およびＣＡＮ－０７－０２については４週前で、イヌＣＡＮ－０７－０３およびＣＡＮ－０７－０４については９週前での培養樹立のために得られた。骨髄１５ｍｌ（各上腕骨から７ｍｌ）は各イヌから得られた。イヌはブトルファノールの注入、続くジアゼパムおよび塩酸ケタミンの混合物（アベコ　カンパニー，インコーポレイテッド、フォートダッジ、アイオワ）の注入により麻酔をかけられた。針挿入部位はポビドンヨードで洗浄されその後アルコールですすぎ落とした。吸引液は、１６ゲージ、２インチの骨髄針を用いて各イヌの両上腕骨顆から得られた。注射器は針に取り付けられ、各上腕骨から骨髄８ｍｌを取り出すために吸引した。骨髄吸引液は無菌的手法で１５ｍｌのポリプロピレン円錐形チューブに移された。本方法に続いてイヌは回復のために温床に置かれた。

【０１１１】

骨髄の一部５ｍｌ乃至１０ｍｌはポリプロピレン培養チューブ中で、ダルベッコリン酸緩

衝生理食塩水（ＤＰＢＳ）中で５０ｍｌに希釈された。細胞懸濁液は室温（ＲＴ）で１０分間、２２００ＲＰＭで遠心された。全有核細胞数は４％酢酸中で測定された。細胞はその後、最終濃度２０ｘ１０^{6}個／ｍｌまでＤＰＢＳ中で希釈された。１０ｍｌまたは２００ｘ１０^{6}個の細胞は５０ｍｌ円錐形チューブ中で２０ｍｌのパーコール（ｓｐ．ｇｒ．１，０７３ｇｍ／ｍｌ）に乗せられ、２０分間１３００ＲＰＭで遠心された。単核細胞を含む細胞界面はＤＰＢＳ中で洗浄され、完全培地中に再懸濁され、回復割合を得るために計数された。細胞はその後完全培地中に希釈され、培養細胞は前に記載されたように樹立され５％二酸化炭素濃度で３７℃のインキュベーター中に置かれた。

【０１１２】

２シストロンＭｕＬＶレトロウイルスベクターの構成

グリーン蛍光タンパク質（ＥＧＦＰ）レトロウイルスはクラゲ、エクオレアビクトリア（クロンテック、カリフォルニア）からＥＧＦＰ－１遺伝子を単離することにより構成された。ＥＧＦＰ遺伝子はレトロウイルスベクターｐＪＭ５７３－ｎｅｏ（帰属プラスミドの名称はｐＯＴ－２４）中にクローン化された。ｐＪＭ５７３－ｎｅｏプラスミドは、修飾されたｐＮ２（ケラー他、１９８５年、Ｎａｔｕｒｅ　３１８巻：１４９ページ）から得られた。ｐＮ２の修飾は次のようであった：マウスレトロウイルスｇａｇ開始部位が枠内終止コドンで置換され、５’ＬＴＲおよび３’ＬＴＲが同一カセット内に構成され、ネオマイシンホスホトランスフェラーゼ遺伝子（ｎｅｏ）および内部リボソームエントリー部位（ＩＲＥＳ）がｐＮ２中に挿入された。ＥＧＦＰ　ｐＯＴ２４プラスミドの模式図は図８に示す。

【０１１３】

組換えレトロウイルスの調製

ｐＯＴ－２４は、製造者によって示されたようにＤＯＴＡＰ（ベーリンガー　マンハイム）を用いてＧＰ＆Ｅ８６環境栄養性産生細胞中に形質移入された。形質移入細胞は、１０％熱失活ＦＢＳ、ペニシリン－ストレプトマイシン（ライフ　テクノロジーズ）および選択標識としての硫酸プロタミン－Ｇ４１８（シグマ）０．５ｍｇ／ｍｌの補足されたＤＭＥＭ－高グルコース（ＨＧ）培地中で生育された。培養細胞は密集度７０％になるまで維持され、その時点で培地は新鮮レトロウイルス培地（Ｇ４１８無し）に交換され、細胞は３２℃で２日間維持された。レトロウイルスを含む培養培地は回収され０．４５μｍフィルターを通して濾過され－７０℃で保存された。両栄養性レトロウイルスは、遠心導入法と続くＧ４１８（０．５ｍｇ／ｍｌ）での選別法を用いてＰＡ３１７細胞を環境栄養性ウイルスに２回形質導入することにより調製された。レトロウイルス上澄みは回収された。貯留ＥＧＦＰウイルスの３Ｔ３細胞に対する力価は１．２ｘ１０^{6}ＣＦＵ／ｍｌであった。ＧＦＰレトロウイルス上澄みは－７０℃で低温保存された。

【０１１４】

ＣＡＮ－０７－０１およびＣＡＮ－０７－０２

パーコール界面から得られた洗浄された単核細胞は完全培地３０ｍｌおよび細胞数１０ｘ１０^{6}個／フラスコを含むＴ－１８５フラスコ１０個中で樹立された。

【０１１５】

培養２、６、９日目にフラスコ中の培地は新鮮完全培地と交換された。初期培養の１２日目に写真を撮り、細胞は継代０（Ｐ０）から継代１（Ｐ１）に移された。培地は吸引されフラスコは８ｍｌＤＰＢＳで２回洗浄された。トリプシン８ｍｌが添加され、フラスコは３７℃インキュベーター中に３分間置かれた。細胞が剥離し、完全培地８ｍｌの添加により反応は止められた。細胞は５０ｍｌ円錐形チューブに移され貯留された。フラスコはＤＰＢＳで洗浄され貯留細胞は室温で５分間、２０００ＲＰＭで遠心された。上澄みは除去され細胞のペレットは完全培地中に再懸濁された。細胞は貯留され、測定され生存可能性が調べられた。細胞は完全培地１８ｍｌおよび細胞数フラスコ当たり０．４ｘ１０^{6}個を含むＴ８０フラスコ１５個中で平板培養された。

【０１１６】

培養１５日目、１８フラスコのうち１５フラスコに最初の形質導入が行われた。培地は除

かれた。レトロウイルス上澄みの一部が解凍され、形質導入混合物を作るためにポリブレンが最終濃度８μｇ／ｍｌまで添加された。細胞培地は形質導入混合物１０ｍｌと交換されフラスコは３２℃で１時間、３０００ＲＰＭで遠心された。遠心後、熱失活胎児ウシ血清（ＦＢＳ）を用いて調製された完全培地１０ｍｌは（形質導入混合物を含む）各フラスコに添加され、フラスコはインキュベーターに戻された。フラスコ３つは形質導入をせず、新鮮培地が交換された。培養１６日目、培地は新鮮完全培地に交換された。培養１７日目に形質導入法が繰り返された。

【０１１７】

培養１８日目、細胞は前に記載したように回収されＰ１からＰ２に移された。細胞３ｘ１０^{6}個は完全培地１００ｍｌ中に添加され三つ組みフラスコ（５００ｍｍ^{2}）中に注入された。三つ組みフラスコ１５個は形質導入細胞で調製され３個は非形質導入細胞で調製された。残りの細胞は低温保存された。凍結溶液は１０％ＤＭＳＯおよび９０％ＦＢＳを含むように調製された。細胞１０ｘ１０^{6}個は凍結溶液１ｍｌ中に再懸濁された。バイアルはラベルを貼られ最低４時間はナルジーン　クリオ　コンテナ中に低温保存された。

【０１１８】

Ｐ２培養２２日目、細胞分布と形態を記録するために写真が撮られ、Ｐ２細胞は回収され前に記載されたように低温保存された。

【０１１９】

ＣＡＮ－０７－０３およびＣＡＮ－０７－０４

パーコール界面で得られ洗浄された単核細胞は、完全培地２０ｍｌおよび細胞１２ｘ１０^{6}個／フラスコを含むＴ－７５フラスコ１５個中に樹立された。

【０１２０】

培養２日目、フラスコおよびディッシュ中の培地は新鮮完全培地と交換された。ｃＭＳＣの初代培養の６日目、最初の形質導入が前に記載したように行われた。フラスコ３個は形質導入されず新鮮培地は６日目に交換された。培養７日目、培地は新鮮培地に交換された。

【０１２１】

培養８日目、形質導入法は繰り返された。培養９日目、写真が撮られ、細胞は前に記載されたようにＰ０からＰ１に進められた。細胞３ｘ１０^{6}個は完全培地１００ｍｌに添加され三つ組みフラスコ中に注入された。三つ組みフラスコ１５個は形質導入細胞で調製され３個は非形質導入細胞で調製された。

【０１２２】

骨髄吸引液１５ｍｌは提供者ＣＡＮ－０７－０１、ＣＡＮ－０７－０２、ＣＡＮ－０７－０３およびＣＡＮ－０７－０４からそれぞれ有核細胞９１０、１２１２、８５６および１９４８ｘ１０^{6}個を産生した。パーコール界面から得られた単核細胞数は６１２、６６６、５８８および４６２ｘ１０^{6}個であり６７．２、５５、６８．７および２３．７％の回復であった。Ｐ１時、細胞生存可能性は平均９７．１（９３．３乃至１００の範囲）％であった。提供者ＣＡＮ－０７－０１およびＣＡＮ－０７－０２のＰ２および提供者ＣＡＮ－０７－０３およびＣＡＮ－０７－０４のＰ１細胞の時、形質導入細胞の細胞の生存可能性は平均９６．７（９６．３乃至９７．９の範囲）％であった。非形質導入細胞は９５．４（９３．３乃至９６．９の範囲）％の生存可能性であった。ｃＭＳＣの低温保存のための回収時には、形質導入細胞の生存可能性は平均９９．４（９７．４乃至１００の範囲）％であり、非形質導入細胞は９９．４（９７．６乃至１００の範囲）％であった。

【０１２３】

継代２後４日目に回収されフラスコ当たり３ｘ１０^{6}個で平板培養された、提供者ＣＡＮ－０７－０１およびＣＡＮ－０７－０２のフラスコ当たりの産生された形質導入ｃＭＳＣは５．９および６．７ｘ１０^{6}個であり、フラスコ当たりの産生された非形質導入ｃＭＳＣは８．４および７．５ｘ１０^{6}個であった。継代１（異なる形質導入および継代設定）後４日目に回収されフラスコ当たり３ｘ１０^{6}個で平板培養された、提供者ＣＡＮ－０７－０３およびＣＡＮ－０７－０４のフラスコ当たりの産生された形質導入ｃＭＳＣは２０

．０および１４．０ｘ１０^{6}個であり、フラスコ当たりの産生された非形質導入ｃＭＳＣは２５．３および１８．０ｘ１０^{6}個であった。

【０１２４】

Ｐ０培養細胞から獲得したｃＭＳＣに対するＣＦＵ分析

ＣＦＵコロニー分析は完全培地１０ｍｌを含む１００ｍｌディッシュ３個内で細胞０．５ｘ１０^{6}個を平板培養することにより初代培養樹立時に調製された。ディッシュは３７℃、５％二酸化炭素濃度でインキュベートされた。培地は２日目、４日目に新鮮培地と交換された。培養１０日目、ＣＦＵ分析ディッシュはＨＢＳＳで２回すすぎ落とされ、１％グルタルアルデヒドで１５分間固定され、ＨＢＳＳで２回すすぎ落とされ風乾された。ディッシュ中のｃＭＳＣはその後０．１％クリスタルバイオレットで染色され、脱イオン水で３回洗浄され風乾された。コロニーは平板培養された細胞１０^{6}個当たりのコロニー形成数を算定するために測定された。

【０１２５】

単核細胞単離および培養樹立の日に平板培養され１０日目に回収されたＣＦＵ分析はイヌＣＡＮ－０７－０１、ＣＡＮ－０７－０２、ＣＡＮ－０７－０３およびＣＡＮ－０７－０４それぞれから細胞１０^{6}個当たり５６、４６．７、１１４および７２コロニーを産生した。

【０１２６】

Ｐ１培養の１３日目、細胞分布と形態を記録するために写真が撮られ、Ｐ１細胞はトリプシン処理により回収され前に記載されたように低温保存された。

【０１２７】

三つ組みフラスコ中の培地は傾瀉され、フラスコはＤＰＢＳ５０ｍｌですすぎ落とされた。ＤＰＢＳの傾瀉後０．２５％トリプシン２３ｍｌが各三つ組みフラスコに添加された。フラスコは３分間３７℃インキュベーター中に置かれた。細胞剥離後２３ｍｌ完全培地が各フラスコに添加された。細胞懸濁液は５０ｍｌ円錐形チューブに移され、フラスコはＨＢＳＳ３０ｍｌで洗浄された。チューブは室温で５分間、２２００ＲＰＭで遠心された。形質導入または非形質導入細胞を含むペレットはそれぞれ貯留され計数された。細胞１ｘ１０^{7}個の一部は、抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ分析による形質導入割合の測定のために取っておかれた。

【０１２８】

回収後、回復され、Ｐ１またはＰ２形質導入され、培養増殖されたｃＭＳＣは５分間１３００ＲＰＭで遠心され、８５％プラスマ－ライトＡ（バクスター　ＩＶ　セラピー）、１０％ＤＭＳＯおよび５％自己由来イヌ血清を含む氷冷不凍溶液中でｃＭＳＣ１ｘ１０^{7}個／ｍｌの１ｍｌ中に再懸濁された。細胞のアリコートは別々の低温バイアルに各１ｍｌずつ分けられた。チューブはイヌ提供者番号および全生存可能細胞数の書かれたラベルを貼られた。ｃＭＳＣｓは細胞バイアルをナルジーン　フリージング　コンテナ中に置いて低温保存され、４時間－７０℃冷凍庫に置かれその後－７０℃で貯蔵庫に移された。

【０１２９】

産物の低温保存のために細胞を回収した時に、細胞１ｘ１０^{7}個のアリコートは形質導入効率の測定のために得られた。形質導入効率は、産物中に取り込まれるジゴキシゲニンを用いた抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ法および第２段階として抗ジゴキシゲニン比色定量分析により分析された。

【０１３０】

ｃＭＳＣ注入産物

注入の１時間乃至２時間前にｃＭＳＣのバイアルは３７℃ウォーターバス中で回転させて解凍され、７０％エタノールが噴霧されバイオセイフティーキャビネット中で開けられた。ｃＭＳＣ産物はＤＭＥＭ－ＬＧ、細胞提供者に自己由来の３０％血清を含む注入培地５０ｍｌ中に懸濁された。ｃＭＳＣ産物の生存可能性は、実際の生存可能投与量を測定するためにトリパンブルー除去により測定された。各ｃＭＳＣ産物のアリコートは酵母菌単離、好気的および嫌気的生育を受けた。ｃＭＳＣは、組織培養プラスチックへの接着および

Ｐ２（ＣＡＮ－０７－０１およびＣＡＮ－０７－０２に対してはＰ３）培地中での増殖能力について評価された。ｃＭＳＣ１ｘ１０^{6}および０．１６ｘ１０^{6}個のアリコートは、３個のＴ－２５プラスチック培養フラスコ中で完全イヌ培養培地中に平板培養された。ｃＭＳＣ１ｘ１０^{6}個を平板培養したフラスコは２４時間後、ｃＭＳＣ０．１６ｘ１０^{6}個を平板培養したフラスコは３日目にトリプシン処理により回収され計数された。

【０１３１】

ＴＢＩに続きｃＭＳＣ懸濁液は、１５分乃至２０分の間５０ｍｌ輸送するために手持ちのハーバード　バード　ミニ　インフューザーを用いて頭側静脈に挿入されたカテーテルを通して注入された。

【０１３２】

生存可能ｃＭＳＣ７．４９、７．３５、１０．０および１０．０（平均８．７）ｘ１０^{6}個／ｋｇの適度に高い投与量は、０日目にイヌＣＡＮ－０７－０１、ＣＡＮ－０７－０２、ＣＡＮ－０７－０３およびＣＡＮ－０７－０４にそれぞれ注入された。これらの投与量は被験者が受容する通常の投与量の４倍乃至１０倍増に相当する。注入された全生存可能ｃＭＳＣは６７．７乃至１２９（平均９３．９）ｘ１０^{6}個の範囲であった。細胞の生存可能性はトリパンブルー除去による測定で９２．１乃至９７．６（平均９４．９）の範囲であった。ｃＭＳＣ注入はＴＢＩ後７１乃至１４６（平均１１０）分の間に行われた。

【０１３３】

注入後の血液サンプリング

血液サンプル（２ｍｌ）はｃＭＳＣ注入の前におよび注入の間すなわち注入開始後５分および１５分、１時間および２時間そして１、２、３および４日目に得られた。細胞溶解産物はピュアジーン^{TM}（ジェントラ　システムズ，インコーポレイテッド）ＤＮＡアイソレーションキットを用いて調製された。このキットは、血流中のＧＦＰ標識ｃＭＳＣのレベルを検出するために産物中に取り込まれるジゴキシゲニンを用いた抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ法および第２段階として抗ジゴキシゲニン比色定量分析を使用する目的で用いられた。

【０１３４】

骨髄回収および移植片注入

移植片として用いられる骨髄はＴＢＩに先立ってＤＬＡ同一同腹子から回収された。吸引液は、１１ゲージ、４－６インチのボールトップステンレススチール回収針を用いて両上腕骨から得られた。回収針は１００ｍｌの組織培養培地１９９およびヘパリン４ｍｌ（４０００Ｕ）を含む真空フラスコのポリビニルチューブに取り付けられた。骨髄は３００μｍおよび２００μｍサイズの小孔を通され輸送パックコンテナ中に４℃で保存された。骨髄は提供者および受容者のラベルを貼られ後日の注入まで保存された。骨髄全有核細胞数（ＢＭ－ＴＮＣ）は、骨髄回収の間に得られた多量の末梢血中に存在する有核細胞を除外することで修正された。

【０１３５】

骨髄の全有核細胞数（ＴＮＣ）は骨髄回収の間に得られた多量の末梢血中に存在するＴＮＣを除去することにより修正された。骨髄の修正投与量は、イヌＣＡＮ－０７－０１、ＣＡＮ－０７－０２、ＣＡＮ－０７－０３およびＣＡＮ－０７－０４に対してそれぞれＴＮＣ４．３、３．５、３．１および２．０（平均３．２）ｘ１０^{8}個／ｋｇであった。無修正骨髄投与量はＴＮＣ５．６、４．２、４．５および２．７（平均４．３）ｘ１０^{8}個／ｋｇであった。

【０１３６】

注入の２０分前に骨髄は室温に置かれた。ｃＭＳＣ注入の一時間後骨髄は、１分乃至２分の間バックに圧力を加えることにより頭側静脈に挿入された蝶形針を通って静脈内に注入された。

【０１３７】

補助介護

５日前に、抗生物質の経口投与（硫酸ネオマイシンおよび硫酸ポリミキシン）は１日３回

行われた。これらの抗生物質の経口投与は、完全好中球数が５００個／ｍｍ^{3}に達するまで投与された。０日目、全身性抗生物質ベイトリルは静脈内に１日２回投与され完全好中球数が一定して１，０００個／ｍｍ^{3}に達するまで続けられた。短時間の放射線毒性の結果としての液体および電解質損失は、食物と水が摂取されるまで１日２回のリンガー液５００ｍｌの皮下投与により補われた。

【０１３８】

種々の血液細胞数

血液サンプル（２ｍｌ）は、ｃＭＳＣの単離のための骨髄吸引の朝、０日目乃至５０日目までおよびそれ以降は２週間に１回研究の終了まで、頸静脈または頭側静脈から回収された。血液はＥＤＴＡを含むバキュテーナー中に移された。１ｍｍ^{3}当たりの全白血球（ＷＢＣ）および血小板数はシスメックスＥ２５００を用いて測定され、種々の細胞数は固定およびライト染色液での染色後指示どおりに測定された。

【０１３９】

剖検

血液サンプルはＣＢＣ、化学２３分析およびＰＣＲ評価のために得られた。イヌはブトルファノールと続くジアゼパムおよび塩酸ケタミンの混合物により鎮静された。鎮静後、生検材料および両側の骨髄吸引液は上腕骨、大腿骨および腸骨稜から得られた。その後、安楽死が鎮静剤ペントバルビタールナトリウムの過剰投与で遂行された。５０日グループのイヌ（ＣＡＮ－０７－０１およびＣＡＮ－０７－０２）は研究４３日目に安楽死させられ、１００日グループのイヌ（ＣＡＮ－０７－０３およびＣＡＮ－０７－０４）は研究１００日目に安楽死させられた。組織の全組合わせは動物の剖検時に回収された。

【０１４０】

組織学的調査のための組織の回収は続いて即座に行われた。組織のサブセットは抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ分析に用いられた。骨髄吸引液および生検材料は抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ分析、更なるＰＣＲ分析のための培養増殖およびＣＦＵ分析に用いられた。

【０１４１】

組織は約１インチ平方の小片に形を整えられ、１０％中性緩衝ホルマリン（ｐＨ６．８－７．２）で満たされた、ラベルの貼られた別々の５０ｍｌ円錐形チューブ中に置かれた。組織はパラフィン中に包埋され、薄切されヘマトキシリンおよびエオシンで染色された。骨髄サンプルは過ヨウ素酸シッフ染色で染色された。

【０１４２】

剖検に先だって得られた骨髄吸引液は各イヌの左右の上腕骨、大腿骨および超骨稜からラベルの貼られた１５ｍｌチューブ中に回収された。組織サンプルのサブセットは剖検時に得られ約１／４インチ平方の小片に形を整えられ、ＰＢＳに浸したガーゼに包まれてラベルの貼られたジップロックバック中に別々に置かれた。骨髄吸引液は氷中に浸けられた。

【０１４３】

ＣＦＵ分析のための骨髄吸引液の調製

各イヌからＰＣＲ分析のために得られた左右上腕骨、大腿骨および腸骨稜の骨髄吸引液の一部は、別々のラベルの貼られた１５ｍｌチューブ中に分けられた。骨髄サンプルは氷中に浸けられた。

【０１４４】

剖検時に得られた骨髄から獲得したｃＭＳＣに対するＣＦＵ分析

剖検時に得られた骨髄から獲得したｃＭＳＣに対して行われたＣＦＵコロニー分析は完全培地１０ｍｌを含む１００ｍｍディッシュ三つ組みに細胞０．５ｘ１０^{6}個を平板培養することにより調製された。ディッシュは３７℃および５％二酸化炭素濃度でインキュベートされた。培地は２日間乃至４日間で新鮮培地に交換された。培養１０日目、ＣＦＵ分析ディッシュはＨＢＳＳで２回すすぎ落とされ１％グルタルアルデヒドで１５分間固定されて、ＨＢＳＳで２回すすぎ落とされ風乾された。ディッシュ中のｃＭＳＣはその後０．１％クリスタルバイオレットで染色され脱イオン水で３回洗浄されて風乾された。コロニー

は、平板培養された細胞10^6個当たりのコロニー数を算定するために算出された。

【０１４５】

ＤＮＡの単離および精製

ＤＮＡは各組織片から単離された。サンプルの残りは－７０℃フリーザー中で低温保存された。ＤＮＡは、サンプルをリン酸緩衝生理食塩水（ＰＢＳ）に置きプロテイナーゼＫ溶液を添加し、５５℃で３時間または組織が溶解するまでインキュベートすることにより単離された。サンプルは続いて３７℃で６０分間ＲＮアーゼで処置された。サンプルは室温に冷やされタンパク質は沈殿した。サンプルは遠心され、水溶層は１００％イソプロパノール中に丁寧に回収された。サンプルは混合され遠心された。ペレットは７０％エタノール中で洗浄された。チューブは遠心され、上澄みは抽出され、ペレットはおおよそ１時間乃至６時間乾燥された。ＤＮＡは室温で一晩水和され、続いて４℃で保存された。

【０１４６】

末梢血および骨髄サンプルは最初にＲＢＣ溶解溶液（塩化アンモニウム緩衝液）で溶解された。ＤＮＡはその後前に記載したように溶解液から単離された。ＤＮＡは、脱イオン水９９８μｌおよびサンプルから得られたＤＮＡ２μｌをキュベット中に添加することにより増量され撹拌された。分光光度計は光学密度（ＯＤ）を測定するために用いられた。ＯＤは２６０および２８０で読み取られ、ＤＮＡ濃度はμｇ／ｍｌで算出された。ＤＮＡ濃度は脱イオン水を用いて１μｇ／ｍｌに調整された。

【０１４７】

抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ

本研究で用いられた抗ＥＧＦＰ　ＤＮＡ　ＰＣＲ　Ｅｌｉｓａ分析は、ＧＦＰに特異的なオリゴヌクレオチドプライマーを利用して注入ｃＭＳＣｓを検出する。遺伝子発現の分析のために、我々はＰＣＲ－ＥＬＩＳＡ（ＤＩＧ標識／検出）キット（ベーリンガー　マンハイム）を利用した。要約すると、ＰＣＲは、増幅産物を標識するためのジゴキシゲニン標識ヌクレオチドの存在下で行われた。次にＰＣＲ産物２５μｌは、ストレプトアビジンがコートされたマイクロタイタプレートにおいて、３７℃の５’－ビオチン化オリゴヌクレオチドプローブ溶液中で変性されハイブリッド形成された。プローブ－ＰＣＲ結合産物は、抗ジゴキシゲニンペルオキシダーゼ複合体によりおよび比色定量基質２，２’アジノビス（３－エチルベンゾチアゾリン－スルホン酸）（ＡＢＴＳ）を用いることにより検出された。滴定基準曲線は、分析に用いられたＤＮＡの量当たりのＤＮＡ濃度を近似するために形質移入対照ｃＭＳＣを用いて作成された。ＤＬＡクラスＩＩゲノムＤＮＡのＰＣＲに対する内部標準との最初の相互作用、その後のＤＮＡ濃縮液の同量の細胞に対する相互作用および形質導入細胞当たりの一つのレトロウイルスの組込みの推定により概算細胞数を得ることができる。

【０１４８】

ＧＦＰのためのＤＮＡの量的測定は全ての骨髄ヘラ／生検材料において示された。

【０１４９】

移植後の血球回復

血小板閾値（３連続の数値）が１０，０００個／mm^3となる平均日数は１２．８（１１－１７の範囲）日、５０，０００個／mm^3となるのは１９．８（１６－２５の範囲）日および１００，０００個／mm^3となるのは２３．０（２０－２７の範囲）日であった。完全好中球細胞の閾値（３連続の数値）が５００個／mm^3となる平均日数は９．３（８－１１の範囲）日および１，０００個／mm^3となるのは１０．５（９－１３の範囲）日であった。

【０１５０】

仮骨髄吸引液

血小板が回復して１mm^3当たり５０，０００個以上の値に一定したとき、仮骨髄吸引液は腸骨稜から回収された。この方法は本研究においてＣＡＮ－０７－０１およびＣＡＮ－０７－０２については２７日目に、ＣＡＮ－０７－０３およびＣＡＮ－０７－０４については２９日目に行われた。

【０１５１】
結果
４３日目に安楽死させられたＣＡＮ－０７－０１およびＣＡＮ－０７－０２から獲得した全組織の組織病理学評価については、結果は異所性結合組織および亜急性のＧＶＨＤに対して陰性であった。
【０１５２】
検出可能なＤＮＡシグナルは注入の１時間の間および２日目に再び観察できた。サンプルの一つはＧＦＰ　ＤＮＡについて注入３日後に量的に測定できた。この時間は、自己由来のイヌ移植研究においてシグナルが２および３日目に見られたという前の観察と一致している。
【０１５３】
ＣＡＮ－０７－０３およびＣＡＮ－０７－０４におけるＧＦＰ＋細胞についての１００日目剖検データは、ＣＡＮ－０７－０３の大腿骨および上腕骨に、ＣＡＮ－０７－０４の上腕骨にＧＦＰシグナル（ＰＣＲ挿入ＤＮＡ１０μｇ当たりの同量の１ＧＦＰ＋細胞）を示した。
【０１５４】
このモデルにおいては、動物の目および耳の充血を観察することにより皮膚移植片対宿主性病（ＧＶＨＤ）を検出することが可能であった。この指標を用いて、間葉肝細胞を受容した動物は間葉肝細胞で処置されなかった対照動物と比較してＧＶＨＤの発症度が低かったことが測定された。
【０１５５】
これらの結果は、同種異系ＭＳＣｓが骨髄造血細胞の迅速な移植を補助できることを証明している。輸液補助は必要でなかった。ＧＶＨＤの臨床徴候は無かった。血小板回復はこれまでの対照よりも早かった。これらは同種異系移植後の同質細胞におけるキメラ現象の証拠である。同種異系ＭＳＣｓを用いることによる移植同種異系組織の選択は、臨床移植の場面で用いることができる移植片材料の範囲を広げる。
【０１５６】
（実施例９）
ＭＳＣ上澄み（ＭＬＲ９５）による混合リンパ球反応の抑制
上澄みの産生
提供者１５５から獲得したＴ細胞は、抗ＣＤ１９および抗ＣＤ１４マイクロビーズ（ミルテニー　バイオテック）を用いた陰性免疫磁気選別法によりＰＢＭＣから精製された。提供者４１３から獲得したＰＢＭＣは３６００ラド（７０ｋＶで１２分）でＸ線照射された。２４ウェル組織培養プレート中で、Ｔ細胞（９ｘ１０^{5}個／ウェル）は３日間Ｘ線照射ＰＢＭＣｓと混合され、その後異なる提供者（２１９、４５９、４６１－全て）から獲得したＭＳＣｓが３日間１．２ｘ１０^{5}個／ウェルで培養細胞中に添加された。対照培養では、同量の培地がＭＳＣｓの代わりに添加された。別々のウェルにおいて、同数のＭＳＣｓは単独で平板培養され３日間培養された。培養３日後（初期ＭＬＲの開始後６日目）細胞はピペッティングにより再懸濁され、細胞懸濁液２００μｌは９６ウェルプレート三つ組みに移され、増殖レベルを測定するために〔^{3}Ｈ〕ＴｄＲ（５Ｃｉ／ｍｍｏｌ、１μＣｉ／ウェル）で１８時間標識された。残りの細胞は１２５０ｒｐｍで１０分間遠心され、上澄みは回収され、分別されて－８０℃で凍結された。
【０１５７】
上澄みによる初期ＭＬＲの抑制
提供者１５５から獲得したＴ細胞は、抗ＣＤ１９および抗ＣＤ１４マイクロビーズ（ミルテニー　バイオテック）を用いた陰性免疫磁気選別法によりＰＢＭＣｓから精製された。提供者４１３または提供者２７３から獲得したＰＢＭＣｓは３６００ラド（７０ｋＶで１２分）でＸ線照射された。９６ウェル組織培養プレート中でＴ細胞（１．５ｘ１０^{5}個／ウェル）は、種々の上澄みの存在または不在下でＰＢＭＣｓ（１．５ｘ１０^{5}個／ウェル）と混合された。この上澄みは培養開始時に各希釈濃度（１／８、１／３２、１／１２８

、１／５１２、１／２０４８および１／８１９２）で添加されたものである。対照培養においては、同量の培地が上澄みの代わりに添加された。培養細胞は６日間インキュベートされ、その後〔^{3}Ｈ〕ＴｄＲ（５Ｃｉ／ｍｍｏｌ、１μＣｉ／ウェル）で１８時間標識された。

【０１５８】

図９はＴ１５５ｘＰＢＭＣ４１３間のＭＬＲの結果を示す。ＭＳＣｓ＋ＭＬＲ（＃１）、ＭＬＲ＋ＭＳＣ２１９（＃２）、ＭＬＲ＋ＭＳＣ４５９（＃３）、ＭＬＲ＋ＭＳＣ４６１およびＭＳＣｓ単独（＃８）、ＭＳＣ２１９単独（＃９）、ＭＳＣ４５９単独（＃１０）、ＭＳＣ４６１単独の上澄みの存在下では、初期ＭＬＲは抑制された。ＭＬＲ単独（＃５）の上澄みの存在下では初期ＭＬＲは抑制されなかった。

【０１５９】

進行中のＭＬＲの抑制

提供者１５５から獲得したＴ細胞は、抗ＣＤ１９および抗ＣＤ１４マイクロビーズ（ミルテニー　バイオテック）を用いた陰性免疫磁気選別法によりＰＢＭＣｓから精製された。提供者４１３から獲得したＰＢＭＣｓ（１．５ｘ１０^{5}個／ウェル）は３６００ラド（７０ｋＶで１２分）でＸ線照射された。９６ウェル組織培養プレート中でＴ細胞（１．５ｘ１０^{5}個／ウェル）はＰＢＭＣｓ（１．５ｘ１０^{5}個／ウェル）と４日間混合され、その後上澄みは種々希釈濃度（１／８、１／３２、１／１２８、１／５１２、１／２０４８および１／８１９２）で培養細胞に添加された。対照培養においては、同量の培地が上澄みの代わりに添加された。培養細胞は２日間（ＭＬＲ開始後６日目）インキュベートされ、その後〔^{3}Ｈ〕ＴｄＲ（５Ｃｉ／ｍｍｏｌ、１μＣｉ／ウェル）で１８時間標識された。

【０１６０】

図１０はＴ１５５ｘＰＢＭＣ４１３間のＭＬＲの結果を示す。ＭＳＣｓ＋ＭＬＲ（＃１）、ＭＬＲ＋ＭＳＣ２１９（＃２）、ＭＬＲ＋ＭＳＣ４５９（＃３）、ＭＬＲ＋ＭＳＣ４６１から獲得した上澄みは進行中のＭＬＲｓの強い抑制を示した。ＭＳＣｓ単独（＃８）、ＭＳＣ２１９単独（＃９）、ＭＳＣ４５９単独（＃１０）、ＭＳＣ４６１単独から獲得した上澄みは投与依存法において抑制し、１／５１２希釈まで顕著な効果を示した。ＭＬＲ単独（＃５）の上澄みの存在下では進行中のＭＬＲは抑制されなかった。

【０１６１】

（実施例１０）

異種間葉幹細胞による混合リンパ球反応の抑制

ヒヒＭＳＣｓによるヒトＭＬＲｓの抑制

ヒト提供者（Ｒ４、Ｒ６、Ｒ７、Ｒ１１）から獲得した応答ＰＢＭＣｓは、マイクロタイタウェル内でＸ線照射（３０００Ｒ）同種異系ヒトＰＢＭＣｓ（Ｓ４、Ｓ６、Ｓ７、Ｓ１１）と混合された。各集団は細胞１．５ｘ１０^{5}個／ウェルである。培養は５％ヒトＡＢ血清を含む標準細胞培養培地中で行われた。提供者８６２４３から獲得したヒヒＭＳＣｓ（ｂＭＳＣｓ）はＭＬＲの開始時に２ｘ１０^{4}個／ウェルで添加された。ＭＳＣｓはＩＦＮγで処置されなかった。リンパ球増殖は、培養７日目にシンチレーション測定のための細胞回収に先立って細胞を^{3}Ｈ－チミジンで１８時間標識することにより測定された。図１１に示された結果は、ヒヒＭＳＣｓが強固なヒトＭＬＲｓを５０％以上抑制したことを示している。

【０１６２】

ヒトまたはヒヒＭＳＣｓによる異種ＭＬＲの抑制

提供者２７３から獲得した応答ヒトＴ細胞（ｈＴ）は、提供者５９５７または提供者５９０９から獲得したＸ線照射（３０００Ｒ）ヒヒＰＢＭＣ（ｂＰＢＭＣ）と５％ヒトＡＢ血清を含む標準細胞培養培地中で培養された。提供者２４４から獲得したヒトＭＳＣｓ（ｈＭＳＣｓ）または提供者６２４３から獲得したヒヒＭＳＣｓ（ｂＭＳＣｓ）は開始時に培養細胞に添加された。リンパ球増殖は、培養７日目にシンチレーション測定のための細胞回収に先立って細胞を^{3}Ｈ－チミジンで１８時間標識することにより測定された。図１２（ｂＰＢＭＣ提供者５９５７）および図１３（ｂＰＢＭＣ５９０９）に示された結果は、

ヒトおよびヒヒＭＳＣｓの両方が異種ヒトｘヒヒＭＬＲを抑制することができることを示している。

【図面の簡単な説明】

【図１】同種異系間葉幹細胞が免疫応答を誘導しないことを示す図。Ａから獲得されたＴ細胞は、投与依存法において異なる量のＢから獲得されたＰＢＭＣｓと混合されたとき増殖した。Ａから獲得されたＴ細胞は、Ｂから獲得された間葉幹細胞との接触に対する応答として増殖しなかった。間葉幹細胞が十分なＴ細胞活性化を提供するように操作されていた場合（間葉幹細胞はＩＦＮ－γで処置され、また補助的刺激分子Ｂ７－１またはＢ７－２を形質導入された）においても増殖しなかった。

【図２】間葉幹細胞が異なる二個体から獲得したリンパ球間の混合リンパ球反応（ＭＬＲ）を積極的に抑制することを示す図。受容者に対して同種異系（第三者または提供者）のｈＭＳＣｓは、ＭＬＲ中の刺激細胞および応答細胞の両方に不適合であった（白抜き部）か、またはＭＬＲ中の刺激細胞に対して適合（提供者）していた（斜線部）。このように、間葉幹細胞はＭＨＣ型に関して特異性を持たずにＭＬＲを抑制した。間葉幹細胞は投与依存法においてＭＬＲを抑制した。

【図３】刺激（同種異系）ＰＢＭＣｓにより特発され、ＭＳＣｓへの露出をせず、その後自己由来ＰＢＭＣｓ、同種異系ＰＢＭＣｓ（刺激細胞または第三者）に露出されまたは細胞に露出されなかった応答Ｔ細胞の二次応答を示す図。

【図４】刺激（同種異系）ＰＢＭＣｓにより活性化され、続いて同種異系ＭＳＣｓ（刺激細胞）と共に培養され、その後自己由来ＰＢＭＣｓ、同種異系ＰＢＭＣｓ（刺激細胞または第三者）に露出されまたは細胞に露出されなかった応答Ｔ細胞の二次応答を示す図。

【図５Ａ】刺激同種異系ＰＢＭＣｓにより前活性化され、活性化後、同種異系（同一提供者（図５Ｂ）、または第三者（図５Ｄ））あるいは自己由来ＭＳＣｓ（図５Ｃ）と共に培養され、その後自己由来または同種異系（同一提供者または第三者）刺激細胞に露出された応答者Ｔ細胞の二次応答が抑制されたことを示す図。

【図５Ｂ】図５Ａと同じ。

【図５Ｃ】図５Ａと同じ。

【図５Ｄ】図５Ａと同じ。

【図６Ａ】イヌモデルにおけるＭＳＣｓによる初期ＭＬＲの抑制を示す図。ａｕｔｏｌ＝自己由来、ｉｄｅｎｔ＝ＤＬＡ同一同腹子、ｕｎｒｅｌ＝無関係

【図６Ｂ】図６Ａと同じ。

【図６Ｃ】図６Ａと同じ。

【図６Ｄ】図６Ａと同じ。

【図７】非粘着ＭＳＣｓによる初期ＭＬＲの抑制を示す図。

【図８】実施例８において用いられるＥＧＦＰ　ｐＯＴ２４プラスミドの模式図を示す図。

【図９】ヒト間葉幹細胞またはヒト間葉幹細胞抑制混合リンパ球反応培養系から産生されたＭＳＣ上澄みの初期混合リンパ球反応に対する抑制効果を示す図。

【図１０】ヒト間葉幹細胞またはヒト間葉幹細胞抑制混合リンパ球反応培養系から産生されたＭＳＣ上澄みの進行中の混合リンパ球反応に対する抑制効果を示す図。

【図１１】ヒヒ間葉幹細胞によるヒト応答Ｔ細胞とヒト刺激ＰＢＭＳ細胞との間の混合リンパ球反応の抑制を示す図。

【図１２】ヒヒまたはヒト間葉幹細胞によるヒト応答Ｔ細胞とヒヒ刺激ＰＢＭＣ細胞（提供者５９５７）との間の混合リンパ球反応の抑制を示す図。

【図１３】ヒヒまたはヒト間葉幹細胞によるヒト応答Ｔ細胞とヒヒ刺激ＰＢＭＣ細胞（提供者５９０９）との間の混合リンパ球反応の抑制を示す図。

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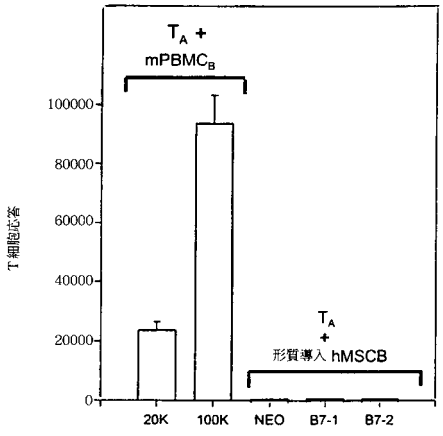

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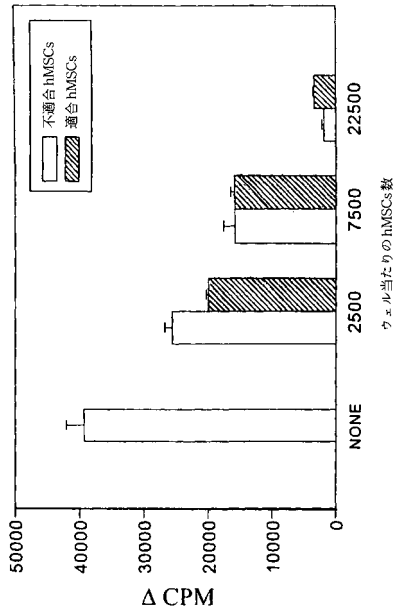

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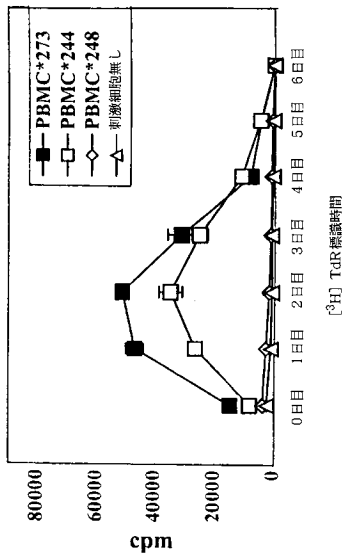

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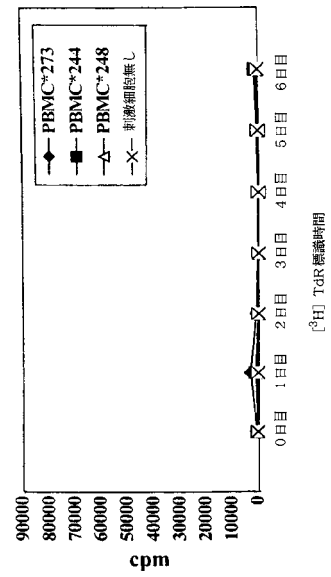

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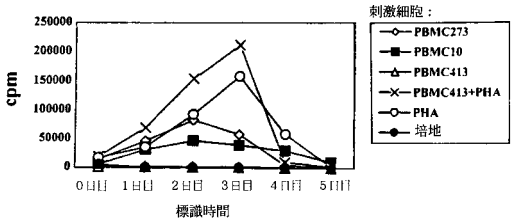

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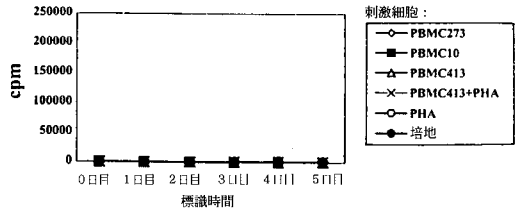

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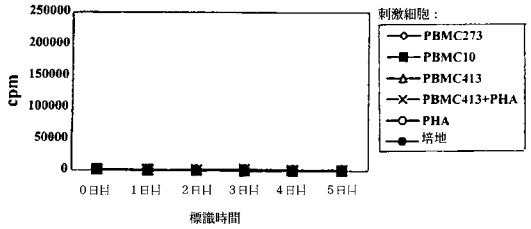

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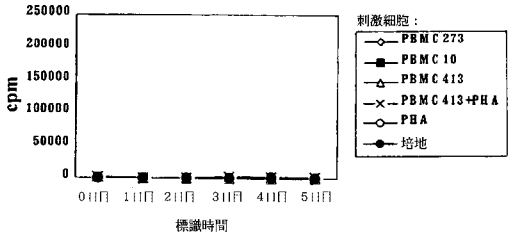

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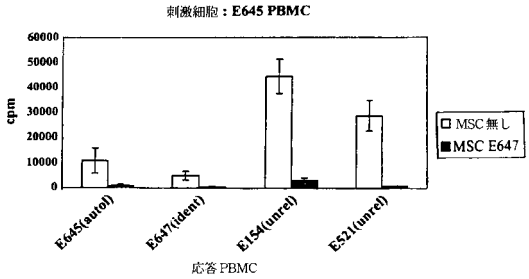

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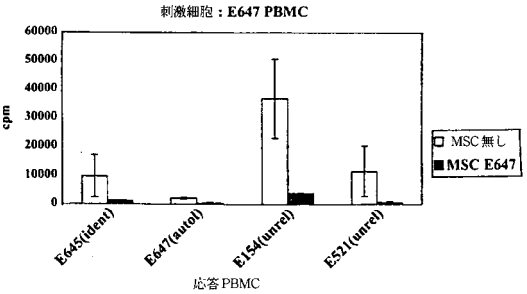

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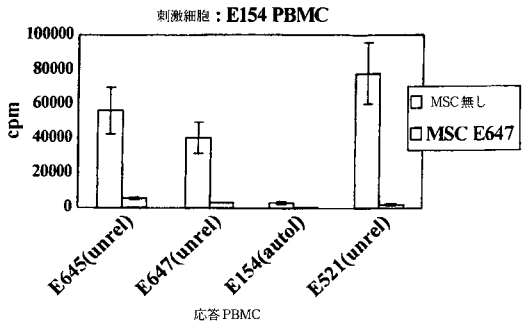

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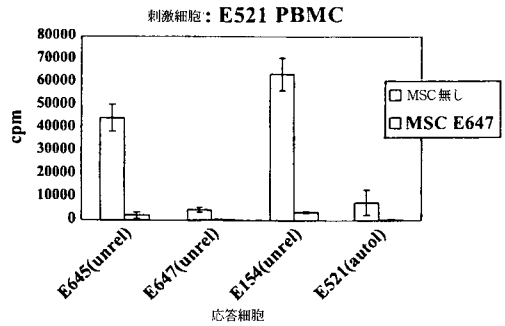

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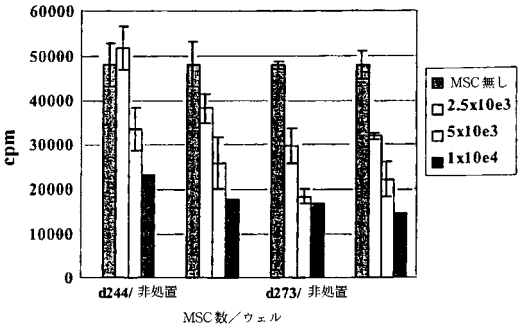

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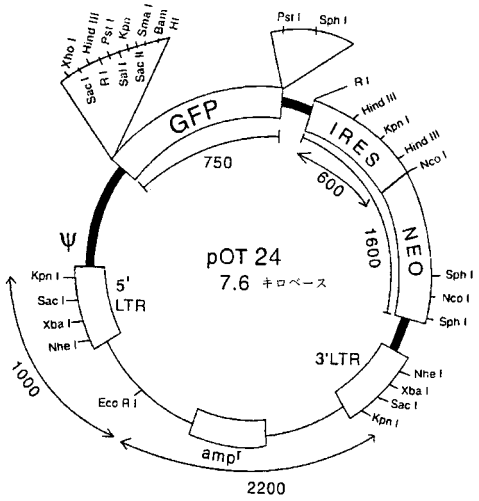
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GFP
IRES
NEO
5' LTR
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ampr
Ψ
750
600
1600
1000
2200
Xho I
Hind III
Psi I
Kpn I
Sal I
Sac II
Sma I
Bam HI
Sac I
R I
Psi I
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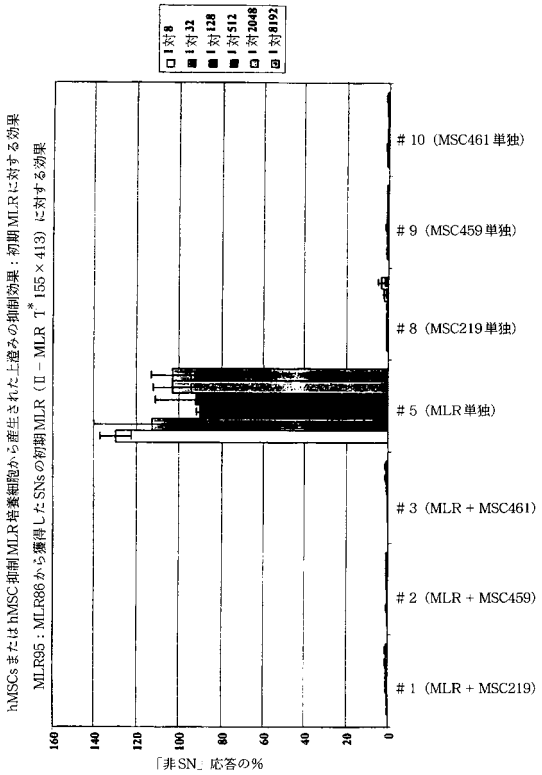
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1対32
1対128
1対512
1対2048
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MLR95：MLR86から獲得したSNsの初期MLR（II－MLR T* 155×413）に対する効果
10（MSC461単独）
9（MSC459単独）
8（MSC219単独）
5（MLR単独）
3（MLR＋MSC461）
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「非SN」応答の%

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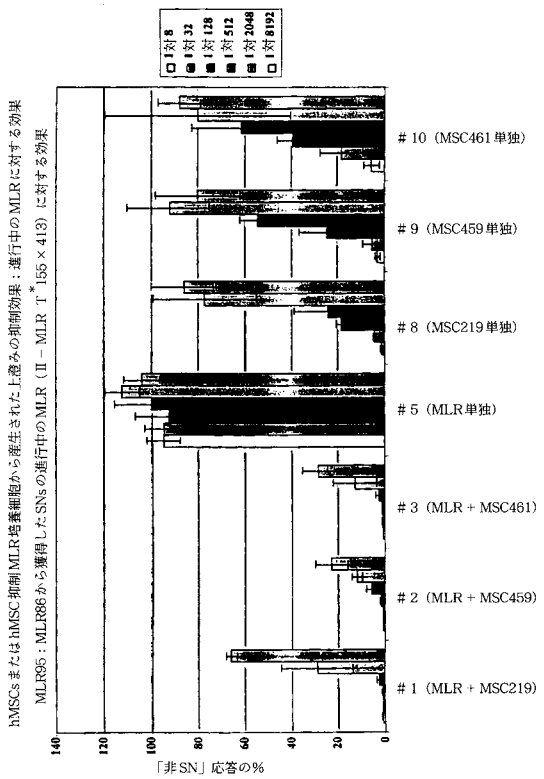
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MLR95：MLR86から獲得したSNsの進行中のMLR（II－MLR T* 155×413）に対する効果
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9（MSC459単独）
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3（MLR＋MSC461）
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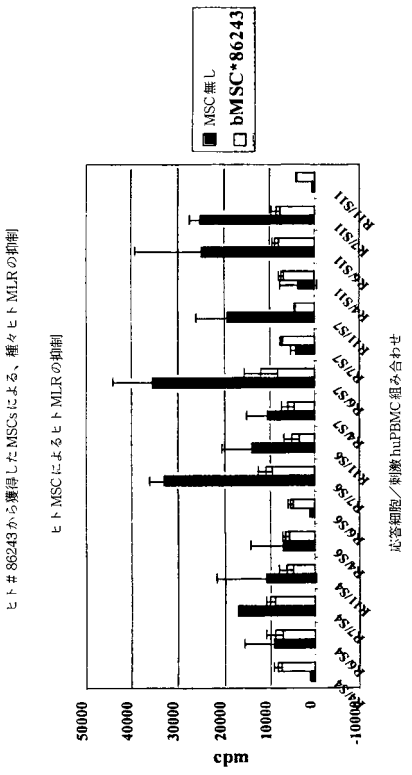
MSC無し
bMSC*86243
ヒト#86243から獲得したMSCsによる、種々ヒトMLRの抑制
ヒトMSCによるヒトMLRの抑制
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cpm
応答細胞／刺激huPBMC組み合わせ

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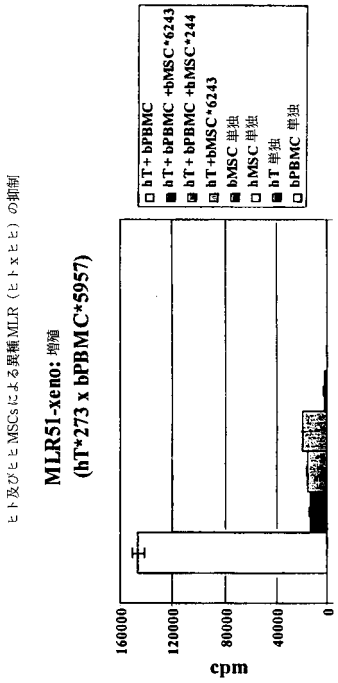
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hT+bPBMC
hT+bPBMC +bMSC*6243
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hMSC 単独
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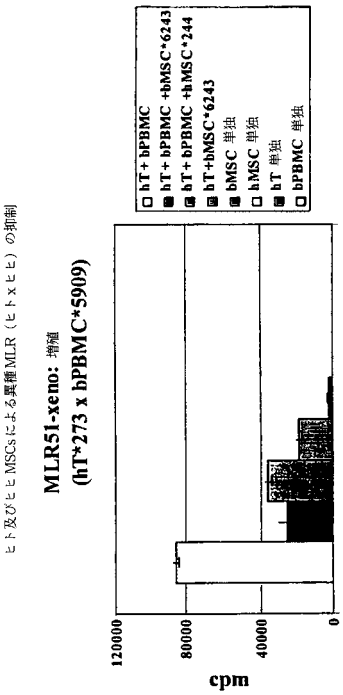
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80000
40000
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cpm

【国際公開パンフレット】

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(54) Title: SUPERNATANT FROM MESENCHYMAL STEM CELLS FOR PREVENTION AND TREATMENT OF IMMUNE RESPONSES IN TRANSPLANTATION

(57) Abstract: A method of reducing an immune response to a transplant in a recipient by treating said recipient with an amount of mesenchymal stem cells effective to reduce or inhibit host rejection of the transplant. The mesenchymal stem cells can be administered before, at the same time as, or after the transplant. Also disclosed is a method of inducing a reduced immune response against a host by foreign tissue, i.e., graft versus host disease, by treatment with mesenchymal stem cells.

SUPERNATANT FROM MESENCHYMAL STEM CELLS FOR PREVENTION AND TREATMENT OF IMMUNE RESPONSES IN TRANSPLANTATION

This application claims priority of U.S. Application Serial No. 09/427,333, filed October 26, 1999, which is a continuation-in-part of U.S. Patent application serial no. 09/267, 536 filed March 12, 1999; is based on and claims priority of U.S. provisional application serial no. 60/078,463 filed March 18, 1998 and U.S. provisional application serial no. 60/089,964 filed June 19, 1998.

The present invention relates to inhibiting a T cell response to an alloantigen and further relates to inhibiting and/or preventing reactivation of previously activated T cells. More particularly, the present invention relates to the field of preventing, reducing or treating an immune response caused by immune effector cells to foreign tissue and/or cells and/or organs. The invention further relates to preventing, reducing or treating transplant rejection and/or graft versus host reaction.

BACKGROUND OF THE INVENTION

Tolerance is the acquired lack of specific responsiveness to an antigen to which an immune response would normally occur. Typically, to induce tolerance, there must be an exposure to a tolerizing antigen, which results in the death or functional inactivation of certain lymphocytes. Complete tolerance is characterized by the lack of a detectable immune response, to the second antigenic challenge. Partial tolerance is typified by the quantitative reduction of an immune response.

The function of the immune system is to eliminate foreign bodies which may contain pathogens, and to maintain unresponsiveness or tolerance against self antigen. T cell tolerance is achieved 1) in the thymus where thymocytes reactive for self-

peptides are eliminated by clonal deletion (central tolerance), and 2) in the periphery by exposure to self-antigens under tolerogenic conditions (peripheral tolerance). Clonal deletion can also result from expression of cell death molecules on antigen presenting cells. Classic examples of death molecules are Fas ligand (FasL) and TRAIL ligand, which ligate their receptors, Fas and DR4, respectively, on activated T cells, inducing apoptosis of the T cells. The interaction of CD27, a member of the TNFR superfamily, and the CD27-ligand (CD70) also induces T cell apoptosis.

Unfortunately, the immune system does not distinguish beneficial intruders, such as transplanted tissue, from those that are harmful, and thus the immune system rejects transplanted tissue or organs. Rejection of transplanted organs is significantly mediated by alloreactive T cells present in the host which recognize donor alloantigens or xenoantigens.

At present, in order to prevent or reduce an immune response against a transplant, patients are treated with powerful immunosuppressive drugs. The infusion of individuals with drugs that prevent or suppress T-cell immune response does inhibit transplant rejection, but can also result in general immune suppression, toxicity and even death due to opportunistic infections. Because of the toxicity and incomplete response rate to conventional treatment of donor tissue rejection, alternative approaches are needed to treat patients who cannot withstand or do not respond to current modes of drug therapy.

Accordingly, there is a need for the prevention and/or reduction of an unwanted immune response by a host to a transplant by immune effector cells as a method to avert host rejection of donor tissue. Also advantageous would be a method to eliminate or reduce an unwanted immune response by a donor tissue against a recipient tissue, known as graft-versus-host disease.

SUMMARY OF THE INVENTION

It has been discovered that mesenchymal stem cells can be used in transplantation to ameliorate a response by the immune system such that an immune response to an antigen(s) will be reduced or eliminated.

In accordance with one aspect of the invention, there is provided a method for reducing or suppressing an immune response caused by T cells responding to an alloantigen, in particular allogeneic tissue, organ or cells, wherein the immune response is reduced or suppressed by the use of mesenchymal stem cells. The mesenchymal stem cells may be autologous to the T cells (obtained from the same host) or allogeneic or xenogeneic to the T cells. In the case of mesenchymal stem cells that are allogeneic to the T cells, the mesenchymal stem cells may be autologous to the cells or tissue to which the T cells are responding (obtained from the same host) or the mesenchymal stem cells may be obtained from a host that is allogeneic to both the source of the T cells and the source of the cells or tissue to which the T cells are responding. Alternatively the mesenchymal stem cells can be obtained from a source that is xenogeneic to either or both the source of the T cells and the source of the cells or tissue to which the T cells are responding.

In accordance with another aspect of the present invention there is provided a process for preventing restimulation of activated T cells (activated against an alloantigen, in particular an allogeneic organ, tissue or cells) by contacting activated T cells with mesenchymal stem cells in an amount effective to prevent and/or reduce a subsequent T cell response to a foreign antigen. The mesenchymal stem cells that are used may be autologous to the T cells and/or allogeneic to the T cells. When using allogeneic mesenchymal stem cells, the mesenchymal stem cells may be obtained from the same host as the tissue or cells that activated the T cells or may be obtained from a host that is allogeneic to both the T cells and the host that provided the cells or tissues that activated the T cells.

In accordance with another aspect of the present invention, mesenchymal stem cells are used to suppress or ameliorate an immune response to a transplant (tissue, organ, cells, etc.) by administering to the transplant recipient mesenchymal stem cells in an amount effective to suppress or ameliorate an immune response against the transplant. The mesenchymal stem cells may be autologous to the transplant recipient or may be allogeneic or xenogeneic to the transplant recipient.

Accordingly, one method of the present invention provides contacting the recipient of donor tissue with mesenchymal stem cells. In one embodiment of this aspect, the method involves administering mesenchymal stem cells to the recipient of donor tissue. The mesenchymal stem cells can be administered to the recipient before or at the same time as the transplant or subsequent to the transplant. The mesenchymal stem cells may be autologous or may be allogeneic to the recipient and can be obtained from the donor. In another aspect of the invention, the allogeneic mesenchymal stem cells can also be obtained from a source other than the donor and such source need not be matched either to the donor type or the recipient type.

In a further embodiment of this method, as part of a transplantation procedure the mesenchymal stem cells are modified to express a molecule that induces cell death. The mesenchymal stem cells can be used to deliver to the immune system a molecule that induces apoptosis of activated T cells carrying a receptor for the molecule. This results in the deletion of activated T lymphocytes and in the suppression of an unwanted immune response to a transplant. In accordance with this aspect of the invention, allogeneic mesenchymal stem cells are modified to express a cell death molecule. The molecule can be exogenous or endogenous to the mesenchymal stem cells. In preferred embodiments of the methods described herein, the mesenchymal stem cells express the cell death molecule Fas ligand or TRAIL ligand.

The mesenchymal stem cells can also be administered to the recipient as part of the transplant. To this objective, the present invention provides a method for reducing or ameliorating an immune response by providing to the recipient donor tissue or organ that is perfused with or includes mesenchymal stem cells obtained from the donor of the organ or tissue or mesenchymal stem cells from a third party or mesenchymal stem cells autologous to the T cells. The mesenchymal stem cells ameliorate an immune response by the recipient's T cells against the foreign tissue when it is transplanted into the recipient.

In a further embodiment of this invention, the mesenchymal stem cells perfused into the organ or tissue also can include a molecule that induces activated T cell death.

In another embodiment, the method of the present invention provides treating a patient who has received a transplant, in order to reduce the severity of or eliminate a rejection episode against the transplant, by administering to the recipient of donor tissue mesenchymal stem cells after the donor tissue has been transplanted into the recipient. The mesenchymal stem cells can be autologous or allogeneic to the recipient. The allogeneic mesenchymal stem cells can be obtained from the donor or from a third party source. The presentation of mesenchymal stem cells to a recipient undergoing an adverse immune response to a transplant induces nonresponsiveness of T cells to further antigenic stimulation thereby reducing or eliminating an adverse response by activated T cells to donor tissue or organ.

In a further aspect of the present invention, there is provided a method of reducing an immune response by donor tissue, organ or cells against a recipient, i.e. graft versus host response, comprising treating the donor tissue, organ or cells with allogeneic (allogeneic to the donor) mesenchymal stem cells *ex vivo* prior to transplantation of the tissue, organ or cells into the recipient. The mesenchymal stem cells reduce the responsiveness of T cells in the transplant that may be subsequently

activated against recipient antigen presenting cells such that the transplant may be introduced into the recipient's (host's) body without the occurrence of, or with a reduction in, an adverse response of the transplant to the host. Thus, what is known as "graft versus host" disease may be averted.

In a preferred embodiment, the donor transplant may be first exposed to recipient or third party tissue or cells *ex vivo*, to activate the T cells in the donor transplant. The donor transplant is then contacted with mesenchymal stem cells autologous or allogeneic to the donor. The mesenchymal stem cells can be recipient or third party mesenchymal stem cells. The mesenchymal stem cells will reduce or inhibit an adverse secondary immune response by T cells in the donor transplant against antigenic stimulation by the recipient when the donor transplant is subsequently placed into the recipient.

Accordingly, the mesenchymal stem cells can be obtained from the recipient, for example, prior to the transplant. The mesenchymal stem cells can be isolated and stored frozen until needed. The mesenchymal stem cells may also be culture-expanded to desired amounts and stored until needed. The mesenchymal stem cells are administered to the recipient in an amount effective to reduce or eliminate an ongoing adverse immune response caused by the donor transplant against the recipient (host). The presentation of the mesenchymal stem cells to the recipient undergoing an adverse immune response caused by a transplant inhibits the ongoing

response and prevents restimulation of the T cells thereby reducing or eliminating an adverse response by activated T cells to recipient tissue.

A further embodiment includes modifying the recipient's mesenchymal stem cells with a molecule that induces activated T cell death.

Thus, in accordance with preferred embodiments of the present invention, human mesenchymal stem cells are employed to treat transplant rejection and or graft versus host disease as a result of a transplant and or to prevent or reduce transplant rejection and or graft versus host disease. Human mesenchymal stem cells may also be employed to facilitate the use of xenogeneic grafts or transplants. It is also within the present invention to use xenogeneic cells, such as non-human primate cells, for the above purposes.

It has further been discovered that the supernatant derived from MSC cultures and MSC/mixed lymphocyte reaction cultures has a suppressive effect on a T cell response to an alloantigen. Thus the present invention further provides a method of use of supernatants.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Allogeneic mesenchymal stem cells do not induce an immune response. T cells from A proliferated in a dose dependent manner when mixed with different amounts of PBMCs from B. T cells from A did not proliferate in response to contact with mesenchymal stem cells from B, even when the mesenchymal stem cells were manipulated to provide full T cell activation (the mesenchymal stem cells were treated with IFN-γ and transduced with costimulatory molecules B7-1 or B7-2).

Fig. 2. Mesenchymal stem cells actively suppressed the mixed lymphocyte reaction (MLR) between lymphocytes from two different individuals. hMSCs allogeneic to recipient (third party or donor) were either mismatched to both the stimulator and responder cells in the (MLR) (open bars); or hMSCs were matched (donor) to the stimulator cells in the MLR (hatched bars). Thus, the mesenchymal stem cells suppressed the MLR without specificity as to MHC type. The mesenchymal stem cells suppressed the MLR in a dose dependent manner.

Fig. 3 shows the secondary response of responder T cells primed by stimulator (allogeneic) PBMCs, not exposed to MSCs, and then exposed to autologous PBMCs, allogeneic PBMCs (stimulator or third party) or no cells.

Fig. 4 shows the secondary response of responder T cells activated by stimulator (allogeneic) PBMCs, and subsequently cultured with allogeneic MSCs (stimulator) and then exposed to autologous PBMCs, allogeneic PBMCs (stimulator or third party) or no cells.

Fig. 5 (Figs 5A-5D) shows that the secondary response of responder T cells previously activated by stimulator allogeneic PBMCs and after activation cultured with allogeneic (same donor (Fig.5B) or third party (Fig. 5D) or autologous MSCs (Fig.5C) and then exposed to autologous or allogeneic (same donor or third party) stimulator cells was suppressed.

Fig. 6 (Figs 6A-6D) shows the suppression of a primary MLR in the canine model by MSCs. autol = autologous; ident = DLA identical litter mates; unrel = unrelated.

Fig. 7 shows the suppression of a primary MLR by non-adherent MSCs.

Fig. 8 shows a schematic map of EGFP pOT24 plasmid used in Example 8.

Fig. 9 shows the suppressive effect of MSC supernatants generated from human mesenchymal stem cells or human mesenchymal stem cell-suppressed mixed lymphocyte reaction cultures on a primary mixed lymphocyte reaction.

Fig. 10 shows the suppressive effect of MSC supernatants generated from human mesenchymal stem cells or human mesenchymal stem cell-suppressed mixed lymphocyte reaction cultures on an ongoing mixed lymphocyte reaction.

Fig. 11 shows the suppression by baboon mesenchymal stem cells of a mixed lymphocyte reaction between human responder T cells and human stimulator PBMC cells.

Fig. 12 shows the suppression by baboon or human mesenchymal stem cells of a mixed lymphocyte reaction between human responder T cells and baboon stimulator PBMC cells (donor 5957).

Fig. 13 shows suppression by baboon or human mesenchymal stem cells of a mixed lymphocyte reaction between human responder T cells and baboon stimulator PBMC cells (donor 5909).

DETAILED DESCRIPTION OF THE INVENTION

As defined herein, an allogeneic mesenchymal stem cell is obtained from a different individual of the same species as the recipient. Donor antigen refers to antigens expressed by the donor tissue to be transplanted into the recipient. Alloantigens are antigens which differ from antigens expressed by the recipient. Donor tissue, organs or cells to be transplanted is the transplant. As examples of transplants may be included skin, bone marrow, and solid organs such as heart,

pancreas, kidney, lung and liver. Thus, an alloantigen is an antigen that is foreign to the recipent.

The inventors have discovered that when mesenchymal stem cells are contacted with allogeneic T lymphocytes, *in vitro*, the allogeneic T cells do not proliferate. Normally, co-culturing cells from different individuals results in a T cell response, manifested by activation and proliferation of the T cells, known as a mixed lymphocyte reaction (MLR).

These unexpected results demonstrate that T cells are not responsive to mismatched mesenchymal stem cells. The lack of a proliferative response to human mesenchymal stem cells by allogeneic T cells was unexpected because human mesenchymal stem cells express surface molecules that should render them immunogenic, i.e., they express allogeneic class I MHC molecules. This discovery indicates that the mesenchymal stem cells are not immunogenic to the immune system.

The inventors further discovered that mesenchymal stem cells can suppress an MLR between allogeneic cells. Mesenchymal stem cells actively reduced the allogeneic T cell response in mixed lymphocyte reactions in a dose dependent manner. In addition, mesenchymal stem cells from different donors did not exhibit specificity of reduced response with regard to MHC type. Thus, mesenchymal stem cells did not need to be MHC matched to the target cell population in the mixed lymphocyte reaction in order to reduce the proliferative response of alloreactive T cells to mesenchymal stem cells. The mesenchymal stem cells can also be xenogeneic to the responder or stimulator cells or both.

The inventors also discovered that the supernatants derived from mesenchymal stem cell cultures can suppress an MLR between allogeneic cells. As used herein, supernatants derived from mesenchymal stem cell cultures, also referred

to herein as "MSC supernatant", can be obtained from mesenchymal stem cells cultured alone or mesenchymal stem cells co-cultured with cell undergoing an immune response, i.e. T cells undergoing a mixed lymphocyte reaction.

Mesenchymal stem cell supernatants actively reduced the allogeneic T cell response in mixed lymphocyte reactions in a dose dependent manner. As with mesenchymal stem cells, supernatants from mesenchymal stem cell cultures from different donors did not exhibit specificity of reduced response with regard to MHC type.

In addition, the supernatants derived from mixed lymphocyte reactions contacted with mesenchymal stem cells can also suppress an MLR between allogeneic cells. These MLR/mesenchymal stem cell supernatants actively reduced the allogeneic T cell response in mixed lymphocyte reactions in a dose dependent manner and did not exhibit specificity of reduced response with regard to MHC type.

It is believed that a soluble factor or compound may be secreted into the mesenchymal stem cell culture medium that has a suppressive effect on mixed lymphocyte reactions. A stronger suppressive effect is seen using supernatants from MSCs exposed to a mixed lymphocyte reaction.

Accordingly, the present invention provides a method of reducing, inhibiting or eliminating an immune response by administering allogeneic mesenchymal stem cells to a recipient of a donor tissue, organ or cells. In one embodiment, the mesenchymal stem cells are administered to the recipient contemporaneously with the transplant. Alternatively, the mesenchymal stem cells can be administered prior to the administration of the transplant. For example, the mesenchymal stem cells can be administered to the recipient about 3 to 7 days before transplantation of the donor tissue. Alternatively, the cells may be administered subsequent to the transplant.

Thus, mesenchymal stem cells can be used to condition a recipient's immune system to donor or foreign tissue by administering to the recipient, prior to, or at the same time as transplantation of the donor tissue, mesenchymal stem cells in an amount effective to reduce or eliminate an immune response against the transplant by, for example, the recipient's T cells. The mesenchymal stem cells affect the T cells of the recipient such that the T cell response is reduced or eliminated when presented with donor or foreign tissue. Thus, host rejection of the transplant may be avoided or the severity thereof reduced.

The inventors have further discovered that when T lymphocytes that have already been exposed to antigenic stimulation, i.e. are activated, are subsequently exposed to mesenchymal stem cells, the T cells do not produce an immune response or produce a reduced immune response, to subsequent antigenic stimulation by allogeneic cells. Thus, mesenchymal stem cells induce a state of hyporesponsiveness of the T cells.

These unexpected results demonstrate that activated T cells were made non-responsive to further allogeneic stimulation by exposure of preactivated T cells to mesenchymal stem cells. The mesenchymal stem cells can be autologous or allogeneic to the T cells.

Accordingly, the present invention provides a method for treating a patient who is undergoing an adverse immune response to a transplant by administering mesenchymal stem cells to such patient in an amount effective to reduce or suppress the immune response. The mesenchymal stem cells are obtained from the tissue donor, the transplant recipient or a third party. As a further alternative, the MSCs may be xenogeneic to the donor, the recipient or both.

The mesenchymal stem cells may further be modified to express a cell death molecule to enhance the elimination of activated T cells. For example, the cell death molecule may be

expressed by the mesenchymal stem cells which have been engineered to express the exogenous cell death molecule.

In another aspect, the present invention provides a method to reduce or inhibit or eliminate an immune response by a donor transplant against a recipient thereof (graft versus host). Accordingly, the invention provides contacting a donor organ or tissue with mesenchymal stem cells prior to transplant. The mesenchymal stem cells ameliorate, inhibit or reduce an adverse response by the donor transplant against the recipient.

In a preferred embodiment, prior to transplant the donor transplant is treated with allogeneic (recipient) tissue or cells which activate the T cells in the donor transplant. The donor transplant is then treated with mesenchymal stem cells, autologous or allogeneic, prior to transplant. The mesenchymal stem cells prevent restimulation, or induce hyporesponsiveness, of the T cells to subsequent antigenic stimulation.

For preconditioning a donor transplant, the mesenchymal stem cells may be further modified to express a cell death molecule such that activated T cells contacted with the mesenchymal stem cells will be eliminated.

Thus, in the context of hematopoietic stem cell transplantation, for example, from the marrow and/or peripheral blood, attack of the host by the graft can be reduced or eliminated. Donor marrow can be pretreated with recipient mesenchymal stem cells prior to implant of the bone marrow or peripheral blood stem cells into the recipient. In a preferred embodiment, the donor marrow is first exposed to recipient tissue/cells and then treated with mesenchymal stem cells. Although not being limited thereto, it is believed that the initial contact with recipient tissue or cells functions to activate the T cells in the marrow. Subsequent treatment with the mesenchymal stem cells inhibits or eliminates further activation of the T cells in the marrow, thereby

reducing or eliminating an adverse affect by the donor tissue, i.e. the therapy reduces or eliminates graft versus host response.

In a further embodiment, a transplant recipient suffering from graft versus host disease may be treated to reduce or eliminate the severity thereof by administering to such recipient mesenchymal stem cells autologous or allogeneic to the donor, which allogeneic cells can be mesenchymal stem cells autologous to the recipient or third party mesenchymal stem cells, in an amount effective to reduce or eliminate a graft rejection of the host. The mesenchymal stem cells inhibit or suppress the activated T cells in the donor tissue from mounting an immune response against the recipient, thereby reducing or eliminating a graft versus host response.

The recipient's mesenchymal stem cells may be obtained from the recipient prior to the transplantation and may be stored and/or culture-expanded to provide a reserve of mesenchymal stem cells in sufficient amounts for treating an ongoing graft attack against host.

In yet another method of the present invention, the donor tissue is exposed to mesenchymal stem cells such that the mesenchymal stem cells integrate into the organ graft itself prior to transplantation. In this situation, an immune response against the graft caused by any alloreactive recipient cells that escaped standard treatment to prevent transplant rejection, e.g., drug-mediated immunosuppression, would be suppressed by the mesenchymal stem cells present in the graft. The mesenchymal stem cells are preferably allogeneic to the recipient and may be donor mesenchymal stem cells or mesenchymal stem cells obtained from other than the donor or recipient. In some cases, mesenchymal stem cells autologous to the recipient may be used to suppress an immune response against the graft.

In a further embodiment of this method, the mesenchymal stem cells are engineered to express cell death molecules such that any alloreactive host T cells will be eliminated upon contact with these mesenchymal stem cells.

It is further believed that in addition to preventing or ameliorating an initial immune response, the mesenchymal stem cells remaining in the local site would also suppress any subsequent T cell response that may occur.

As used herein, a "cell death molecule" is a molecule that interacts or binds with its cognate receptor on a stimulated T cell inducing T cell death or apoptosis. Fas mediates apoptosis of recently activated T cells which are again exposed to stimulation (van Parijs et al., *Immunity* 4: 321-328 (1996)). Fas is a type I membrane receptor that when crosslinked by its cognate ligand induces apoptosis in a wide variety of cells. The interaction between the Fas molecule (CD95) on target T cells and its ligand Fas L on mesenchymal stem cells results in receptor aggregation, which transduces signals leading to apoptosis of the target cell. The Fas system has been shown to be involved in a number of cell functions *in vivo* including negative selection of thymocytes, maintaining immune privilege sites within the body, and cytotoxic T-lymphocyte (CTL)-mediated cytotoxicity (Green and Ware, *Proc Natl Acad Sci,* 94(12):5986-90 (1997)).

Other members of the tumor necrosis factor receptor (TNFR) family have roles in programmed cell death. TRAIL ligand, which interacts with its receptor DR4 can induce apoptosis in a variety of transformed cell lines (G. Pan et al. *Science,* 277:815-818 (1997)); and the expression of CD27 and its ligand CD70 (Prasad et al., *Proc Natl Acad Sci,* 94:6346-6351 (1997)) also induces apoptosis. Fas expression is restricted to stimulated T cells and sites of immune privilege. TRAIL is detected in many normal tissues.

Both Trail-ligand and CD27, but not Fas-ligand, are expressed on unmanipulated human mesenchymal stem cells. Activated, but not resting, T cells express the Trail receptor and CD70. Most of the T cells found in the body are in the resting state; T cells are activated when they encounter cells both in the context of MHC and the appropriate co-stimulatory molecule such as B7-1 or B7-2.

Thus, the engagement of cell death receptors on activated T cells with their ligands expressed on the mesenchymal stem cells results in T cell death via apoptosis. Ligands and their receptors other than those specifically mentioned above, either present within the mesenchymal stem cell or introduced into the mesenchymal stem cell can perform this function. Therefore, mesenchymal stem cells administered to an individual delete activated T cells, reducing the severity or incidence of transplant rejection disease.

In accordance with the methods of the present invention described herein, it is contemplated that the mesenchymal stem cells of the present invention can be used in conjunction with current modes of treating donor tissue rejection or graft versus host disease. An advantage of such use is that by ameliorating the severity of the immune response in a transplant recipient, the amount of drug used in treatment and/or the frequency of administration of drug therapy can be reduced, resulting in alleviation of general immune suppression and unwanted side effects.

It is further contemplated that only a single treatment with the mesenchymal stem cells of the present invention may be required, eliminating the need for chronic immunosuppressive drug therapy. Alternatively, multiple administrations of mesenchymal stem cells may be employed.

Accordingly, the invention described herein provides for preventing or treating transplant rejection by administering the mesenchymal stem cells in a prophylactic or therapeutically effective amount for the prevention or treatment or amelioration of

transplant rejection of an organ, tissue or cells from the same species, or a xenograft organ or tissue transplant and or graft versus host disease.

Administration of a single dose of mesenchymal stem cells may be effective to reduce or eliminate the T cell response to tissue allogeneic to the T cells or to "non-self" tissue, particularly in the case where the T lymphocytes retain their nonresponsive character (i.e., tolerance or anergy) to allogeneic cells after being separated from the mesenchymal stem cells.

The dosage of the mesenchymal stem cells varies within wide limits and will, of course be fitted to the individual requirements in each particular case. In general, in the case of parenteral administration, it is customary to administer from about 0.01 to about 5 million cells per kilogram of recipient body weight. The number of cells used will depend on the weight and condition of the recipient, the number of or frequency of administrations, and other variables known to those of skill in the art. The mesenchymal stem cells can be administered by a route which is suitable for the tissue, organ or cells to be transplanted. They can be administered systemically, i.e., parenterally, by intravenous injection or can be targeted to a particular tissue or organ, such as bone marrow. The human mesenchymal stem cells can be administered via a subcutaneous implantation of cells or by injection of stem cell into connective tissue, for example muscle.

The cells can be suspended in an appropriate diluent, at a concentration of from about 0.01 to about 5 x 10^6 cells/ ml. Suitable excipients for injection solutions are those that are biologically and physiologically compatible with the cells and with the recipient, such as buffered saline solution or other suitable excipients. The composition for administration must be formulated, produced and stored according to standard methods complying with proper sterility and stability.

Although the invention is not limited thereof, mesenchymal stem cells can be isolated, preferably from bone marrow, purified, and expanded in culture, i.e. *in vitro*, to obtain sufficient numbers of cells for use in the methods described herein. Mesenchymal stem cells, the formative pluripotent blast cells found in the bone, are normally present at very low frequencies in bone marrow (1:100,000) and other mesenchymal tissues. See, Caplan and Haynesworth, U.S. Patent No. 5,486,359. Gene transduction of mesenchymal stem cells is disclosed in Gerson et al U.S. Patent No. 5,591,625.

Unless otherwise stated, genetic manipulations are performed as described in Sambrook and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

It should be understood that the methods described herein may be carried out in a number of ways and with various modifications and permutations thereof that are well known in the art. It may also be appreciated that any theories set forth as to modes of action or interactions between cell types should not be construed as limiting this invention in any manner, but are presented such that the methods of the invention can be more fully understood.

The following examples further illustrate aspects of the present invention. However, they are in no way a limitation of the teachings or disclosure of the present invention as set forth herein.

Example 1

Absence of Alloreactivity of Mesenchymal Stem Cells

The mixed lymphocyte reaction measures the compatibility of the donor's surface antigens and is an indication of the likelihood of rejection of donor tissue. Cell surface antigens responsible for eliciting transplant rejection are class I and class

II MHC antigens. T cells are alloreactive to foreign MHC antigens. Class I and II MHC molecules stimulate the mixed lymphocyte reaction.

Normal human volunteers were leukopheresed on a COBE SPECTRA ™ apheresis system (COBE, Lakewood, CO). 1 x 10^5 T cells from individual A (T_A) were cultured in flat bottom microtiter wells with mitomycin C treated allogeneic PBMCs (to prevent proliferation of PBMCs to T cells) from individual B ($mPBMC_B$) for 7 days. The $mPBMC_B$s were seeded at 20K and 100K. The cultures were pulsed with ^{3}H-thymidine for the last 18 hours of the culture period to measure T cell proliferation. The results shown in Figure 1 indicate that the T_A cells recognized the $PBMC_B$ as being foreign. (See bars under "T_A + $mPBMC_B$".) With more $PBMC_B$s present, the more the T cells proliferated.

2 x10^4 human mesenchymal stem cells (hMSCs) from the same donor as the PBMCs were co-incubated with 1x10^5 T cells from individual A (T_A). The cells were cultured in flat-bottom microtiter wells for a total of 7 days. Cultures were pulsed with ^{3}H-thymidine for the last 18 hours of the culture period to measure T cell proliferation. Two days prior to coculture with the T cells, the human mesenchymal stem cells were seeded into microtiter wells at the number given above (confluent) and treated with IFN-γ (50 units/ml) to stimulate surface antigen expression on MSCs. Non-transduced hMSCs or hMSCs transduced with human B7-1 or human B7-2 costimulation molecules were incubated with the T cells. Control cells were transduced with Neo.

The results shown in Fig. 1 (See Figure 1 "T_A + transduced hMSCs") demonstrate that the T lymphocytes were nonresponsive (did not proliferate) to the human mesenchymal stem cells, i.e., they were not recognized as being foreign.

The results also show that the lack of response to the mesenchymal stem cells was not due to genetic compatibility between the individuals since the T cells did

recognize peripheral blood mononuclear cells ($PBMC_B$) from the hMSC donor as being foreign.

Example 2

Suppression of Mixed Lymphocyte Reaction

To determine whether mesenchymal stem cells actively suppressed the allogeneic response, mixed lymphocyte reactions (MLR) were set up in tissue culture plates, with or without adherent mesenchymal stem cells obtained from 2 different donors: one donor matched the stimulator cells in the MLR and the other donor was unrelated to either the stimulator or responder cells.

10^5 PBMCs from individual A ($PBMC_A$) were mixed with 10^5 target individual B's PBMC's ($PBMC_B$). The $PBMC_B$s were irradiated with 3000 rads X irradiation to prevent their proliferation due to activation by $PBMC_A$s. Thus, only $PBMC_A$s would proliferate. When $PBMC_A$s and $PBMC_B$s were mixed, a mixed lymphocyte reaction occurred wherein the $PBMC_A$ cells (responder cells) were activated by the surface antigens on the $PBMC_B$s (stimulator cells). The cultures were incubated over an interval of 7 days and were pulsed with ^3H-thymidine during the final 18 hours. In the presence of the $PBMC_B$s, the $PBMC_A$s proliferated giving counts of 40,000. See Figure 2, 1st bar, ("NONE" refers to no mesenchymal stem cells present.).

However, when $PBMC_A$s and $PBMC_B$s were mixed in the presence of mesenchymal stem cells, the mixed lymphocyte reaction was suppressed. 10^5 $PBMC_A$s were mixed with 10^5 $PBMC_B$s in microtiter plate wells coated with an adherent monolayer of human mesenchymal stem cells. The mesenchymal stem cells were plated in the wells in amounts ranging from 7500 to 22,500 mesenchymal stem cells per well. Two mesenchymal stem cell populations were tested: human mesenchymal stem cells were obtained from an individual B and human mesenchymal stem cells were obtained from an individual that did not match either

individual A's or B's MHC type (a third party). The cultures were incubated over an interval of 7 days and were pulsed with ^{3}H-thymidine during the final 18 hours. In the presence of the human mesenchymal stem cells, the MLR was suppressed. See Figure 2. Thus, regardless of the MHC origin of the mesenchymal stem cells, the mesenchymal stem cells suppressed the mixed lymphocyte reaction.

The results shown in Figure 2 also indicate that the human mesenchymal stem cells decreased the mixed lymphocyte reaction in a dose-dependent manner. Mesenchymal stem cells from either donor suppressed proliferation equally well, which indicated that there was no specificity of suppression with respect to MHC type. These results demonstrate that

mesenchymal stem cells actively suppressed the mixed lymphocyte reaction when the cells were cultured together.

Example 3

Unresponsiveness in Secondary Mixed Lymphocyte Reaction

These experiments were performed to determine whether suppression of pre-activated T cells by MSCs resulted in specific unresponsiveness during secondary stimulation.

A. T cells from donor 248 (d 248) were primed by allogeneic PBMCs from donor 273 (d 273) for 7 days, then cultured for 3 additional days alone or in the presence of IFN-γ-treated MSCs from the same donor (d273). Cells were then re-stimulated by the same donor (d273), autologous (d248) or "third party" (d244) PBMCs.

Lymphocyte preparation

Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation on Ficoll-Paque (Pharmacia). Aliquots of cells were frozen in 90% FCS with 10% DMSO and stored in liquid nitrogen. After thawing, the cells were washed twice with MSC medium (DMEM with low glucose and 10% FCS) and re-suspended in assay medium (ISCOVE'S with 25 mM Hepes, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 5.5×10^{-5}M 2-mercaptoethanol (all reagents from GibcoBLR) and 5% human AB serum (Sigma, MLR tested)).

To prepare the T cell-enriched fraction, PBMCs were depleted of monocytes and B cells by immunomagnetic negative selection. PBMCs were incubated with mouse anti-human CD19 and CD14 mAbs (no azide/low endotoxin (NA/LE) format) followed by biotin-conjugated goat anti-mouse IgG (multiple adsorption) Ab (all

reagents from Pharmingen) and streptavidin microbeads (Miltenyi Biotec). Cells were then separated using a magnetic cell sorter (MACS, Miltenyi Biotec). The T cell-enriched fraction contained about 70-90% CD3+ cells.

MSC culture.

Human MSCs were isolated from bone marrow as described in U.S.Patent No. 5,486,359 and were maintained in culture with MSC medium and were used at passages from 3 to 6. Cells were lifted using 0.05% Trypsin/EDTA solution, washed once with MSC medium and plated at 70-80% confluent density which was $1x10^6$ /plate for 10 cm tissue culture dish. The day after plating, IFN-γ (Boehringer Mannheim) at 500 U/ml was added and the cells were incubated an additional 3 days. Before transferring T cells, MSC plates were washed 4 times with HBSS, 1 time with ISCOVES, and assay medium was added at 10 ml/well in 10 cm tissue culture dishes.

Primary (1°) MLR.

T cells (d 248) were activated by irradiated PBMCs (d 273). PBMCs used for stimulation were X-ray irradiated with 3,000 rad using Cabinet X ray system (Faxitron X ray, Buffalo Grove, IL). For primary stimulation, 2×10^7 responders were mixed with 2×10^7 stimulators in 20 mls assay medium in 10 cm tissue culture dishes. The cells were incubated at 37°C in 5% CO_2 atmosphere for 7 days.

Activated T cell/MSC cultures

T cells activated in the 1° MLR were collected, washed once with MSC medium and re-suspended in assay medium at 10^6/ml in 10 ml and were added to 10 cm tissue culture dishes containing autologous or allogeneic MSCs or medium alone, and incubated for an additional 3 days.

Restimulation assay

T cells cultured with MSCs or media were collected, washed once with MSC media, and restimulated with irradiated PBMCs from the original donor, an unrelated

donor or autologous PBMCs. For the assay, 5 x 10^4 primed responders and 5 x 10^4 irradiated stimulators were incubated in 96-well plates. Assays were performed in triplicate. Cultures were pulsed with 1 μCi of [^{3}H] thymidine (Amersham) for 18 hours before harvesting. Cultures were collected using Harvester 96 (Tomtec), filters were analyzed using Microbeta Trilux liquid scintillation and luminescence counter (E.G.&G Wallac). Data are presented as mean cpm ± SD of three replicates.

T cells cultured alone (positive control) showed an accelerated response to "same donor" re-stimulation with peak at day 2. "Third party" response was also accelerated, practically with the same kinetics as "same donor", but with a lower maximum and a slightly delayed start. (Fig 3). T cells cultured on allogeneic MSCs subsequently showed no response either to "same donor" or "third party" PBMCs during 6 days of culture (Fig.4).

Example 4

Unresponsiveness in Secondary Mixed Lymphocyte Reaction

T cells from donor 413 were stimulated with irradiated PBMCs from donor 273 for 7 days (1.5 x 10^6 ml each, bulk 20 ml cultures). MSCs from different donors 413, 418 and 273 were plated in 10 cm tissue culture dishes at 1x10^6/dish, pretreated with IFN-γ for 3 days and washed prior to mixing with preactivated T cells.

T cells preactivated in the MLR for 7 days were incubated alone or with MSCs for an additional 3 days (1.0 x 10^6/ml T cells, 10 ml/dish). After 3 days of incubation with MSCs, T cells were collected and re-stimulated with irradiated PBMC 273 (original donor), 413 (autologous), PBMC10 (third party) or PHA (5 μg/ml) in the presence or absence of autologous (d413) PBMC. Cells were added at 5 x 10^4/well, cultures were pulsed with [^{3}H]thymidine at indicated time points for an additional 18 hours.

The results indicate that treatment of activated T cells with autologous (d413) (Fig. 5C), same donor (d273) (Fig. 5B) and third party (d418) (Fig. 5D) MSCs induced unresponsiveness to antigenic stimulation in the T cells. The control culture (Fig. 5A) with no MSC treatment showed restimulation of the cells upon exposure to allogeneic PBMCs.

Example 5

Suppression of Primary MLR by Canine MSCs

Canine PBMCs were purified from peripheral blood by centrifugation on Ficoll-Paque gradient (1.077). Stimulator PBMCs were X-ray irradiated 2200 rad (7 min 70 kV). 10^5 irradiated stimulators were mixed with 10^5 responder PBMCs in 96-well plates in the presence or absence of pre-plated canine MSC (E647, 2 x 10^4/well). Cultures were incubated for 6 days and pulsed with [^{3}H]TdR (5Ci/mmol, 1μCi/well) for an additional 16 hours. Results are shown in Figures 6A-6D. E647 and E645 were litter mates (DLA identical). The results showed that autologous as well as allogeneic MSCs suppressed the primary MLR.

Example 6

Suppression of Primary MLR by Non-Adherent MSCs

T cells from d273 (2 x 10^5/well) were mixed with irradiated PBMCs from d244 (2 x 10^5/well) and different numbers of MSCs. MSCs from dD244 or d273 were pre-treated with IFN-γ (900 U/ml for 3 days) or left untreated, trypsinized on the day of experiment and added at the same time as T cells and PBMCs. Cultures were incubated for 7 days,[^{3}H]TdR (5Ci/mmol, 1 μCi/well) was added for an additional 16-18 hours. Results are shown in Figure 7 and demonstrate that non-adherent MSCs also suppressed a primary MLR.

Example 7

Allogeneic MSCs Support Skin Allograft Survival

Study Population

Juvenile baboons (*papio anubis*) were studied. Male and non-pregnant female baboons weighed 7-20 kg and were between 3–16 years of age. They were screened for tuberculosis papilloma virus, titered for cytomegalovirus (CMV), and tested with the primate viral screen consisting of testing for simian virus, and including fecal floatation and smears. Donor and recipient pairs were determined by major histocompatibility complex (MHC)-disparity through PCR typing. During the study period, the baboons were housed in an individual area beside a companion animal.

Donor Bone Marrow Harvest for MSC Isolation and Culture-Expansion

Needle marrow aspirates were obtained from the iliac crest for isolation and culture-expansion of the MSCs. The marrow aspirate was obtained from an alternate side once a week for four consecutive weeks. The volume of the aspirate was determined by an estimate of 10% of the animal's blood volume. Blood volume (liters) is estimated to be 7% of body weight. A 10 kg baboon then, would have an estimated blood volume of 0.7 liters. An aspirate of 10% of the blood volume would then be 70 milliliters.

Prior to the procedure, 500 mg cefazolin was administered intramuscularly (IM) for perioperative antibacterial prophylaxis. Baboons were sedated and anesthetized for the procedure with ketamine at 10 mg/kg IM, and xylazine 1 mg/kg IM. The sites of needle insertion were scrubbed with povidone-iodine and then rinsed with alcohol. Aspirates were obtained from the iliac crest using a 16-gauge, 2-inch bone marrow needle. A syringe was attached to the needle, and suction was applied to remove the marrow. For postoperative pain, the analgesic Buprenorphine was given at 0.03 mg/kg IM Q12 x 2 doses.

Shipment of Donor Bone Marrow Aspirates

Bone marrow aspirates were transferred from the syringe to a sterile Vacutainer® containing sodium heparin. The tubes were placed in a Styrofoam™

container and shipped at room temperature (RT) by overnight delivery to the cell processing facility.

Isolation and Culture Establishment of MSCs

Five to 10 ml aliquots of bone marrow were diluted to 50 ml in Dulbecco's Phosphate Buffered Saline (DPBS) in a polypropylene culture tube. The cell suspensions were centrifuged at 2200 RPM for 10 minutes at room temperature (RT). Total nucleated cell counts were determined in 4% acetic acid. Cells were then diluted in DPBS for a final concentration of 20 x 10^6 cells/ml. Ten ml or 200 x 10^6 cells were loaded onto 20 ml of Percoll (sp.gr. 1.073 gm/ml) in a 50 ml conical tube and underwent centrifugation at 1300 RPM for 20 minutes. The cell interface containing mononuclear cells was washed in DPBS, resuspended in complete media, and counted to obtain a recovery. The washed mononuclear cells obtained at the Percoll interface were cells were then established in T-185 flasks containing 30 ml of complete media and 15-20 x 10^6 cells/flask (8.1 x 10^4 MSC/cm^2) and placed in a 37°C incubator at 5% CO_2.

Harvest of MSC

The media in the triple flasks was decanted, and the flasks were rinsed with 50 ml DPBS. After decanting the DPBS, 23 ml of 0.05% trypsin was added to each triple flask. The flasks were placed in a 37°C incubator for 3 minutes. After cell detachment, 23 ml complete medium was added to each flask. The cell suspensions were transferred to 50 ml conical tubes and the flasks were washed with 30 ml HBSS. The tubes were centrifuged at 2200 RPM for 5 minutes at RT.

Formulation/Packaging

The harvested MSCs were formulated at approximately 10 x 10^6 cells per ml in

cryoprotectant solution consisting of 85% Plasma-Lyte A (Baxter IV Therapy), 10% DMSO, and
5% MSC-donor serum, and cryopreserved in bags containing 15-20 ml.

Labeling/Storage/Shipment
Cells were cryopreserved using a controlled-rate freezer (Cryomed, Forma Scientific) at 1-2° per minute to –90°C. The samples were then transferred to a liquid nitrogen storage freezer in the vapor phase (-120 to –150°C).

Dose
To achieve an MSC dose of 20 x 10^6 cells/kg, the final product was prepared at 115% of the dose required on infusion day.

Skin harvest
Prior to surgery the baboon was given cefazolin at 500 mg IM as a perioperative antibacterial prophylaxis. The baboon was sedated with ketamine at 10 mg/kg IM and anesthetized by intravenous Thiopental induction, a 1-2% isofluorane inhalational anesthetic. Skin was harvested from the anterior abdominal wall, placed on a pre-labeled moistened saline gauze pad. The wound defect was then closed. The baboon was returned to the colony after awakening. For postoperative pain, the analgesic Buprenorphine was administered at Q12 x 2 doses and Ancef daily for 2 days.

Recipient Skin Transplant and MSC Infusion
Prior to surgery the baboon was given cefazolin at 500 mg IM as an antibacterial prophylaxis perioperatively. The baboon was sedated and anesthetized with ketamine at 10 mg/kg IM and intravenous Thiopental induction, a 1-2% isofluorane inhalational anesthetic. Skin was harvested from the anterior abdominal wall and placed on a pre-labeled moistened saline gauze pad. This skin was divided into two grafts; one was used as the third party control for another recipient baboon and one was used as an autologous control for this same animal. The animal was then placed in a prone position. Three, 3 x 2-cm sections of skin were removed from the dorsum, along the spine, between the scapulae. The previously harvested skin grafts

from the MSC donor, a third party donor and self were defatted, trimmed to fit the skin defects created, and sutured in place.

After grafting, the baboon received an intravenous infusion of MSC at a dose of 20 x 10^6 donor MSC/kg. Peripheral blood samples were obtained at pre-MSC, 1 hour, and days 1-3 post-MSC; marrow aspirates were obtained on day 0 post-MSC, day 3, 14, and 30.

For postoperative pain, the analgesic Buprenorphine was administered at Q12 x 2 doses and Ancef daily for 2 days. The animal was observed daily, and the grafts were photographed every other day beginning on post-graft day 7.

Physical Examinations and Diagnostic Testing

Each baboon was sedated with ketamine 10mg/kg IM for examination. While sedated, two-three milliliters of marrow were obtained from the iliac crest by needle aspiration and collected in sodium heparin on days 4, 13, and 30, the end of study. A skin biopsy was harvested on the same day that marrow aspirates were obtained.

RESULTS

Effects of MSC Infusion on Skin Allograft Survival

Untreated control animals (N=2) had a mean skin allograft survival time of 8.0± 0 days. The infusion of unrelated-MSC-donor MSCs donor (N=2) resulted in a prolongation of skin graft survival time to a mean survival time of 11.5±0.71 days (Mann-Whitney U Test, $P<0.05$). The infusion of unrelated-third-party donor MSC on donor allografts (N=4) resulted in a significant prolongation of skin graft survival times to a mean survival time of 12.3±0.96 days (Mann-Whitney U Test, $P<0.003$).

Recipients 6140 and 6200 received allografts from the MSC donor 6243, from each other (a third party graft), and from themselves (an autograft). Twenty-four hours prior to skin graft harvesting from the MSC donor, 6243, MSCs from 6243 were injected under the anterior abdominal skin which had been delineated for grafting. After grafting, the recipients were administered an intravenous infusion of

20 x 10^6 MSC/kg (6243). Both third-party allografts were rejected on day 13. The MSC-donor (6243) allografts were found to be hemorrhagic on day 4, a finding usually attributed to a technical failure. On pathologic examination, keratin was noted to have been insinuated in a track- like fashion, below the dermis: the nature of these tracks suggests these were formed by the needle at the time of subcutaneous MSC injection. The presence of these cells had elicited a tremendous inflammatory response. This inflammatory response precluded the ability of the skin grafts to adhere/"take" properly and these grafts were completely necrosed by day 7. The autografts were not rejected.

Recipients 6654 and 6659 received allografts from the MSC donor 6593, from each other (a third-party graft), and from themselves (an autograft). After grafting, the recipients were administered intravenous infusions of 20 x 10^6 MSC/kg. The MSC-donor allografts were rejected on days 11 and 12, and the third-party donor allografts were rejected on days 11 and 12. The autografts were not rejected.

Similarly, recipients 6663 and 6658 received allografts from the MSC donor 6656, from each other (a third-party graft), and from themselves (an autograft). After grafting, the recipients were administered intravenous infusions of 20 x 10^6 MSC/kg. The MSC-donor allografts were rejected on day 11, and the third-party donor allografts were rejected on days 10 and 12. The autografts were not rejected.

Recipients 6532 and 6720 in the control arm of the study received auto- and allografts without the administration of MSC by infusion or injection. Their allografts were rejected on day 8. The autografts were not rejected.

There were no identifiable toxicities associated with allogeneic MSC infusion and no adverse clinical sequelae in the subsequent 30-day follow-up interval. Blood samples were obtained at pre-MSC, 1 and 2 hours, and days 1, 2, and 3 after grafting

and MSC infusion. Marrow aspirates were obtained on days 4 and 13 after grafting and MSC infusion.

These results demonstrate that a single infusion of allogeneic baboon MSCs can delay rejection of allogeneic skin grafts. No other immunosuppressive therapy was administered. One dose of allogeneic or third party MSCs increased the time to rejection by 50% (standard rejection time in this model is 8 days (*See* Goodman et al. *Am Surg* 62(6):435-42 (1996)).

Example 8

The purpose of the study was to demonstrate the feasibility and safety in dogs of the infusion of a moderately high dose of donor dog leukocyte antigen (DLA)-identical littermate canine mesenchymal stem cell (cMSC) at 10 x 10^6 cells/kg in an allogeneic marrow graft setting. A secondary objective was to examine the distribution and function of donor neo- and GFP-marked cMSC at 50 and 100 days post-transplant.

MATERIALS AND METHODS

Experimental animals

Beagles were used for the study. Two male and two female DLA-identical littermates were used in the study, aged 7 or 9 months on day 0. The method for typing used involves the use of highly polymorphic microsatellite markers to follow inheritance of the Class II DRB region in the Dog Leukocyte Antigen (DLA), the canine equivalent of the major histocompatability complex. Microsatellites are small di- tri- or tetra nucleotide repeats, which show sufficient length variation in alleles that they may be used to follow the inheritance of chromosomal segments through multigeneration crosses. Segregation of alleles is typically monitored using a single-step polymerase chain reaction with primers derived from unique sequences of DNA that surround each repeat. In addition, mixed leukocyte reactions were performed on

the DLA-identical littermate pairs chosen for study to provide confirmation of the PCR microsatellite marker assay results.

Study Design

The dogs underwent transplantation with cMSC and bone marrow from the same DLA-identical littermate donor. The marrow graft was harvested from each of the two DLA-identical littermates on day 0 prior to total body irradiation (TBI) and exchanged. Myeloablation was induced by exposing the dogs on day 0 to a single TBI dose of 920 centigray (cGy) (midline air exposure from two opposing ^{60}Co sources delivered at a rate of 7 cGy (9.3R)/min. Culture-expanded cMSC isolated from a donor marrow aspirate at 4 or more weeks prior to transplantation, were transduced with Papp@OT-24, containing the genes for green fluorescence protein (GFP) and neomycin phosphotransferase (neo). The cMSC were cryopreserved after passage 1 (P1) or passage 2 (P2). Following TBI, the cMSC were thawed and delivered intravenously via a portable infusion pump over a 15-minute time period. Within one to two hours after cMSC infusion the bone marrow graft was infused intravenously at a dose of $\geq 1 \times 10^8$ total nucleated cell (TNC)/kg.

Cyclosporin was administered to all four dogs for graft-versus-host-disease (GVHD) prophylaxis intravenously on days 0 through 5 at a dose of 10 mg/kg BID (20 mg/kg/day) (Sandimmune® Injection Solution, Sandoz Pharmaceuticals Corporation). On days 6 through 50 (end of study) for group I.1.a, or 6 through 100 for group I.1.b, cyclosporin was administered at 10 mg/kg BID PO, (20 mg/kg/day) (Neoral® Soft Gelatin Capsules, Sandoz Pharmaceuticals Corporation). The usual supportive care with oral antibiotics for the recipient began on day –5 and systemic antibiotics started on day 0 and continued until engraftment was achieved. Fluid support was given as necessary. No platelet transfusions were required for any of the four dogs during recovery. Standard canine procedures require that a whole blood transfusion to be administered if the platelet count consistently drops below $10,000/mm^3$, or if the treatment staff observes signs of bleeding. Platelet transfusions,

if necessary, were to be administered as 50-ml of whole irradiated (2000 cGy) blood from a random donor. Engraftment was established as the time of the first of three consecutive measurements of >500 absolute neutrophil cells mm^3, >1,000 /mm^3, and platelets >10,000 /mm^3, 50,000 /mm^3, and >100,000.

To follow hematopoietic recovery, complete blood counts (CBCs) were obtained from day 0 through day 50, and biweekly thereafter for the 100-day study group. Serum chemistry analysis was performed on days 0, 2, and weekly thereafter. Peripheral blood samples were taken on day 0 pre-MSC infusion, 5- and 15-minutes, 1- and 2-hours, and 1-, 2-, 3-, and 4-day time points for DNA isolation. The DNA was evaluated for the presence of GFP marked cells by an Anti-EGFP DNA PCR Elisa with digoxigenin incorporated into the product and a second step anti-digoxigenin colorimetric assay. A marrow aspirate was obtained when the platelet counts consistently reached 50,000/mm^3 and examined for the presence of GFP marked cells using the same PCR method. CMSC cultures were established to examine colony forming units (CFU), and to expand the cMSC for further Anti-EGFP PCR analysis. Upon necropsy, peripheral blood, bone marrow aspirates, and bone marrow biopsies were obtained for Anti-EGFP PCR analysis. CFU assays were performed on the bone marrow aspirates, and the Anti-EGFP PCR analysis was performed on culture-expanded cMSC. An histological analysis was performed for the presence of GFP in various tissues.

cMSC isolation, culture-expansion, transduction and cryopreservation

Bilateral bone marrow aspirates were obtained for cMSC isolation and culture establishment on week –4 for dogs CAN-07-01 and CAN-07-02 and on week –9 for dogs CAN-07-03 and CAN-07-04. Fifteen ml of marrow (7 ml from each humerus) were obtained from each dog. Dogs were anesthetized by the injection of Butorphanol followed by injection of a mixture of Diazepam and ketamine hydrochloride (Aveco Co., Inc., Fort Dodge, IA). The sites of needle insertion were scrubbed with povidone-iodine and then rinsed with alcohol. Aspirates were obtained

from each humeral condyle of each dog using a 16-gauge, 2-inch bone marrow needle. A syringe was attached to the needle, and suction was applied to remove 8 ml of marrow from each humerus. Bone marrow aspirates were transferred to 15 ml polypropylene conical tubes using sterile technique. Following the procedure, the dog was then placed on a warming pad to recover.

Five to 10 ml aliquots of bone marrow were diluted to 50 ml in Dulbecco's Phosphate Buffered Saline (DPBS) in a polypropylene culture tube. The cell suspensions underwent centrifugation at 2200 RPM for 10 minutes at room temperature (RT). Total nucleated cell counts were determined in 4% acetic acid. Cells were then diluted in DPBS for a final concentration of 20×10^6 cells/ml. Ten ml or 200×10^6 cells were loaded onto 20 ml of Percoll (sp.gr. 1.073 gm/ml) in a 50 ml conical tube and underwent centrifugation at 1300 RPM for 20 minutes. The cell interface containing mononuclear cells was washed in DPBS, resuspended in complete media, and counted to obtain a recovery percentage. The cells were then diluted in complete media, cultures were established as described below, and placed in a 37°C incubator at 5% CO_2.

Construction of bicistronic MuLV retroviral vector

The green fluorescent protein (EGFP) retrovirus was constructed by isolating EGFP-1 gene from the jellyfish *Aequorea victoria* (Clontech, CA). EGFP gene was cloned into retroviral vector pJM573-neo (resulting plasmid was named pOT-24). The plasmid pJM573-neo was derived from pN2 (*Keller et. Al., 1985, Nature 318:149*) with the following modifications: murine retroviral *gag* initiation site was substituted with an in-frame stop codon; 5'LTR and 3'LTR were constructed into the same cassette; neomycin phosphotransferase gene (*neo*) and an internal ribosomal entry site (*IRES*) were inserted into pN2. A schematic map of EGFP pOT24 plasmid is shown in Figure 8.

Preparation of Recombinant Retrovirus

pOT-24 was transfected into GP&E86 ecotropic producer cells using DOTAP (Boehringer Manheim) as suggested by manufacturer. The transfected cells were grown in DMEM-high glucose (HG) medium supplemented with 10% heat inactivated FBS, Penicillin-Streptomycin (Life Technologies) and 0.5mg/ml of protamine sulfate-G418 (Sigma) as a selective marker. Cultures were maintained up to 70% confluency at which point medium was replaced with fresh retroviral media (without G418) and cells were maintained at 32°C for 2 days. The culture medium containing the retrovirus was collected, filtered through 0.45 μm filter and stored at -70°C. Amphotropic retrovirus was prepared by transducing PA317 cells twice with ecotropic virus using a centrifugal transduction procedure followed by selection with G418 (0.5 mg/ml). Retroviral supernatant was collected. The titer of the pooled EGFP retrovirus on 3T3 cells was $1.2x10^6$ CFU/ml. GFP-retroviral supernatants were cryopreserved at -70°C.

CAN-07-01 and CAN-07-02

The washed mononuclear cells obtained at the Percoll interface were established in 10, T-185 flasks containing 30 ml of complete media and 10×10^6 cells/flask.

On days 2, 6, and 9 of culture, the media in the flasks was replaced entirely with fresh complete media. On day 12 of the primary culture photographs were taken, and the cells were taken from passage 0 (PO) to passage 1 (P1). The media was aspirated and the flasks were washed twice with 8 ml DPBS. Eight ml of trypsin was added, and flasks were placed in a 37°C incubator for 3 minutes. When the cells had lifted, the reaction was stopped by the addition of 8 ml of complete media. The cells were transferred and pooled into 50 ml conical tubes. The flasks were washed with DPBS and the pooled cells were centrifuged at RT at 2000 RPM for 5 minutes. The supernatant was removed and the cell pellets were resuspended in complete media. The cells were pooled, counted and examined for viability. Cells were plated into 15, T80 flasks containing 18 ml of complete medium and 0.4×10^6 cells per flask.

On day 15 in culture, the first transduction was performed on 15 of the 18 flasks. The media was removed. Aliquots of the retroviral supernatant were thawed and polybrene was added to a final concentration of 8 μg/ml to make the transduction cocktail. The cell medium was replaced with 10 ml of the transduction cocktail, and the flasks were centrifuged at 3000 RPM for 1 hour at 32°C. After centrifugation, 10 ml of complete media prepared using heat inactivated fetal bovine serum (FBS) was added to each flask (with the transduction cocktail) and the flasks were returned to the incubator. Three flasks were not transduced, and fresh media was replaced. On day 16 of culture, the media was replaced with fresh complete media. On day 17 of culture the transduction procedure was repeated.

On day 18 of culture, the cells were harvested as described above and taken from P1 to P2. Three x 10^6 cells were added to 100 ml of complete medium, and poured into triple-flasks (500 cm^2). Fifteen triple-flasks were prepared with transduced cells and three were prepared with untransduced cells. Any remaining cells were cryopreserved. A freeze solution was prepared containing 10% DMSO and 90% FBS. Ten x 10^6 cells were resuspended in 1 ml of freezing solution. The vials were labeled and cryopreserved in a Nalgene Cryo container for a minimum of 4 hours at –70°C, and stored at –70°C.

On day 22 of P2 culture, photographs were taken to record the cell distribution and morphology and the P2 cells were harvested and cryopreserved as described below.

CAN-07-03 and CAN-07-04

The washed mononuclear cells obtained at the Percoll interface were established in 15, T-75 flasks containing 20 ml of complete media and 12 x 10^6 cells/flask.

On day 2 of culture, the media in the flasks and in the dishes was replaced entirely with fresh complete media. On day 6 of primary culture for cMSC, the first transduction was performed as described above. Three flasks were not transduced, and fresh media was replaced on day 6. On day 7 of culture, the media was replaced with fresh media.

On day 8 of culture the transduction procedure was repeated. On day 9 in culture, photographs were taken, and the cells were passaged from P0 to P1 as described above. Three x 10^6 cells were added to 100 ml of complete medium, and poured into triple flasks. Fifteen triple flasks were prepared with transduced cells and three were prepared with untransduced cells.

The 15 ml bone marrow aspirates yielded 910, 1212, 856, and 1948 x 10^6 nucleated cells for donors CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively. Mononuclear cell counts obtained from the Percoll interface were 612, 666, 588, and 462 x 10^6, resulting in recoveries of 67.2, 55, 68.7, and 23.7%. Upon P1, the cell viability was a mean of 97.1 (range 93.3 to 100)%. Upon P2 for donors CAN-07-01 and CAN-07-02, and P1 cells for donors CAN-07-03 and CAN-07-04, the cell viability of the transduced cells was a mean of 96.7 (range 96.3 to 97.9)%. The untransduced cells were 95.4 (range 93.3 to 96.9)% viable. Upon harvest for cryopreservation of the cMSC, the viability of the transduced cells was a mean of 99.4 (range 97.4 to 100)% and the untransduced cells were 99.4 (range 97.6 to 100)% viable (Table 4).

The transduced cMSC yield per flask for donors CAN-07-01 and CAN-07-02, harvested 4 days after passage 2 and plated at 3 x 10^6 per flask was 5.9 and 6.7 x 10^6, and the untransduced cMSC yield per flask was 8.4 and 7.5 x 10^6. The transduced cMSC yield per flask for donors CAN-07-03 and CAN-07-04, harvested 4 days after passage 1 (different transduction and passage design) and plated at 3 x 10^6 per flask

was 20.0 and 14.0 x 10^6, and the untransduced cMSC yield per flask was 25.3 and 18.0 x 10^6.

CFU Assays on cMSC from P0 Cultures

CFU colony assays were prepared at the time of primary culture establishment by plating 0.5 x 10^6 cells in triplicate in 100 mm dishes containing 10 ml complete media. The dishes were incubated at 37°C and 5% CO_2. The media was replaced with fresh media each 2 to 4 days. On day 10 in culture, the CFU assay dishes were rinsed with HBSS twice, fixed with 1% gluteraldehyde for 15 minutes, rinsed with HBSS twice, and air dried. The cMSC in the dishes were then stained with 0.1% crystal violet, rinsed with deionized water three times, and air dried. Colonies were counted to calculate the number of colonies forming per 10^6 cells plated.

CFU assays plated on day of mononuclear cell isolation and culture establishment and harvested on day 10 yielded 56, 46.7, 114, and 72 colonies per 10^6 cells for dogs CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively.

On day 13 of P1 culture, photographs were taken to record the cell distribution and morphology and the P1 cells were harvested by trypsinization and cryopreserved as described below.

The media in the triple flasks was decanted, and the flasks were rinsed with 50 ml DPBS. After decanting the DPBS, 23 ml of 0.25% trypsin was added to each triple flask. The flasks were placed in a 37°C incubator for 3 minutes. After cell detachment, 23 ml complete medium was added to each flask. The cell suspensions were transferred to 50 ml conical tubes and the flasks were washed with 30 ml HBSS. The tubes were centrifuged at 2200 RPM for 5 minutes at RT. The pellets containing the transduced or untransduced cells, respectively, were pooled and counted. One aliquot of 1 x 10^7 cells was set aside for determination of the transduction percentage by an Anti-EGFP DNA PCR Elisa assay.

After harvest, the recovered P1 or P2 transduced and culture-expanded cMSCs centrifuged at 1300 RPM for 5 minutes and resuspended in 1 ml aliquots with 1 x 10^7 cMSC/ml in ice-cold cryoprotectant solution containing 85% Plasma-Lyte A (Baxter IV Therapy), 10% DMSO, and 5% autologous canine serum. Cell aliquots were dispensed into separate cryo-vials containing 1 ml each. The tubes were labeled with the canine donor number and total viable cell count. The cMSCs were cryopreserved by placing the cell vials into a Nalgene freezing container and placed in a -70°C freezer for 4 hours, then moved to storage at –70°C.

Upon cell harvest for cryopreservation of the product, aliquots of 1 x 10^7 cells were obtained for determination of the transduction efficiency. The transduction efficiency was analyzed by an Anti EGFP DNA PCR Elisa with digoxigenin incorporation into the product and a second step Anti-digoxigenin colorimetric assay.

CMSC Infusion Product

One to two hours before infusion, the vials of cMSC were thawed by swirling in a 37° water bath, sprayed with 70% ethanol, and opened in a biosafety cabinet. The cMSC product was suspended in 50 ml of infusion medium containing DMEM-LG plus 30% serum autologous to the cell donor. The viability of the cMSC product was determined by exclusion of trypan blue to determine the actual viable dose. An aliquot of each cMSC product was submitted for yeast isolate, aerobic, and non-aerobic growth. The cMSCs were evaluated for the ability to attach to tissue culture plastic and to proliferate in P2 (P3 for CAN-07-01 and CAN-07-02) culture. Aliquots of 1 x 10^6 and 0.16 x 10^6 cMSC were plated into complete canine culture medium in triplicate in T-25 plastic culture flasks. After 24 hours, the flasks plated with 1 x 10^6 cMSC and on day three, the flasks plated with 0.16 x 10^6 cMSC were harvested by trypsinization and counted.

Following TBI, the cMSC suspension was infused via a catheter inserted into the cephalic vein using a hand-held Harvard Bard Mini Infuser to deliver the 50 ml over a 15-20 minute period.

Moderately high doses of 7.49, 7.35, 10.0, and 10.0 (mean 8.7) x 10^6 viable cMSC/kg were infused on day 0 to dogs CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively. These doses represent a 4- to 10-fold increase over the typical dose that a patient would receive. Total viable cMSC infused ranged from 67.7 to 129 (mean 93.9) x 10^6 cMSC. The viability of the cells ranged from 92.1 to 97.6 (mean 94.9) as determined by trypan blue exclusion. CMSC Infusions were given between 71 and 146 (mean 110) minutes post-TBI.

Blood Sampling Post-Infusion

Blood samples (2 ml) were obtained before (pre) and during the cMSC infusion at five and fifteen minutes after the start of the infusion, as well as 1- and 2-hour, and 1-, 2-, 3-, and 4-day time points. Cell lysates were prepared using the Puregene™ (Gentra Systems, Inc.) DNA Isolation Kit for use in an Anti EGFP DNA PCR Elisa with digoxigenin incorporated into the product and a second step Anti-digoxigenin colorimetric assay to detect of the level of GFP marked cMSC in the bloodstream.

Bone marrow harvest and graft infusion

Bone marrow to be used as the transplant graft was harvested from the DLA-identical littermate prior to TBI. Aspirates were obtained from each humerus using an 11-gauge, 4-6 inch ball-top stainless steel marrow harvest needle, attached to polyvinyl tubing originating from a vacuum flask containing 100 ml Tissue Culture Medium 199 and 4 ml (4000 U) heparin. The marrow is passed through 300- and 200-um pore size, and stored at 4°C in a transfer pack container, labeled with the donor and recipient, until infusion later that day. The bone marrow total nucleated cell count (BM-TNC) of the marrow is corrected to exclude any nucleated cells which

would be present in the volume of peripheral blood obtained during the marrow harvest.

The total nucleated cell count (TNC) of the bone marrow was corrected to exclude any TNC which would be present in the volume of peripheral blood obtained during the marrow harvest. Corrected doses of marrow were 4.3, 3.5, 3.1, and 2.0 (mean 3.2) x 10^8 TNC/kg to dogs CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively. Uncorrected bone marrow doses were 5.6, 4.2, 4.5, and 2.7 (mean 4.3) x 10^8 TNC/kg.

Twenty minutes prior to infusion, the marrow was placed at room temperature. One hour after the cMSC infusion, the marrow was infused intravenously through a butterfly needle inserted into the cephalic vein, by exerting pressure on the bag over 1 to 2 minutes.

Supportive care

On day –5, oral antibiotics (neomycin sulfate and polymyxin sulfate) were given three times daily. These oral antibiotics were administered until absolute neutrophil counts reached 500/mm^3. On day 0, the systemic antibiotic Baytril was administered intravenously twice daily and continued until absolute neutrophil counts reached 1,000/mm^3 consistently. Fluid and electrolytes lost as results of transient radiation toxicity were replaced by subcutaneous administration of 500 ml of Ringers Solution, twice daily until food and water were accepted.

Differential blood cell counts

Blood samples (2 ml) were collected from either the jugular or cephalic vein on the mornings of the marrow aspirate for isolation of cMSC, days 0 through 50 and biweekly thereafter through the end of study. The blood was transferred into a vacutainer containing EDTA. Total white blood cell (WBC) and platelet counts per

mm^3 are measured using a Sysmex E2500 and differential cell counts were determined manually after fixation and staining with Wrights stain.

Necropsy

Blood samples were obtained for CBC, Chemistry 23 analysis, and PCR evaluation. The dogs were sedated with Butorphanol followed by a mixture of Diazepam and ketamine hydrochloride. After sedation, biopsies and bilateral bone marrow aspirates were obtained from the humerii, femora, and iliac crests. Euthanasia was then completed with an overdose of the sedative sodium pentobarbital. The day-50 group of dogs (CAN-07-01 and CAN-07-02) were euthanised on day 43 in the study; the day-100 group of dogs (CAN-07-03 and CAN-07-04) were euthanised on day 100 in the study. Complete sets of the tissues were collected upon necropsy of the animals.

The collection of tissues for histological examination followed immediately. A subset of tissues was used for Anti-EGFP DNA PCR Elisa analysis. Bone marrow aspirates and biopsies were used for Anti-EGFP DNA PCR Elisa analysis, culture expansion for further PCR analysis, and CFU assays

The tissues were trimmed to about 1 inch square pieces and placed into separate labeled 50 ml conical tubes filled with 10% Neutral Buffered Formalin (pH 6.8 - 7.2). The tissues were embedded in paraffin, sectioned and stained with Hematoxylin and Eosin. Bone marrow samples were stained with Periodic Acid Schiff's stain.

Bone marrow aspirates obtained prior to necropsy were collected in 15 ml labeled tubes from the left and right humerii, femora, and iliac crests from each dog. A subset of the tissue samples were obtained during necropsy and trimmed to about 1/4 inch square pieces, wrapped in

PBS-soaked gauze and placed separately in a labeled zip-lock bag. The bone marrow aspirates were held on ice.

Preparation of bone marrow aspirates for CFU assay

Aliquots of the bone marrow aspirates from the left and right humerus, femur, and iliac crest from each canine obtained for PCR analysis were aliquoted into separate 15 ml labeled tubes. The bone marrow samples were held on ice.

CFU assay on cMSC from bone marrow obtained at necropsy

CFU colony assays performed on cMSC obtained from bone marrow obtained at necropsy were prepared by plating 0.5 x 10^6 cells in triplicate in 100-mm dishes containing 10 ml complete media. The dishes were incubated at 37°C and 5% CO_2. The media was replaced with fresh media each 2-4 days. On day 10 in culture, the CFU assay dishes were rinsed with HBSS twice, fixed with 1% gluteraldehyde for 15 minutes, rinsed with HBSS twice, and air dried. The cMSC in the dishes were then stained with 0.1% crystal violet, rinsed with deionized water three times, and air dried. Colonies were counted to calculate the number of colonies per 10^6 cells plated.

Isolation and purification of DNA

DNA was isolated from a part of each tissue. The remaining piece of the sample was cryopreserved and stored in -70°C freezer. DNA was isolated by placing samples in Phosphate Buffered Saline (PBS), adding proteinase K solution, and incubating at 55°C for 3 hrs, or until the tissue has dissolved. The samples were subsequently treated with RNase at 37°C for 60 min. The samples were cooled to room temperature and the protein was precipitated. The samples were centrifuged and the aqueous phase was gently collected in 100% isopropanol. The samples were mixed and centrifuged and the pellet was washed in 70% ethanol. The tubes were centrifuged and the supernatant was drained off and the pellets were allowed to dry for approximately 1 to 6 hrs. The DNA was allowed to hydrate overnight at room temperature and was subsequently stored at 4°C.

Peripheral blood and bone marrow samples were first lysed with RBC lysis solution (Ammonium Chloride Buffer). DNA was then isolated from the lysates as described above. DNA was quantified by the addition of 998 µl deionized H_20 and 2 µl DNA from the sample into a cuvette and vortexed. A spectrophotometer was used to determine the optical density (OD). The OD was read at 260 and 280, and the concentration of DNA was calculated for µg/ml. The DNA concentration was adjusted to 1 µg/ml using deionized water.

Anti-EGFP DNA PCR Elisa

The anti-EGFP DNA PCR Elisa assay used in these studies detects infused cMSCs utilizing oligonucleotide primers specific for GFP. For analysis of gene expression, we utilized PCR-ELISA (DIG labeling/detection) kit (Boehringer Mannheim). Briefly, PCR was performed in the presence of digoxigenin-labeled nucleotides to label the amplified product. Next, 25 µl of the PCR product was denatured and allowed to hybridize in solution to 5'-biotinylated oligonucleotide probe at 37°C in streptavidin-coated microtiter plate. The bound probe-PCR product was detected by an anti-digoxigenin peroxidase conjugate and by use of the colorimetric substrate 2,2'Azinobis (3-ethylbenzthiazoline-sulfonic Acid) (ABTS). Titration standard curves were generated using transfected control cMSC to approximate concentration of DNA per quantity of DNA used in the assay. By first correlating with an internal standard for PCR of the DLA Class II genomic DNA, and then correlating DNA concentration to cell equivalents, and assuming one retrovirus integration event per transduced cell, an estimation of cell number can be obtained.

Quantitative measurements of DNA for GFP were noted in all bone marrow scoops/biopsies.

Post-transplant blood cell recovery

The mean day to a threshold (to 3 consecutive values) of platelets to 10,000/mm^3 was 12.8 (range 11-17), to 50,000/mm^3 was 19.8 (range 16-25), and to 100,000/mm^3 23.0 (range 20-27). The mean day to a threshold value (to 3 consecutive days) of absolute neutrophil cells to 500/mm^3 was 9.3 (range 8-11), and to 1,000/mm^3 was 10.5 (range 9-13).

Interim bone marrow aspirates

When platelets recovered consistently to values greater than 50,000 per mm^3, an interim bone marrow aspirate was collected from the iliac crest. This procedure was performed on day 27 in study for CAN-07-01 and CAN-07-02, and on day 29 for CAN-07-03 and CAN-07-04.

RESULTS

Upon histopathological evaluation of all tissues from CAN-07-01 and CAN-07-02, euthanised on day 43, findings were negative for ectopic connective tissue and for subacute GVHD.

Detectable DNA signal could be found within 1 hour of infusion and again at 2 days. One sample could be quantitatively measured at 3 days post infusion for GFP DNA. This timepoint is consistent with the previous observations in the autologous canine transplant study in which signal was found at 2 and 3 days

Day 100 necropsy data in CAN-07-03 and CAN-07-04 for GFP+ cells showed GFP signal (1 GFP+ cell equivalent per 10 micrograms PCR input DNA) in the femur and humerus of CAN-07-03 and in the humerus of CAN-07-04.

In this model it was possible to detect skin graft-versus-host-disease (GVHD) by observing the redness of the eyes and ears of the animals. Using this indicator, it was determined that the animals that received mesenchymal stem cells had a lower incidence of and/or lower severity of GVHD compared to the control animals that were not treated with mesenchymal stem cells.

These results demonstrate that allogeneic MSCs can support the rapid engraftment of bone marrow hematopoietic cells. No transfusion support was needed. There was no clinical evidence of GVHD. Platelet recovery was faster than in historical controls. There was evidence of chimerism in stromal cells after allogeneic transplantation. The option to engraft allogeneic tissue by using allogeneic MSCs broadens the range of transplant material usable in clinical transplant scenarios.

Example 9

Suppression of Mixed Lymphocyte Reaction by MSC Supernatant (MLR95)

Generation of supernatants: T cells from donor 155 were purified from PBMC by negative immunomagnetic selection with anti-CD19 and anti-CD14 MicroBeads (Miltenyi Biotec). PBMC from donor 413 were X-ray irradiated with 3600 rad (12 min at 70 kV). In 24-well tissue culture plates T cells ($9x10^5$/well) were mixed with irradiated PBMCs for 3 days, then MSCs from different donors (219, 459, 461 - all at passage 5) were added into the cultures at 1.2 $x10^5$ cells/well for 3 additional days. In control cultures, the same volume of medium was added instead of MSCs. In separate wells, the same number of MSCs were plated alone, and cultured for 3 additional days. After 3 days of culture (on day 6 after initiation of the primary MLR), the cells were resuspended by pipetting, and 200 µl of the cell suspensions were transferred into a 96-well plate in triplicate, and pulsed with [H^3]TdR (5 Ci/mmol, 1 µCi/well) for 18 hours to determine the level of proliferation. The remaining cells were centrifuged at 1250 rpm for 10 minutes, the supernatants were collected, aliquoted and kept frozen at -80°C.

Suppression of primary MLR by supernatants: T cells from donor 155 were purified from PBMCs by negative immunomagnetic selection with anti-CD19 and anti-CD14 MicroBeads (Miltenyi Biotec). PBMCs from donor 413 or from donor 273 were X-ray irradiated with 3600 rad (12 min at 70 kV). In 96-well tissue culture plates T cells (1.5 x 10^5/well) were mixed with PBMCs (1.5 x 10^5/well) in the presence or absence of different supernatants that were added at the initiation of cultures at the dilutions (1/8, 1/32, 1/128, 1/512, 1/2048 and 1/8192). In control cultures, the same volume of medium was added instead of supernatants. Cultures were incubated for 6 days, then pulsed with [H^3]TdR (5 Ci/mmol, 1 µCi/well) for 18 hours.

Figure 9 shows the results of the MLR between T 155 x PBMC 413. Primary MLRs in the presence of supernatants of MSCs + MLR, (#1) MLR +MSC219 (#2) MLR +MSC459 (#3) MLR +MSC461, and MSCs alone, (#8) MSC219 alone (#9) MSC459 alone (#10) MSC461 alone, were suppressed. Primary MLR in the presence of supernatant of MLR alone (#5) was not suppressed.

Suppression of an on-going MLR T cells from donor 155 were purified from PBMCs by negative immunomagnetic selection with anti-CD19 and anti-CD14 MicroBeads (Miltenyi Biotec). PBMCs from donor 413 (1.5 x 10^5/well) were X-ray irradiated with 3600 rad (12 min at 70 kV). In 96-well tissue culture plates T cells (1.5 X 10^5/well) were mixed with PBMCs (1.5 x 10^5/well) for 4 days, then supernatants were added into the cultures at different dilutions (1/8, 1/32, 1/128, 1/512, 1/2048 and 1/8192). In control cultures, the same volume of medium was added instead of supernatants. Cultures were incubated for 2 additional days (6 days after initiation of the MLR), then pulsed with [H^3]TdR (5 Ci/mmol, 1 μCi/well) for 18 h.

Figure 10 shows the results of the MLR between T 155 x PBMC 413. Supernatants from all MSCs + MLR (#1) MLR +MSC219 (#2) MLR +MSC459 (#3) MLR +MSC461 showed strong suppression of ongoing MLRs. Supernatants from MSCs alone (#8) MSC219 alone (#9) MSC459 alone (#10) MSC461 alone suppressed in a dose dependent manner with a significant effect up to 1/512 dilution. Ongoing MLR in the presence of supernatant of MLR alone (#5) was not suppressed.

Example 10

Suppression of Mixed Lymphocyte Reaction by Xenogeneic Mesenchymal Stem Cells

Suppression of Human MLRs by Baboon MSCs. Responder PBMCs from various human donors (R4, R6, R7, R11) were mixed with irradiated (3000R) allogeneic human PBMCs (S4, S6, S7, S11) in microtiter wells are 1.5 x 10^5 cells/well

for each population. Cultures were performed in standard cell culture medium containing 5% human AB serum. Baboon MSCs (bMSCs) from donor 86243 were added at 2 x 10^4 /well at the initiation of the MLR. The MSCs were not treated with IFNγ. Lymphoproliferation was determined on day 7 of culture by pulsing the cells with ^{3}H-thymidine for the final 18 hours prior to cell harvest for scintillation counting. The results illustrated in Figure 11 show that baboon MSCs suppressed robust human MLRs by greater than 50%.

Suppression of Xenogeneic MLR by Human or Baboon MSCs. Responder human T cells (hT) from donor 273 were cultured with irradiated (3000R) baboon PBMC (bPBMC) from donor 5957 or donor 5909 in standard cell culture medium containing 5% human AB serum. Human MSCs (hMSCs) from donor 244 or baboon MSCs (bMSC) from donor 6243 were added to the cultures at initiation. Lymphoproliferation was determined on day 7 of culture by pulsing the cells with ^{3}H-thymidine for the final 18 hours prior to cell harvest for scintillation counting. The results illustrated in Figure 12 (bPBMC donor 5957) and Figure 13 (bPBMC 5909) show that both human and baboon MSCs can suppress the xenogeneic human x baboon MLR.

What Is Claimed Is:

1. A method for reducing an immune response against an alloantigen, comprising contacting immune effector cells with a supernatant from a mesenchymal stem cell culture in an amount effective to reduce the immune response.

2. The method of claim 1 wherein the effector cells are T cells.

3. A method for preventing or reducing reactivation of activated T cells comprising contacting T cells that have been previously activated by an alloantigen with a supernatant from a mesenchymal stem cell culture in an amount effective to suppress re-stimulation of said activated T cells.

4. A method of reducing an immune response to a donor transplant, comprising treating the recipient with a supernatant from a mesenchymal stem cell culture in an amount effective to reduce an immune response in the recipient to the transplant.

5. The method of claim 4 wherein the mesenchymal stem cells are autologous to the recipient.

6. The method of claim 4 wherein the mesenchymal stem cells are allogeneic to the recipient.

7. The method of claim 6 wherein the mesenchymal stem cells are obtained from the donor of the transplant.

8. The method of claim 4 wherein the mesenchymal stem cells are allogeneic to both the donor of the transplant and the recipient.

9. The method of claim 4 wherein the mesenchymal stem cells are xenogeneic to both the donor of the transplant and the recipient.

10. The method of claim 4 wherein the transplant is skin.

11. The method of claim 4 wherein the supernatant is administered to the recipient prior to administration of the transplant.

12. The method of claim 4 wherein the supernatant is administered concurrently with administration of the transplant.

13. The method of claim 4 wherein the supernatant is administered as a part of the transplant.

14. The method of claim 4 wherein the supernatant is administered after the transplant.

15. The method of claim 4 wherein the supernatant is administered to the transplant recipient to treat rejection of the transplant by the recipient.

16. The method of claim 4 further comprising administering to the recipient immunosuppressive agents.

17. The method of claim 4 wherein the transplant is a solid organ.

18. The method of claim 17 wherein the solid organ is selected from heart, pancreas, kidney, lung or liver.

19. A method of treating a transplant recipient for graft versus host disease, comprising treating the recipient of a donor transplant with a supernatant from a

mesenchymal stem cell culture in an amount effective to reduce an immune response against the recipient by the transplant.

20. The method of claim 19 wherein the mesenchymal stem cells are autologous to the recipient.

21. The method of claim 19 wherein the mesenchymal stem cells are autologous to the donor transplant.

22. The method of claim 19 wherein the mesenchymal stem cells are allogeneic to both the donor and recipient.

23. The method of claim 19 further comprising administering to the recipient immunosuppressive agents.

24. A composition for reducing an adverse immune response against a donor transplant, comprising a supernatant from a mesenchymal stem cell culture in an amount effective to inhibit or reduce an adverse immune response against a donor transplant, and a pharmaceutical carrier.

25. The composition of claim 24 wherein the mesenchymal stem cells are autologous to the recipient.

26. The composition of claim 24 wherein the mesenchymal stem cells are autologous to the donor.

27. The composition of claim 24 wherein the mesenchymal stem cells are allogeneic to both the recipient and the donor.

28. The composition of claim 24 wherein the mesenchymal stem cells are xenogeneic to both the recipient and the donor.

29. A composition for reducing an adverse immune response against a graft recipient caused by a graft, comprising a supernatant from a mesenchymal stem cell culture in an amount effective to reduce the adverse immune response against the graft recipient caused by the graft, and a pharmaceutical carrier.

30. The composition of claim 29 wherein the mesenchymal stem cells are autologous to the recipient.

31. The composition of claim 29 wherein the mesenchymal stem cells are autologous to the donor.

32. The composition of claim 29 wherein the mesenchymal stem cells are allogeneic to both the recipient and the donor.

33. The composition of claim 29 wherein the mesenchymal stem cells are xenogeneic to both the recipient and the donor.

34. The process of Claim 1 wherein the supernatant is obtained from mesenchymal stem cells co-cultured with T cells undergoing a mixed lymphocyte reaction.

35. A method for reducing an immune response against an alloantigen, comprising contacting immune effector cells with xenogeneic mesenchymal stem cells in an amount effective to reduce the immune response.

36. A method for reducing an immune response of effector cells against an alloantigen, comprising contacting effector cells with a supernatant of mesenchymal

stem cells in an amount effective to reduce an immune response against an alloantigen whereby said effector cells upon contact with an alloantigen have a reduced immune response against said alloantigen.

37. The method of claim 36 wherein said effector cells are T cells previously activated and said immune response is the reactivation of said T cells.

38. The method of claim 36 wherein said immune response to be reduced is an *in vitro* immune response.

39. The method of claim 36 wherein said immune response to be reduced is an *in vivo* immune response.

40. The method of claim 36 wherein said immune response to be reduced is an immune response to a donor transplant.

41. The method of claim 40 wherein said donor transplant is a xenogeneic donor transplant.

FIG. 1

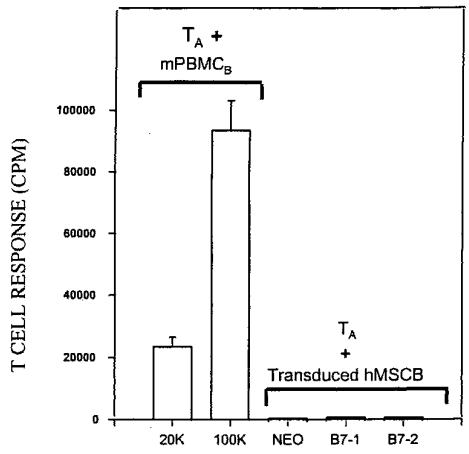

T CELL RESPONSE (CPM)
100000
80000
60000
40000
20000
0
T_A +
$mPBMC_B$
T_A
+
Transduced hMSCB
20K
100K
NEO
B7-1
B7-2

FIG. 2

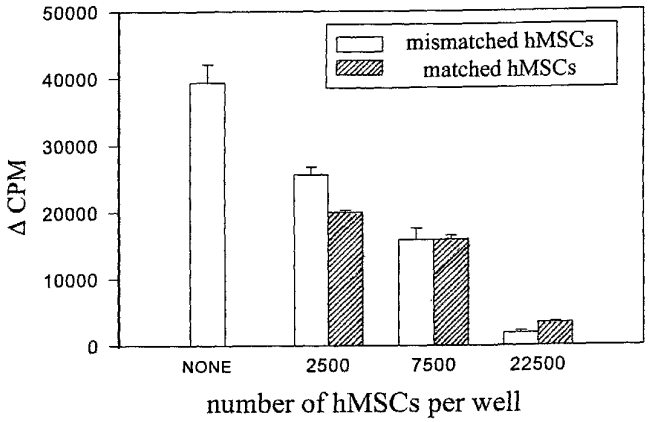

50000
40000
30000
20000
10000
0
Δ CPM
mismatched hMSCs
matched hMSCs
NONE
2500
7500
22500
number of hMSCs per well

FIG. 3

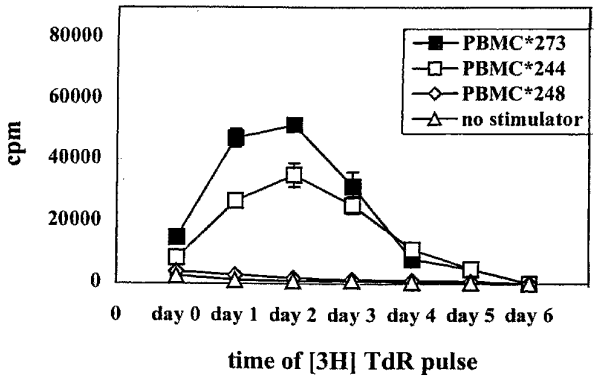

80000
60000
40000
20000
0
cpm
PBMC*273
PBMC*244
PBMC*248
no stimulator
0 day 0 day 1 day 2 day 3 day 4 day 5 day 6
time of [3H] TdR pulse

FIG. 4

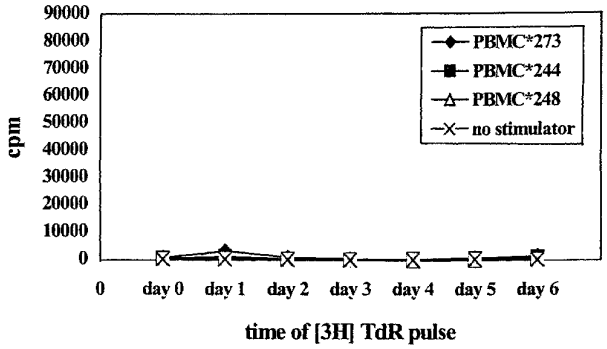

90000
80000
70000
60000
50000
40000
30000
20000
10000
0
cpm
PBMC*273
PBMC*244
PBMC*248
no stimulator
0
day 0
day 1
day 2
day 3
day 4
day 5
day 6
time of [3H] TdR pulse

FIG. 5A

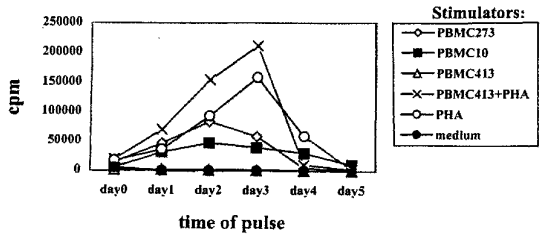

Stimulators:
PBMC273
PBMC10
PBMC413
PBMC413+PHA
PHA
medium
cpm
250000
200000
150000
100000
50000
0
day0
day1
day2
day3
day4
day5
time of pulse

FIG. 5B

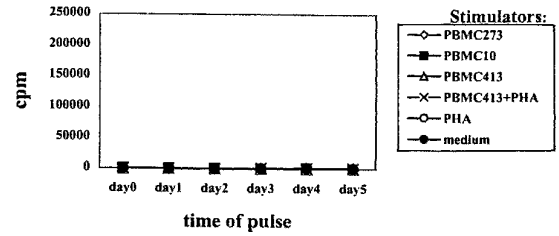

Stimulators:
PBMC273
PBMC10
PBMC413
PBMC413+PHA
PHA
medium
cpm
250000
200000
150000
100000
50000
0
day0
day1
day2
day3
day4
day5
time of pulse

FIG. 5C

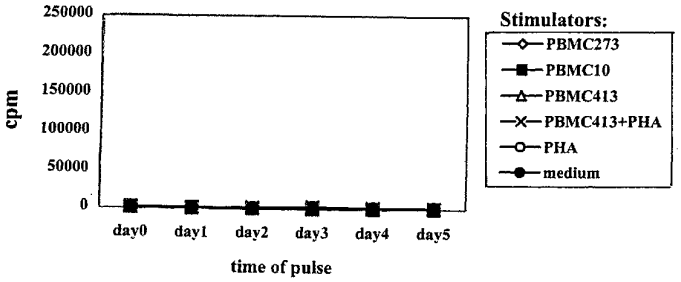

250000
200000
150000
100000
50000
0
cpm
day0
day1
day2
day3
day4
day5
time of pulse
Stimulators:
PBMC273
PBMC10
PBMC413
PBMC413+PHA
PHA
medium

FIG. 5D

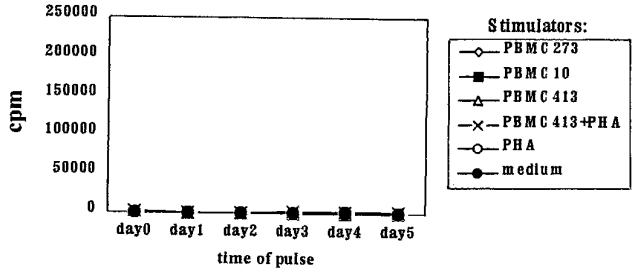

250000
200000
150000
100000
50000
0
cpm
day0
day1
day2
day3
day4
day5
time of pulse
Stimulators:
PBMC273
PBMC10
PBMC413
PBMC413+PHA
PHA
medium

FIG. 6A

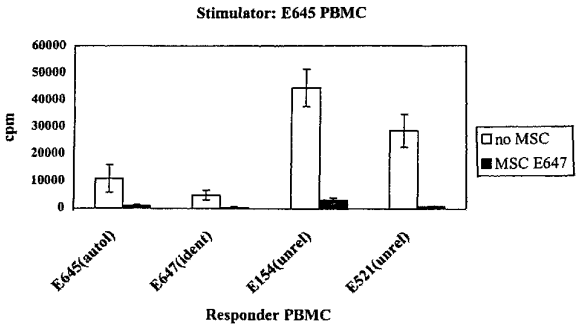

Stimulator: E645 PBMC
60000
50000
40000
30000
20000
10000
0
cpm
no MSC
MSC E647
E645(autol)
E647(ident)
E154(unrel)
E521(unrel)
Responder PBMC

FIG. 6B

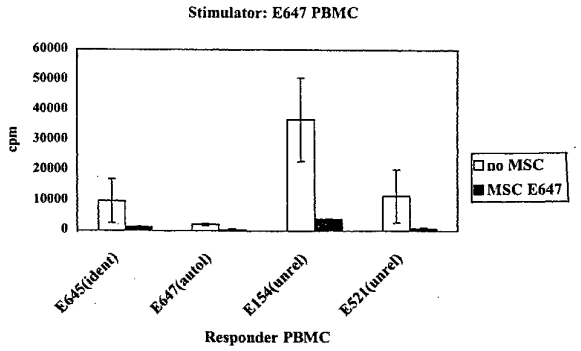

Stimulator: E647 PBMC
60000
50000
40000
30000
20000
10000
0
cpm
no MSC
MSC E647
E645(ident)
E647(autol)
E154(unrel)
E521(unrel)
Responder PBMC

FIG. 6C

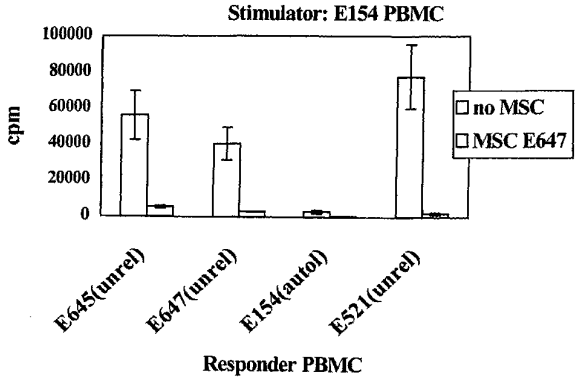

Stimulator: E154 PBMC
100000
80000
60000
40000
20000
0
cpm
no MSC
MSC E647
E645(unrel)
E647(unrel)
E154(autol)
E521(unrel)
Responder PBMC

FIG. 6D

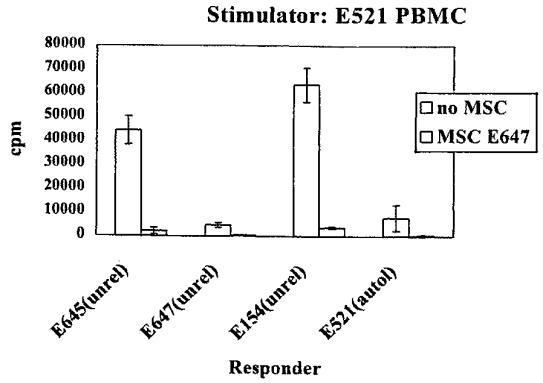

Stimulator: E521 PBMC
80000
70000
60000
50000
40000
30000
20000
10000
0
cpm
no MSC
MSC E647
E645(unrel)
E647(unrel)
E154(unrel)
E521(autol)
Responder

FIG. 7

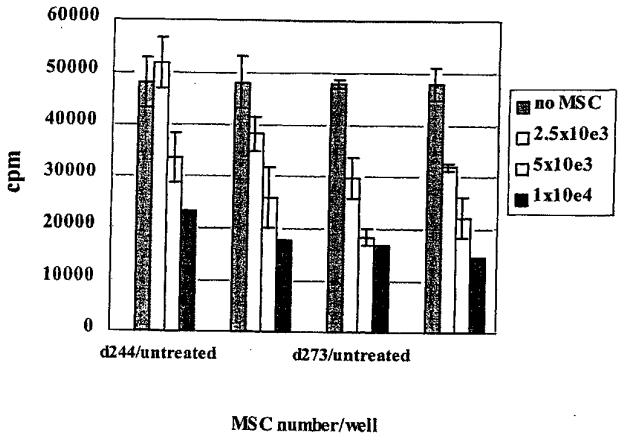

60000
50000
40000
30000
20000
10000
0
cpm
d244/untreated
d273/untreated
no MSC
2.5x10e3
5x10e3
1x10e4
MSC number/well

FIG. 8

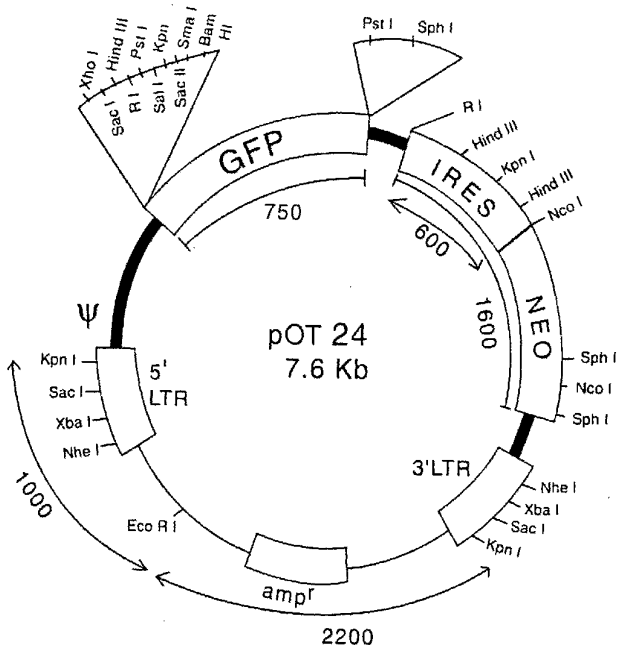

Xho I
Hind III
Pst I
Kpn
Sma I
Bam HI
Sac I
R I
Sal I
Sac II
Pst I
Sph I
GFP
750
R I
Hind III
Kpn I
Hind III
Nco I
IRES
600
1600
NEO
Sph I
Nco I
Sph I
pOT 24
7.6 Kb
Ψ
Kpn I
Sac I
Xba I
Nhe I
5'
LTR
3'LTR
Nhe I
Xba I
Sac I
Kpn I
1000
Eco R I
ampr
2200

Figure 9

Suppressive Effect of Supernatants Generated from hMSCs or hMSC-Suppressed MLR Cultures: Effect on Primary MLR

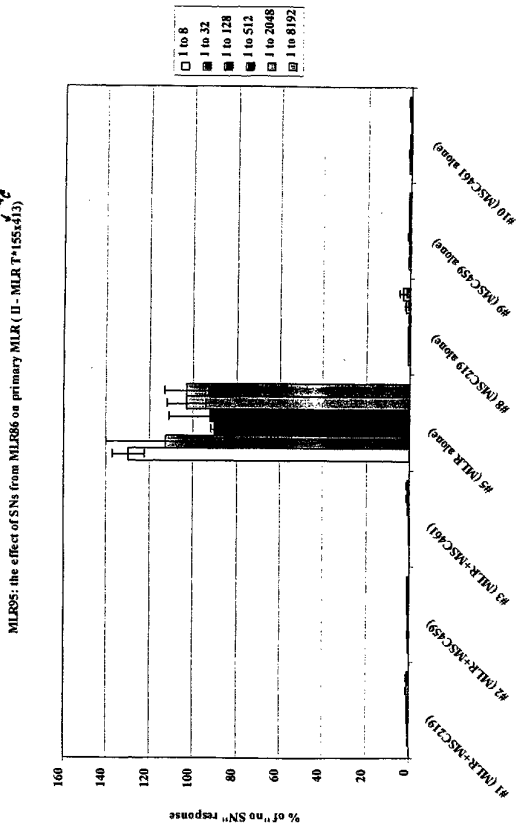


Figure 10

Suppressive Effect of Supernatants Generated from hMSCs or hMSC-Suppressed MLR Cultures: Effect on Ongoing MLR

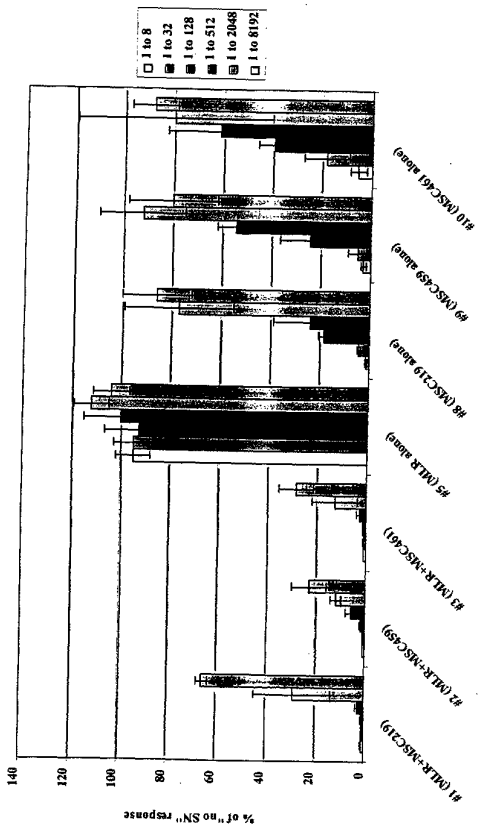


Figure 11

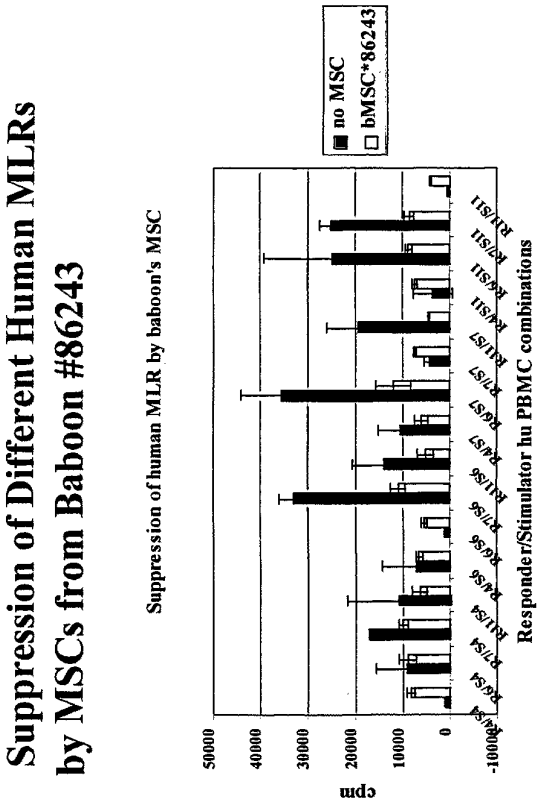

Suppression of Different Human MLRs by MSCs from Baboon #86243
Suppression of human MLR by baboon's MSC
no MSC
bMSC*86243
cpm
50000
40000
30000
20000
10000
0
-10000
Responder/Stimulator hu PBMC combinations
R4/S4
R6/S4
R7/S4
R11/S4
R4/S6
R6/S6
R7/S6
R11/S6
R4/S7
R6/S7
R7/S7
R11/S7
R4/S11
R6/S11
R7/S11
R11/S11

Figure 12

Suppression of Xenogeneic MLR (Human X Baboon) by Human and Baboon MSCs

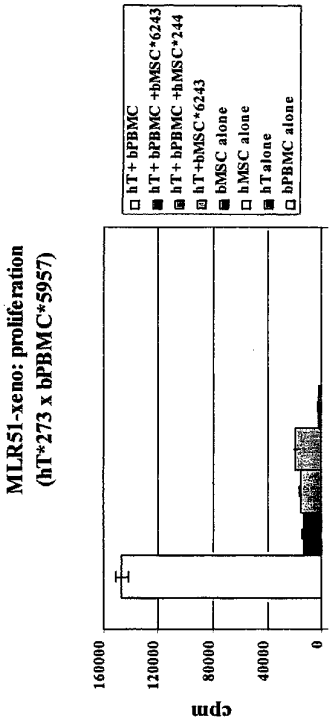
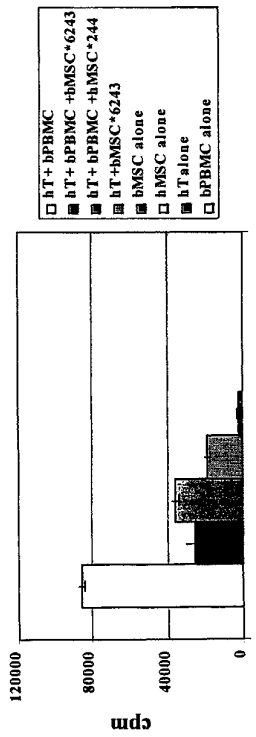


Figure 13

Suppression of Xenogeneic MLR (Human X Baboon) by Human and Baboon MSCs



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(54) Title: SUPERNATANT FROM MESENCHYMAL STEM CELLS FOR PREVENTION AND TREATMENT OF IMMUNE RESPONSES IN TRANSPLANTATION

(57) Abstract: A method of reducing an immune response to a transplant in a recipient by treating said recipient with an amount of mesenchymal stem cells effective to reduce or inhibit host rejection of the transplant. The mesenchymal stem cells can be administered before, at the same time as, or after the transplant. Also disclosed is a method of inducing a reduced immune response against a host by foreign tissue, i.e., graft versus host disease, by treatment with mesenchymal stem cells.

SUPERNATANT FROM MESENCHYMAL STEM CELLS FOR PREVENTION AND TREATMENT OF IMMUNE RESPONSES IN TRANSPLANTATION

This application claims priority of U.S. Application Serial No. 09/427,333, filed October 26, 1999, which is a continuation-in-part of U.S. Patent application serial no. 09/267, 536 filed March 12, 1999; is based on and claims priority of U.S. provisional application serial no. 60/078,463 filed March 18, 1998 and U.S. provisional application serial no. 60/089,964 filed June 19, 1998.

The present invention relates to inhibiting a T cell response to an alloantigen and further relates to inhibiting and/or preventing reactivation of previously activated T cells. More particularly, the present invention relates to the field of preventing, reducing or treating an immune response caused by immune effector cells to foreign tissue and/or cells and/or organs. The invention further relates to preventing, reducing or treating transplant rejection and/or graft versus host reaction.

BACKGROUND OF THE INVENTION

Tolerance is the acquired lack of specific responsiveness to an antigen to which an immune response would normally occur. Typically, to induce tolerance, there must be an exposure to a tolerizing antigen, which results in the death or functional inactivation of certain lymphocytes. Complete tolerance is characterized by the lack of a detectable immune response, to the second antigenic challenge. Partial tolerance is typified by the quantitative reduction of an immune response.

The function of the immune system is to eliminate foreign bodies which may contain pathogens, and to maintain unresponsiveness or tolerance against self antigen. T cell tolerance is achieved 1) in the thymus where thymocytes reactive for self-

peptides are eliminated by clonal deletion (central tolerance), and 2) in the periphery by exposure to self-antigens under tolerogenic conditions (peripheral tolerance). Clonal deletion can also result from expression of cell death molecules on antigen presenting cells. Classic examples of death molecules are Fas ligand (FasL) and TRAIL ligand, which ligate their receptors, Fas and DR4, respectively, on activated T cells, inducing apoptosis of the T cells. The interaction of CD27, a member of the TNFR superfamily, and the CD27-ligand (CD70) also induces T cell apoptosis.

Unfortunately, the immune system does not distinguish beneficial intruders, such as transplanted tissue, from those that are harmful, and thus the immune system rejects transplanted tissue or organs. Rejection of transplanted organs is significantly mediated by alloreactive T cells present in the host which recognize donor alloantigens or xenoantigens.

At present, in order to prevent or reduce an immune response against a transplant, patients are treated with powerful immunosuppressive drugs. The infusion of individuals with drugs that prevent or suppress T-cell immune response does inhibit transplant rejection, but can also result in general immune suppression, toxicity and even death due to opportunistic infections. Because of the toxicity and incomplete response rate to conventional treatment of donor tissue rejection, alternative approaches are needed to treat patients who cannot withstand or do not respond to current modes of drug therapy.

Accordingly, there is a need for the prevention and/or reduction of an unwanted immune response by a host to a transplant by immune effector cells as a method to avert host rejection of donor tissue. Also advantageous would be a method to eliminate or reduce an unwanted immune response by a donor tissue against a recipient tissue, known as graft-versus-host disease.

SUMMARY OF THE INVENTION

It has been discovered that mesenchymal stem cells can be used in transplantation to ameliorate a response by the immune system such that an immune response to an antigen(s) will be reduced or eliminated.

In accordance with one aspect of the invention, there is provided a method for reducing or suppressing an immune response caused by T cells responding to an alloantigen, in particular allogeneic tissue, organ or cells, wherein the immune response is reduced or suppressed by the use of mesenchymal stem cells. The mesenchymal stem cells may be autologous to the T cells (obtained from the same host) or allogeneic or xenogeneic to the T cells. In the case of mesenchymal stem cells that are allogeneic to the T cells, the mesenchymal stem cells may be autologous to the cells or tissue to which the T cells are responding (obtained from the same host) or the mesenchymal stem cells may be obtained from a host that is allogeneic to both the source of the T cells and the source of the cells or tissue to which the T cells are responding. Alternatively the mesenchymal stem cells can be obtained from a source that is xenogeneic to either or both the source of the T cells and the source of the cells or tissue to which the T cells are responding.

In accordance with another aspect of the present invention there is provided a process for preventing restimulation of activated T cells (activated against an alloantigen, in particular an allogeneic organ, tissue or cells) by contacting activated T cells with mesenchymal stem cells in an amount effective to prevent and/or reduce a subsequent T cell response to a foreign antigen. The mesenchymal stem cells that are used may be autologous to the T cells and/or allogeneic to the T cells. When using allogeneic mesenchymal stem cells, the mesenchymal stem cells may be obtained from the same host as the tissue or cells that activated the T cells or may be obtained from a host that is allogeneic to both the T cells and the host that provided the cells or tissues that activated the T cells.

In accordance with another aspect of the present invention, mesenchymal stem cells are used to suppress or ameliorate an immune response to a transplant (tissue, organ, cells, etc.) by administering to the transplant recipient mesenchymal stem cells in an amount effective to suppress or ameliorate an immune response against the transplant. The mesenchymal stem cells may be autologous to the transplant recipient or may be allogeneic or xenogeneic to the transplant recipient.

Accordingly, one method of the present invention provides contacting the recipient of donor tissue with mesenchymal stem cells. In one embodiment of this aspect, the method involves administering mesenchymal stem cells to the recipient of donor tissue. The mesenchymal stem cells can be administered to the recipient before or at the same time as the transplant or subsequent to the transplant. The mesenchymal stem cells may be autologous or may be allogeneic to the recipient and can be obtained from the donor. In another aspect of the invention, the allogeneic mesenchymal stem cells can also be obtained from a source other than the donor and such source need not be matched either to the donor type or the recipient type.

In a further embodiment of this method, as part of a transplantation procedure the mesenchymal stem cells are modified to express a molecule that induces cell death. The mesenchymal stem cells can be used to deliver to the immune system a molecule that induces apoptosis of activated T cells carrying a receptor for the molecule. This results in the deletion of activated T lymphocytes and in the suppression of an unwanted immune response to a transplant. In accordance with this aspect of the invention, allogeneic mesenchymal stem cells are modified to express a cell death molecule. The molecule can be exogenous or endogenous to the mesenchymal stem cells. In preferred embodiments of the methods described herein, the mesenchymal stem cells express the cell death molecule Fas ligand or TRAIL ligand.

The mesenchymal stem cells can also be administered to the recipient as part of the transplant. To this objective, the present invention provides a method for reducing or ameliorating an immune response by providing to the recipient donor tissue or organ that is perfused with or includes mesenchymal stem cells obtained from the donor of the organ or tissue or mesenchymal stem cells from a third party or mesenchymal stem cells autologous to the T cells. The mesenchymal stem cells ameliorate an immune response by the recipient's T cells against the foreign tissue when it is transplanted into the recipient.

In a further embodiment of this invention, the mesenchymal stem cells perfused into the organ or tissue also can include a molecule that induces activated T cell death.

In another embodiment, the method of the present invention provides treating a patient who has received a transplant, in order to reduce the severity of or eliminate a rejection episode against the transplant, by administering to the recipient of donor tissue mesenchymal stem cells after the donor tissue has been transplanted into the recipient. The mesenchymal stem cells can be autologous or allogeneic to the recipient. The allogeneic mesenchymal stem cells can be obtained from the donor or from a third party source. The presentation of mesenchymal stem cells to a recipient undergoing an adverse immune response to a transplant induces nonresponsiveness of T cells to further antigenic stimulation thereby reducing or eliminating an adverse response by activated T cells to donor tissue or organ.

In a further aspect of the present invention, there is provided a method of reducing an immune response by donor tissue, organ or cells against a recipient, i.e. graft versus host response, comprising treating the donor tissue, organ or cells with allogeneic (allogeneic to the donor) mesenchymal stem cells *ex vivo* prior to transplantation of the tissue, organ or cells into the recipient. The mesenchymal stem cells reduce the responsiveness of T cells in the transplant that may be subsequently

activated against recipient antigen presenting cells such that the transplant may be introduced into the recipient's (host's) body without the occurrence of, or with a reduction in, an adverse response of the transplant to the host. Thus, what is known as "graft versus host" disease may be averted.

In a preferred embodiment, the donor transplant may be first exposed to recipient or third party tissue or cells *ex vivo*, to activate the T cells in the donor transplant. The donor transplant is then contacted with mesenchymal stem cells autologous or allogeneic to the donor. The mesenchymal stem cells can be recipient or third party mesenchymal stem cells. The mesenchymal stem cells will reduce or inhibit an adverse secondary immune response by T cells in the donor transplant against antigenic stimulation by the recipient when the donor transplant is subsequently placed into the recipient.

Accordingly, the mesenchymal stem cells can be obtained from the recipient, for example, prior to the transplant. The mesenchymal stem cells can be isolated and stored frozen until needed. The mesenchymal stem cells may also be culture-expanded to desired amounts and stored until needed. The mesenchymal stem cells are administered to the recipient in an amount effective to reduce or eliminate an ongoing adverse immune response caused by the donor transplant against the recipient (host). The presentation of the mesenchymal stem cells to the recipient undergoing an adverse immune response caused by a transplant inhibits the ongoing

response and prevents restimulation of the T cells thereby reducing or eliminating an adverse response by activated T cells to recipient tissue.

A further embodiment includes modifying the recipient's mesenchymal stem cells with a molecule that induces activated T cell death.

Thus, in accordance with preferred embodiments of the present invention, human mesenchymal stem cells are employed to treat transplant rejection and or graft versus host disease as a result of a transplant and or to prevent or reduce transplant rejection and or graft versus host disease. Human mesenchymal stem cells may also be employed to facilitate the use of xenogeneic grafts or transplants. It is also within the present invention to use xenogeneic cells, such as non-human primate cells, for the above purposes.

It has further been discovered that the supernatant derived from MSC cultures and MSC/mixed lymphocyte reaction cultures has a suppressive effect on a T cell response to an alloantigen. Thus the present invention further provides a method of use of supernatants.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Allogeneic mesenchymal stem cells do not induce an immune response. T cells from A proliferated in a dose dependent manner when mixed with different amounts of PBMCs from B. T cells from A did not proliferate in response to contact with mesenchymal stem cells from B, even when the mesenchymal stem cells were manipulated to provide full T cell activation (the mesenchymal stem cells were treated with IFN-γ and transduced with costimulatory molecules B7-1 or B7-2).

Fig. 2. Mesenchymal stem cells actively suppressed the mixed lymphocyte reaction (MLR) between lymphocytes from two different individuals. hMSCs allogeneic to recipient (third party or donor) were either mismatched to both the stimulator and responder cells in the (MLR) (open bars); or hMSCs were matched (donor) to the stimulator cells in the MLR (hatched bars). Thus, the mesenchymal stem cells suppressed the MLR without specificity as to MHC type. The mesenchymal stem cells suppressed the MLR in a dose dependent manner.

Fig. 3 shows the secondary response of responder T cells primed by stimulator (allogeneic) PBMCs, not exposed to MSCs, and then exposed to autologous PBMCs, allogeneic PBMCs (stimulator or third party) or no cells.

Fig. 4 shows the secondary response of responder T cells activated by stimulator (allogeneic) PBMCs, and subsequently cultured with allogeneic MSCs (stimulator) and then exposed to autologous PBMCs, allogeneic PBMCs (stimulator or third party) or no cells.

Fig. 5 (Figs 5A-5D) shows that the secondary response of responder T cells previously activated by stimulator allogeneic PBMCs and after activation cultured with allogeneic (same donor (Fig.5B) or third party (Fig. 5D) or autologous MSCs (Fig.5C) and then exposed to autologous or allogeneic (same donor or third party) stimulator cells was suppressed.

Fig. 6 (Figs 6A-6D) shows the suppression of a primary MLR in the canine model by MSCs. autol = autologous; ident = DLA identical litter mates; unrel = unrelated.

Fig. 7 shows the suppression of a primary MLR by non-adherent MSCs.

Fig. 8 shows a schematic map of EGFP pOT24 plasmid used in Example 8.

Fig. 9 shows the suppressive effect of MSC supernatants generated from human mesenchymal stem cells or human mesenchymal stem cell-suppressed mixed lymphocyte reaction cultures on a primary mixed lymphocyte reaction.

Fig. 10 shows the suppressive effect of MSC supernatants generated from human mesenchymal stem cells or human mesenchymal stem cell-suppressed mixed lymphocyte reaction cultures on an ongoing mixed lymphocyte reaction.

Fig. 11 shows the suppression by baboon mesenchymal stem cells of a mixed lymphocyte reaction between human responder T cells and human stimulator PBMC cells.

Fig. 12 shows the suppression by baboon or human mesenchymal stem cells of a mixed lymphocyte reaction between human responder T cells and baboon stimulator PBMC cells (donor 5957).

Fig. 13 shows suppression by baboon or human mesenchymal stem cells of a mixed lymphocyte reaction between human responder T cells and baboon stimulator PBMC cells (donor 5909).

DETAILED DESCRIPTION OF THE INVENTION

As defined herein, an allogeneic mesenchymal stem cell is obtained from a different individual of the same species as the recipient. Donor antigen refers to antigens expressed by the donor tissue to be transplanted into the recipient. Alloantigens are antigens which differ from antigens expressed by the recipient. Donor tissue, organs or cells to be transplanted is the transplant. As examples of transplants may be included skin, bone marrow, and solid organs such as heart,

pancreas, kidney, lung and liver. Thus, an alloantigen is an antigen that is foreign to the recipent.

The inventors have discovered that when mesenchymal stem cells are contacted with allogeneic T lymphocytes, *in vitro*, the allogeneic T cells do not proliferate. Normally, co-culturing cells from different individuals results in a T cell response, manifested by activation and proliferation of the T cells, known as a mixed lymphocyte reaction (MLR).

These unexpected results demonstrate that T cells are not responsive to mismatched mesenchymal stem cells. The lack of a proliferative response to human mesenchymal stem cells by allogeneic T cells was unexpected because human mesenchymal stem cells express surface molecules that should render them immunogenic, i.e., they express allogeneic class I MHC molecules. This discovery indicates that the mesenchymal stem cells are not immunogenic to the immune system.

The inventors further discovered that mesenchymal stem cells can suppress an MLR between allogeneic cells. Mesenchymal stem cells actively reduced the allogeneic T cell response in mixed lymphocyte reactions in a dose dependent manner. In addition, mesenchymal stem cells from different donors did not exhibit specificity of reduced response with regard to MHC type. Thus, mesenchymal stem cells did not need to be MHC matched to the target cell population in the mixed lymphocyte reaction in order to reduce the proliferative response of alloreactive T cells to mesenchymal stem cells. The mesenchymal stem cells can also be xenogeneic to the responder or stimulator cells or both.

The inventors also discovered that the supernatants derived from mesenchymal stem cell cultures can suppress an MLR between allogeneic cells. As used herein, supernatants derived from mesenchymal stem cell cultures, also referred

to herein as "MSC supernatant", can be obtained from mesenchymal stem cells cultured alone or mesenchymal stem cells co-cultured with cell undergoing an immune response, i.e. T cells undergoing a mixed lymphocyte reaction.

Mesenchymal stem cell supernatants actively reduced the allogeneic T cell response in mixed lymphocyte reactions in a dose dependent manner. As with mesenchymal stem cells, supernatants from mesenchymal stem cell cultures from different donors did not exhibit specificity of reduced response with regard to MHC type.

In addition, the supernatants derived from mixed lymphocyte reactions contacted with mesenchymal stem cells can also suppress an MLR between allogeneic cells. These MLR/mesenchymal stem cell supernatants actively reduced the allogeneic T cell response in mixed lymphocyte reactions in a dose dependent manner and did not exhibit specificity of reduced response with regard to MHC type.

It is believed that a soluble factor or compound may be secreted into the mesenchymal stem cell culture medium that has a suppressive effect on mixed lymphocyte reactions. A stronger suppressive effect is seen using supernatants from MSCs exposed to a mixed lymphocyte reaction.

Accordingly, the present invention provides a method of reducing, inhibiting or eliminating an immune response by administering allogeneic mesenchymal stem cells to a recipient of a donor tissue, organ or cells. In one embodiment, the mesenchymal stem cells are administered to the recipient contemporaneously with the transplant. Alternatively, the mesenchymal stem cells can be administered prior to the administration of the transplant. For example, the mesenchymal stem cells can be administered to the recipient about 3 to 7 days before transplantation of the donor tissue. Alternatively, the cells may be administered subsequent to the transplant.

Thus, mesenchymal stem cells can be used to condition a recipient's immune system to donor or foreign tissue by administering to the recipient, prior to, or at the same time as transplantation of the donor tissue, mesenchymal stem cells in an amount effective to reduce or eliminate an immune response against the transplant by, for example, the recipient's T cells. The mesenchymal stem cells affect the T cells of the recipient such that the T cell response is reduced or eliminated when presented with donor or foreign tissue. Thus, host rejection of the transplant may be avoided or the severity thereof reduced.

The inventors have further discovered that when T lymphocytes that have already been exposed to antigenic stimulation, i.e. are activated, are subsequently exposed to mesenchymal stem cells, the T cells do not produce an immune response or produce a reduced immune response, to subsequent antigenic stimulation by allogeneic cells. Thus, mesenchymal stem cells induce a state of hyporesponsiveness of the T cells.

These unexpected results demonstrate that activated T cells were made non-responsive to further allogeneic stimulation by exposure of preactivated T cells to mesenchymal stem cells. The mesenchymal stem cells can be autologous or allogeneic to the T cells.

Accordingly, the present invention provides a method for treating a patient who is undergoing an adverse immune response to a transplant by administering mesenchymal stem cells to such patient in an amount effective to reduce or suppress the immune response. The mesenchymal stem cells are obtained from the tissue donor, the transplant recipient or a third party. As a further alternative, the MSCs may be xenogeneic to the donor, the recipient or both.

The mesenchymal stem cells may further be modified to express a cell death molecule to enhance the elimination of activated T cells. For example, the cell death molecule may be

expressed by the mesenchymal stem cells which have been engineered to express the exogenous cell death molecule.

In another aspect, the present invention provides a method to reduce or inhibit or eliminate an immune response by a donor transplant against a recipient thereof (graft versus host). Accordingly, the invention provides contacting a donor organ or tissue with mesenchymal stem cells prior to transplant. The mesenchymal stem cells ameliorate, inhibit or reduce an adverse response by the donor transplant against the recipient.

In a preferred embodiment, prior to transplant the donor transplant is treated with allogeneic (recipient) tissue or cells which activate the T cells in the donor transplant. The donor transplant is then treated with mesenchymal stem cells, autologous or allogeneic, prior to transplant. The mesenchymal stem cells prevent restimulation, or induce hyporesponsiveness, of the T cells to subsequent antigenic stimulation.

For preconditioning a donor transplant, the mesenchymal stem cells may be further modified to express a cell death molecule such that activated T cells contacted with the mesenchymal stem cells will be eliminated.

Thus, in the context of hematopoietic stem cell transplantation, for example, from the marrow and/or peripheral blood, attack of the host by the graft can be reduced or eliminated. Donor marrow can be pretreated with recipient mesenchymal stem cells prior to implant of the bone marrow or peripheral blood stem cells into the recipient. In a preferred embodiment, the donor marrow is first exposed to recipient tissue/cells and then treated with mesenchymal stem cells. Although not being limited thereto, it is believed that the initial contact with recipient tissue or cells functions to activate the T cells in the marrow. Subsequent treatment with the mesenchymal stem cells inhibits or eliminates further activation of the T cells in the marrow, thereby

reducing or eliminating an adverse affect by the donor tissue, i.e. the therapy reduces or eliminates graft versus host response.

In a further embodiment, a transplant recipient suffering from graft versus host disease may be treated to reduce or eliminate the severity thereof by administering to such recipient mesenchymal stem cells autologous or allogeneic to the donor, which allogeneic cells can be mesenchymal stem cells autologous to the recipient or third party mesenchymal stem cells, in an amount effective to reduce or eliminate a graft rejection of the host. The mesenchymal stem cells inhibit or suppress the activated T cells in the donor tissue from mounting an immune response against the recipient, thereby reducing or eliminating a graft versus host response.

The recipient's mesenchymal stem cells may be obtained from the recipient prior to the transplantation and may be stored and/or culture-expanded to provide a reserve of mesenchymal stem cells in sufficient amounts for treating an ongoing graft attack against host.

In yet another method of the present invention, the donor tissue is exposed to mesenchymal stem cells such that the mesenchymal stem cells integrate into the organ graft itself prior to transplantation. In this situation, an immune response against the graft caused by any alloreactive recipient cells that escaped standard treatment to prevent transplant rejection, e.g., drug-mediated immunosuppression, would be suppressed by the mesenchymal stem cells present in the graft. The mesenchymal stem cells are preferably allogeneic to the recipient and may be donor mesenchymal stem cells or mesenchymal stem cells obtained from other than the donor or recipient. In some cases, mesenchymal stem cells autologous to the recipient may be used to suppress an immune response against the graft.

In a further embodiment of this method, the mesenchymal stem cells are engineered to express cell death molecules such that any alloreactive host T cells will be eliminated upon contact with these mesenchymal stem cells.

It is further believed that in addition to preventing or ameliorating an initial immune response, the mesenchymal stem cells remaining in the local site would also suppress any subsequent T cell response that may occur.

As used herein, a "cell death molecule" is a molecule that interacts or binds with its cognate receptor on a stimulated T cell inducing T cell death or apoptosis. Fas mediates apoptosis of recently activated T cells which are again exposed to stimulation (van Parijs et al., *Immunity* 4: 321-328 (1996)). Fas is a type I membrane receptor that when crosslinked by its cognate ligand induces apoptosis in a wide variety of cells. The interaction between the Fas molecule (CD95) on target T cells and its ligand Fas L on mesenchymal stem cells results in receptor aggregation, which transduces signals leading to apoptosis of the target cell. The Fas system has been shown to be involved in a number of cell functions *in vivo* including negative selection of thymocytes, maintaining immune privilege sites within the body, and cytotoxic T-lymphocyte (CTL)-mediated cytotoxicity (Green and Ware, *Proc Natl Acad Sci*, 94(12):5986-90 (1997)).

Other members of the tumor necrosis factor receptor (TNFR) family have roles in programmed cell death. TRAIL ligand, which interacts with its receptor DR4 can induce apoptosis in a variety of transformed cell lines (G. Pan et al. *Science*, 277:815-818 (1997)); and the expression of CD27 and its ligand CD70 (Prasad et al., *Proc Natl Acad Sci*, 94:6346-6351 (1997)) also induces apoptosis. Fas expression is restricted to stimulated T cells and sites of immune privilege. TRAIL is detected in many normal tissues.

Both Trail-ligand and CD27, but not Fas-ligand, are expressed on unmanipulated human mesenchymal stem cells. Activated, but not resting, T cells express the Trail receptor and CD70. Most of the T cells found in the body are in the resting state; T cells are activated when they encounter cells both in the context of MHC and the appropriate co-stimulatory molecule such as B7-1 or B7-2.

Thus, the engagement of cell death receptors on activated T cells with their ligands expressed on the mesenchymal stem cells results in T cell death via apoptosis. Ligands and their receptors other than those specifically mentioned above, either present within the mesenchymal stem cell or introduced into the mesenchymal stem cell can perform this function. Therefore, mesenchymal stem cells administered to an individual delete activated T cells, reducing the severity or incidence of transplant rejection disease.

In accordance with the methods of the present invention described herein, it is contemplated that the mesenchymal stem cells of the present invention can be used in conjunction with current modes of treating donor tissue rejection or graft versus host disease. An advantage of such use is that by ameliorating the severity of the immune response in a transplant recipient, the amount of drug used in treatment and/or the frequency of administration of drug therapy can be reduced, resulting in alleviation of general immune suppression and unwanted side effects.

It is further contemplated that only a single treatment with the mesenchymal stem cells of the present invention may be required, eliminating the need for chronic immunosuppressive drug therapy. Alternatively, multiple administrations of mesenchymal stem cells may be employed.

Accordingly, the invention described herein provides for preventing or treating transplant rejection by administering the mesenchymal stem cells in a prophylactic or therapeutically effective amount for the prevention or treatment or amelioration of

transplant rejection of an organ, tissue or cells from the same species, or a xenograft organ or tissue transplant and or graft versus host disease.

Administration of a single dose of mesenchymal stem cells may be effective to reduce or eliminate the T cell response to tissue allogeneic to the T cells or to "non-self" tissue, particularly in the case where the T lymphocytes retain their nonresponsive character (i.e., tolerance or anergy) to allogeneic cells after being separated from the mesenchymal stem cells.

The dosage of the mesenchymal stem cells varies within wide limits and will, of course be fitted to the individual requirements in each particular case. In general, in the case of parenteral administration, it is customary to administer from about 0.01 to about 5 million cells per kilogram of recipient body weight. The number of cells used will depend on the weight and condition of the recipient, the number of or frequency of administrations, and other variables known to those of skill in the art. The mesenchymal stem cells can be administered by a route which is suitable for the tissue, organ or cells to be transplanted. They can be administered systemically, i.e., parenterally, by intravenous injection or can be targeted to a particular tissue or organ, such as bone marrow. The human mesenchymal stem cells can be administered via a subcutaneous implantation of cells or by injection of stem cell into connective tissue, for example muscle.

The cells can be suspended in an appropriate diluent, at a concentration of from about 0.01 to about 5 x 10^6 cells/ ml. Suitable excipients for injection solutions are those that are biologically and physiologically compatible with the cells and with the recipient, such as buffered saline solution or other suitable excipients. The composition for administration must be formulated, produced and stored according to standard methods complying with proper sterility and stability.

Although the invention is not limited thereof, mesenchymal stem cells can be isolated, preferably from bone marrow, purified, and expanded in culture, i.e. *in vitro*, to obtain sufficient numbers of cells for use in the methods described herein. Mesenchymal stem cells, the formative pluripotent blast cells found in the bone, are normally present at very low frequencies in bone marrow (1:100,000) and other mesenchymal tissues. See, Caplan and Haynesworth, U.S. Patent No. 5,486,359. Gene transduction of mesenchymal stem cells is disclosed in Gerson et al U.S. Patent No. 5,591,625.

Unless otherwise stated, genetic manipulations are performed as described in Sambrook and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

It should be understood that the methods described herein may be carried out in a number of ways and with various modifications and permutations thereof that are well known in the art. It may also be appreciated that any theories set forth as to modes of action or interactions between cell types should not be construed as limiting this invention in any manner, but are presented such that the methods of the invention can be more fully understood.

The following examples further illustrate aspects of the present invention. However, they are in no way a limitation of the teachings or disclosure of the present invention as set forth herein.

Example 1

Absence of Alloreactivity of Mesenchymal Stem Cells

The mixed lymphocyte reaction measures the compatibility of the donor's surface antigens and is an indication of the likelihood of rejection of donor tissue. Cell surface antigens responsible for eliciting transplant rejection are class I and class

II MHC antigens. T cells are alloreactive to foreign MHC antigens. Class I and II MHC molecules stimulate the mixed lymphocyte reaction.

Normal human volunteers were leukopheresed on a COBE SPECTRA ™ apheresis system (COBE, Lakewood, CO). 1 x 10^5 T cells from individual A (T_A) were cultured in flat bottom microtiter wells with mitomycin C treated allogeneic PBMCs (to prevent proliferation of PBMCs to T cells) from individual B ($mPBMC_B$) for 7 days. The $mPBMC_B$s were seeded at 20K and 100K. The cultures were pulsed with ^{3}H-thymidine for the last 18 hours of the culture period to measure T cell proliferation. The results shown in Figure 1 indicate that the T_A cells recognized the $PBMC_B$ as being foreign. (See bars under "T_A + $mPBMC_B$".) With more $PBMC_B$s present, the more the T cells proliferated.

2 x10^4 human mesenchymal stem cells (hMSCs) from the same donor as the PBMCs were co-incubated with 1x10^5 T cells from individual A (T_A). The cells were cultured in flat-bottom microtiter wells for a total of 7 days. Cultures were pulsed with ^{3}H-thymidine for the last 18 hours of the culture period to measure T cell proliferation. Two days prior to coculture with the T cells, the human mesenchymal stem cells were seeded into microtiter wells at the number given above (confluent) and treated with IFN-γ (50 units/ml) to stimulate surface antigen expression on MSCs. Non-transduced hMSCs or hMSCs transduced with human B7-1 or human B7-2 costimulation molecules were incubated with the T cells. Control cells were transduced with Neo.

The results shown in Fig. 1 (See Figure 1 "T_A + transduced hMSCs") demonstrate that the T lymphocytes were nonresponsive (did not proliferate) to the human mesenchymal stem cells, i.e., they were not recognized as being foreign.

The results also show that the lack of response to the mesenchymal stem cells was not due to genetic compatibility between the individuals since the T cells did

recognize peripheral blood mononuclear cells ($PBMC_B$) from the hMSC donor as being foreign.

Example 2

Suppression of Mixed Lymphocyte Reaction

To determine whether mesenchymal stem cells actively suppressed the allogeneic response, mixed lymphocyte reactions (MLR) were set up in tissue culture plates, with or without adherent mesenchymal stem cells obtained from 2 different donors: one donor matched the stimulator cells in the MLR and the other donor was unrelated to either the stimulator or responder cells.

10^5 PBMCs from individual A ($PBMC_A$) were mixed with 10^5 target individual B's PBMC's ($PBMC_B$). The $PBMC_B$s were irradiated with 3000 rads X irradiation to prevent their proliferation due to activation by $PBMC_A$s. Thus, only $PBMC_A$s would proliferate. When $PBMC_A$s and $PBMC_B$s were mixed, a mixed lymphocyte reaction occurred wherein the $PBMC_A$ cells (responder cells) were activated by the surface antigens on the $PBMC_B$s (stimulator cells). The cultures were incubated over an interval of 7 days and were pulsed with ^{3}H-thymidine during the final 18 hours. In the presence of the $PBMC_B$s, the $PBMC_A$s proliferated giving counts of 40,000. See Figure 2, 1st bar, ("NONE" refers to no mesenchymal stem cells present.).

However, when $PBMC_A$s and $PBMC_B$s were mixed in the presence of mesenchymal stem cells, the mixed lymphocyte reaction was suppressed. 10^5 $PBMC_A$s were mixed with 10^5 $PBMC_B$s in microtiter plate wells coated with an adherent monolayer of human mesenchymal stem cells. The mesenchymal stem cells were plated in the wells in amounts ranging from 7500 to 22,500 mesenchymal stem cells per well. Two mesenchymal stem cell populations were tested: human mesenchymal stem cells were obtained from an individual B and human mesenchymal stem cells were obtained from an individual that did not match either

individual A's or B's MHC type (a third party). The cultures were incubated over an interval of 7 days and were pulsed with ^{3}H-thymidine during the final 18 hours. In the presence of the human mesenchymal stem cells, the MLR was suppressed. See Figure 2. Thus, regardless of the MHC origin of the mesenchymal stem cells, the mesenchymal stem cells suppressed the mixed lymphocyte reaction.

The results shown in Figure 2 also indicate that the human mesenchymal stem cells decreased the mixed lymphocyte reaction in a dose-dependent manner. Mesenchymal stem cells from either donor suppressed proliferation equally well, which indicated that there was no specificity of suppression with respect to MHC type. These results demonstrate that

mesenchymal stem cells actively suppressed the mixed lymphocyte reaction when the cells were cultured together.

Example 3

Unresponsiveness in Secondary Mixed Lymphocyte Reaction

These experiments were performed to determine whether suppression of pre-activated T cells by MSCs resulted in specific unresponsiveness during secondary stimulation.

A. T cells from donor 248 (d 248) were primed by allogeneic PBMCs from donor 273 (d 273) for 7 days, then cultured for 3 additional days alone or in the presence of IFN-γ-treated MSCs from the same donor (d273). Cells were then re-stimulated by the same donor (d273), autologous (d248) or "third party" (d244) PBMCs.

Lymphocyte preparation

Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation on Ficoll-Paque (Pharmacia). Aliquots of cells were frozen in 90% FCS with 10% DMSO and stored in liquid nitrogen. After thawing, the cells were washed twice with MSC medium (DMEM with low glucose and 10% FCS) and re-suspended in assay medium (ISCOVE'S with 25 mM Hepes, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 5.5×10^{-5}M 2-mercaptoethanol (all reagents from GibcoBLR) and 5% human AB serum (Sigma, MLR tested)).

To prepare the T cell-enriched fraction, PBMCs were depleted of monocytes and B cells by immunomagnetic negative selection. PBMCs were incubated with mouse anti-human CD19 and CD14 mAbs (no azide/low endotoxin (NA/LE) format) followed by biotin-conjugated goat anti-mouse IgG (multiple adsorption) Ab (all

reagents from Pharmingen) and streptavidin microbeads (Miltenyi Biotec). Cells were then separated using a magnetic cell sorter (MACS, Miltenyi Biotec). The T cell-enriched fraction contained about 70-90% CD3+ cells.

MSC culture.

Human MSCs were isolated from bone marrow as described in U.S.Patent No. 5,486,359 and were maintained in culture with MSC medium and were used at passages from 3 to 6. Cells were lifted using 0.05% Trypsin/EDTA solution, washed once with MSC medium and plated at 70-80% confluent density which was $1x10^6$ /plate for 10 cm tissue culture dish. The day after plating, IFN-γ (Boehringer Mannheim) at 500 U/ml was added and the cells were incubated an additional 3 days. Before transferring T cells, MSC plates were washed 4 times with HBSS, 1 time with ISCOVES, and assay medium was added at 10 ml/well in 10 cm tissue culture dishes.

Primary (1°) MLR.

T cells (d 248) were activated by irradiated PBMCs (d 273). PBMCs used for stimulation were X-ray irradiated with 3,000 rad using Cabinet X ray system (Faxitron X ray, Buffalo Grove, IL). For primary stimulation, 2×10^7 responders were mixed with 2×10^7 stimulators in 20 mls assay medium in 10 cm tissue culture dishes. The cells were incubated at 37°C in 5% CO_2 atmosphere for 7 days.

Activated T cell/MSC cultures

T cells activated in the 1° MLR were collected, washed once with MSC medium and re-suspended in assay medium at 10^6/ml in 10 ml and were added to 10 cm tissue culture dishes containing autologous or allogeneic MSCs or medium alone, and incubated for an additional 3 days.

Restimulation assay

T cells cultured with MSCs or media were collected, washed once with MSC media, and restimulated with irradiated PBMCs from the original donor, an unrelated

donor or autologous PBMCs. For the assay, 5 x 10^4 primed responders and 5 x 10^4 irradiated stimulators were incubated in 96-well plates. Assays were performed in triplicate. Cultures were pulsed with 1 μCi of [^{3}H] thymidine (Amersham) for 18 hours before harvesting. Cultures were collected using Harvester 96 (Tomtec), filters were analyzed using Microbeta Trilux liquid scintillation and luminescence counter (E.G.&G Wallac). Data are presented as mean cpm ± SD of three replicates.

T cells cultured alone (positive control) showed an accelerated response to "same donor" re-stimulation with peak at day 2. "Third party" response was also accelerated, practically with the same kinetics as "same donor", but with a lower maximum and a slightly delayed start. (Fig 3). T cells cultured on allogeneic MSCs subsequently showed no response either to "same donor" or "third party" PBMCs during 6 days of culture (Fig.4).

Example 4

Unresponsiveness in Secondary Mixed Lymphocyte Reaction

T cells from donor 413 were stimulated with irradiated PBMCs from donor 273 for 7 days (1.5 x 10^6 ml each, bulk 20 ml cultures). MSCs from different donors 413, 418 and 273 were plated in 10 cm tissue culture dishes at 1x10^6/dish, pretreated with IFN-γ for 3 days and washed prior to mixing with preactivated T cells.

T cells preactivated in the MLR for 7 days were incubated alone or with MSCs for an additional 3 days (1.0 x 10^6/ml T cells, 10 ml/dish). After 3 days of incubation with MSCs, T cells were collected and re-stimulated with irradiated PBMC 273 (original donor), 413 (autologous), PBMC10 (third party) or PHA (5 μg/ml) in the presence or absence of autologous (d413) PBMC. Cells were added at 5 x 10^4/well, cultures were pulsed with [^{3}H]thymidine at indicated time points for an additional 18 hours.

The results indicate that treatment of activated T cells with autologous (d413) (Fig. 5C), same donor (d273) (Fig. 5B) and third party (d418) (Fig. 5D) MSCs induced unresponsiveness to antigenic stimulation in the T cells. The control culture (Fig. 5A) with no MSC treatment showed restimulation of the cells upon exposure to allogeneic PBMCs.

Example 5

Suppression of Primary MLR by Canine MSCs

Canine PBMCs were purified from peripheral blood by centrifugation on Ficoll-Paque gradient (1.077). Stimulator PBMCs were X-ray irradiated 2200 rad (7 min 70 kV). 10^5 irradiated stimulators were mixed with 10^5 responder PBMCs in 96-well plates in the presence or absence of pre-plated canine MSC (E647, 2 x 10^4/well). Cultures were incubated for 6 days and pulsed with [^{3}H]TdR (5Ci/mmol, 1μCi/well) for an additional 16 hours. Results are shown in Figures 6A-6D. E647 and E645 were litter mates (DLA identical). The results showed that autologous as well as allogeneic MSCs suppressed the primary MLR.

Example 6

Suppression of Primary MLR by Non-Adherent MSCs

T cells from d273 (2 x 10^5/well) were mixed with irradiated PBMCs from d244 (2 x 10^5/well) and different numbers of MSCs. MSCs from dD244 or d273 were pre-treated with IFN-γ (900 U/ml for 3 days) or left untreated, trypsinized on the day of experiment and added at the same time as T cells and PBMCs. Cultures were incubated for 7 days,[^{3}H]TdR (5Ci/mmol, 1 μCi/well) was added for an additional 16-18 hours. Results are shown in Figure 7 and demonstrate that non-adherent MSCs also suppressed a primary MLR.

Example 7

Allogeneic MSCs Support Skin Allograft Survival

Study Population

Juvenile baboons (*papio anubis*) were studied. Male and non-pregnant female baboons weighed 7-20 kg and were between 3–16 years of age. They were screened for tuberculosis papilloma virus, titered for cytomegalovirus (CMV), and tested with the primate viral screen consisting of testing for simian virus, and including fecal floatation and smears. Donor and recipient pairs were determined by major histocompatibility complex (MHC)-disparity through PCR typing. During the study period, the baboons were housed in an individual area beside a companion animal.

Donor Bone Marrow Harvest for MSC Isolation and Culture-Expansion

Needle marrow aspirates were obtained from the iliac crest for isolation and culture-expansion of the MSCs. The marrow aspirate was obtained from an alternate side once a week for four consecutive weeks. The volume of the aspirate was determined by an estimate of 10% of the animal's blood volume. Blood volume (liters) is estimated to be 7% of body weight. A 10 kg baboon then, would have an estimated blood volume of 0.7 liters. An aspirate of 10% of the blood volume would then be 70 milliliters.

Prior to the procedure, 500 mg cefazolin was administered intramuscularly (IM) for perioperative antibacterial prophylaxis. Baboons were sedated and anesthetized for the procedure with ketamine at 10 mg/kg IM, and xylazine 1 mg/kg IM. The sites of needle insertion were scrubbed with povidone-iodine and then rinsed with alcohol. Aspirates were obtained from the iliac crest using a 16-gauge, 2-inch bone marrow needle. A syringe was attached to the needle, and suction was applied to remove the marrow. For postoperative pain, the analgesic Buprenorphine was given at 0.03 mg/kg IM Q12 x 2 doses.

Shipment of Donor Bone Marrow Aspirates

Bone marrow aspirates were transferred from the syringe to a sterile Vacutainer® containing sodium heparin. The tubes were placed in a Styrofoam™

container and shipped at room temperature (RT) by overnight delivery to the cell processing facility.

Isolation and Culture Establishment of MSCs

Five to 10 ml aliquots of bone marrow were diluted to 50 ml in Dulbecco's Phosphate Buffered Saline (DPBS) in a polypropylene culture tube. The cell suspensions were centrifuged at 2200 RPM for 10 minutes at room temperature (RT). Total nucleated cell counts were determined in 4% acetic acid. Cells were then diluted in DPBS for a final concentration of 20 x 10^6 cells/ml. Ten ml or 200 x 10^6 cells were loaded onto 20 ml of Percoll (sp.gr. 1.073 gm/ml) in a 50 ml conical tube and underwent centrifugation at 1300 RPM for 20 minutes. The cell interface containing mononuclear cells was washed in DPBS, resuspended in complete media, and counted to obtain a recovery. The washed mononuclear cells obtained at the Percoll interface were cells were then established in T-185 flasks containing 30 ml of complete media and 15-20 x 10^6 cells/flask (8.1 x 10^4 MSC/cm^2) and placed in a 37°C incubator at 5% CO_2.

Harvest of MSC

The media in the triple flasks was decanted, and the flasks were rinsed with 50 ml DPBS. After decanting the DPBS, 23 ml of 0.05% trypsin was added to each triple flask. The flasks were placed in a 37°C incubator for 3 minutes. After cell detachment, 23 ml complete medium was added to each flask. The cell suspensions were transferred to 50 ml conical tubes and the flasks were washed with 30 ml HBSS. The tubes were centrifuged at 2200 RPM for 5 minutes at RT.

Formulation/Packaging

The harvested MSCs were formulated at approximately 10 x 10^6 cells per ml in

cryoprotectant solution consisting of 85% Plasma-Lyte A (Baxter IV Therapy), 10% DMSO, and
5% MSC-donor serum, and cryopreserved in bags containing 15-20 ml.

Labeling/Storage/Shipment
Cells were cryopreserved using a controlled-rate freezer (Cryomed, Forma Scientific) at 1-2° per minute to –90°C. The samples were then transferred to a liquid nitrogen storage freezer in the vapor phase (-120 to –150°C).

Dose
To achieve an MSC dose of 20 x 10^6 cells/kg, the final product was prepared at 115% of the dose required on infusion day.

Skin harvest
Prior to surgery the baboon was given cefazolin at 500 mg IM as a perioperative antibacterial prophylaxis. The baboon was sedated with ketamine at 10 mg/kg IM and anesthetized by intravenous Thiopental induction, a 1-2% isofluorane inhalational anesthetic. Skin was harvested from the anterior abdominal wall, placed on a pre-labeled moistened saline gauze pad. The wound defect was then closed. The baboon was returned to the colony after awakening. For postoperative pain, the analgesic Buprenorphine was administered at Q12 x 2 doses and Ancef daily for 2 days.

Recipient Skin Transplant and MSC Infusion
Prior to surgery the baboon was given cefazolin at 500 mg IM as an antibacterial prophylaxis perioperatively. The baboon was sedated and anesthetized with ketamine at 10 mg/kg IM and intravenous Thiopental induction, a 1-2% isofluorane inhalational anesthetic. Skin was harvested from the anterior abdominal wall and placed on a pre-labeled moistened saline gauze pad. This skin was divided into two grafts; one was used as the third party control for another recipient baboon and one was used as an autologous control for this same animal. The animal was then placed in a prone position. Three, 3 x 2-cm sections of skin were removed from the dorsum, along the spine, between the scapulae. The previously harvested skin grafts

from the MSC donor, a third party donor and self were defatted, trimmed to fit the skin defects created, and sutured in place.

After grafting, the baboon received an intravenous infusion of MSC at a dose of 20 x 10^6 donor MSC/kg. Peripheral blood samples were obtained at pre-MSC, 1 hour, and days 1-3 post-MSC; marrow aspirates were obtained on day 0 post-MSC, day 3, 14, and 30.

For postoperative pain, the analgesic Buprenorphine was administered at Q12 x 2 doses and Ancef daily for 2 days. The animal was observed daily, and the grafts were photographed every other day beginning on post-graft day 7.

Physical Examinations and Diagnostic Testing

Each baboon was sedated with ketamine 10mg/kg IM for examination. While sedated, two-three milliliters of marrow were obtained from the iliac crest by needle aspiration and collected in sodium heparin on days 4, 13, and 30, the end of study. A skin biopsy was harvested on the same day that marrow aspirates were obtained.

RESULTS

Effects of MSC Infusion on Skin Allograft Survival

Untreated control animals (N=2) had a mean skin allograft survival time of 8.0± 0 days. The infusion of unrelated-MSC-donor MSCs donor (N=2) resulted in a prolongation of skin graft survival time to a mean survival time of 11.5±0.71 days (Mann-Whitney U Test, $P<0.05$). The infusion of unrelated-third-party donor MSC on donor allografts (N=4) resulted in a significant prolongation of skin graft survival times to a mean survival time of 12.3±0.96 days (Mann-Whitney U Test, $P<0.003$).

Recipients 6140 and 6200 received allografts from the MSC donor 6243, from each other (a third party graft), and from themselves (an autograft). Twenty-four hours prior to skin graft harvesting from the MSC donor, 6243, MSCs from 6243 were injected under the anterior abdominal skin which had been delineated for grafting. After grafting, the recipients were administered an intravenous infusion of

20 x 10^6 MSC/kg (6243). Both third-party allografts were rejected on day 13. The MSC-donor (6243) allografts were found to be hemorrhagic on day 4, a finding usually attributed to a technical failure. On pathologic examination, keratin was noted to have been insinuated in a track- like fashion, below the dermis: the nature of these tracks suggests these were formed by the needle at the time of subcutaneous MSC injection. The presence of these cells had elicited a tremendous inflammatory response. This inflammatory response precluded the ability of the skin grafts to adhere/"take" properly and these grafts were completely necrosed by day 7. The autografts were not rejected.

Recipients 6654 and 6659 received allografts from the MSC donor 6593, from each other (a third-party graft), and from themselves (an autograft). After grafting, the recipients were administered intravenous infusions of 20 x 10^6 MSC/kg. The MSC-donor allografts were rejected on days 11 and 12, and the third-party donor allografts were rejected on days 11 and 12. The autografts were not rejected.

Similarly, recipients 6663 and 6658 received allografts from the MSC donor 6656, from each other (a third-party graft), and from themselves (an autograft). After grafting, the recipients were administered intravenous infusions of 20 x 10^6 MSC/kg. The MSC-donor allografts were rejected on day 11, and the third-party donor allografts were rejected on days 10 and 12. The autografts were not rejected.

Recipients 6532 and 6720 in the control arm of the study received auto- and allografts without the administration of MSC by infusion or injection. Their allografts were rejected on day 8. The autografts were not rejected.

There were no identifiable toxicities associated with allogeneic MSC infusion and no adverse clinical sequelae in the subsequent 30-day follow-up interval. Blood samples were obtained at pre-MSC, 1 and 2 hours, and days 1, 2, and 3 after grafting

and MSC infusion. Marrow aspirates were obtained on days 4 and 13 after grafting and MSC infusion.

These results demonstrate that a single infusion of allogeneic baboon MSCs can delay rejection of allogeneic skin grafts. No other immunosuppressive therapy was administered. One dose of allogeneic or third party MSCs increased the time to rejection by 50% (standard rejection time in this model is 8 days (*See* Goodman et al. *Am Surg* 62(6):435-42 (1996)).

Example 8

The purpose of the study was to demonstrate the feasibility and safety in dogs of the infusion of a moderately high dose of donor dog leukocyte antigen (DLA)-identical littermate canine mesenchymal stem cell (cMSC) at 10 x 10^6 cells/kg in an allogeneic marrow graft setting. A secondary objective was to examine the distribution and function of donor neo- and GFP-marked cMSC at 50 and 100 days post-transplant.

MATERIALS AND METHODS

Experimental animals

Beagles were used for the study. Two male and two female DLA-identical littermates were used in the study, aged 7 or 9 months on day 0. The method for typing used involves the use of highly polymorphic microsatellite markers to follow inheritance of the Class II DRB region in the Dog Leukocyte Antigen (DLA), the canine equivalent of the major histocompatability complex. Microsatellites are small di- tri- or tetra nucleotide repeats, which show sufficient length variation in alleles that they may be used to follow the inheritance of chromosomal segments through multigeneration crosses. Segregation of alleles is typically monitored using a single-step polymerase chain reaction with primers derived from unique sequences of DNA that surround each repeat. In addition, mixed leukocyte reactions were performed on

the DLA-identical littermate pairs chosen for study to provide confirmation of the PCR microsatellite marker assay results.

Study Design

The dogs underwent transplantation with cMSC and bone marrow from the same DLA-identical littermate donor. The marrow graft was harvested from each of the two DLA-identical littermates on day 0 prior to total body irradiation (TBI) and exchanged. Myeloablation was induced by exposing the dogs on day 0 to a single TBI dose of 920 centigray (cGy) (midline air exposure from two opposing ^{60}Co sources delivered at a rate of 7 cGy (9.3R)/min. Culture-expanded cMSC isolated from a donor marrow aspirate at 4 or more weeks prior to transplantation, were transduced with Papp@OT-24, containing the genes for green fluorescence protein (GFP) and neomycin phosphotransferase (neo). The cMSC were cryopreserved after passage 1 (P1) or passage 2 (P2). Following TBI, the cMSC were thawed and delivered intravenously via a portable infusion pump over a 15-minute time period. Within one to two hours after cMSC infusion the bone marrow graft was infused intravenously at a dose of $\geq 1 \times 10^8$ total nucleated cell (TNC)/kg.

Cyclosporin was administered to all four dogs for graft-versus-host-disease (GVHD) prophylaxis intravenously on days 0 through 5 at a dose of 10 mg/kg BID (20 mg/kg/day) (Sandimmune® Injection Solution, Sandoz Pharmaceuticals Corporation). On days 6 through 50 (end of study) for group I.1.a, or 6 through 100 for group I.1.b, cyclosporin was administered at 10 mg/kg BID PO, (20 mg/kg/day) (Neoral® Soft Gelatin Capsules, Sandoz Pharmaceuticals Corporation). The usual supportive care with oral antibiotics for the recipient began on day –5 and systemic antibiotics started on day 0 and continued until engraftment was achieved. Fluid support was given as necessary. No platelet transfusions were required for any of the four dogs during recovery. Standard canine procedures require that a whole blood transfusion to be administered if the platelet count consistently drops below $10,000/mm^3$, or if the treatment staff observes signs of bleeding. Platelet transfusions,

if necessary, were to be administered as 50-ml of whole irradiated (2000 cGy) blood from a random donor. Engraftment was established as the time of the first of three consecutive measurements of >500 absolute neutrophil cells mm^3, >1,000 /mm^3, and platelets >10,000 /mm^3, 50,000 /mm^3, and >100,000.

To follow hematopoietic recovery, complete blood counts (CBCs) were obtained from day 0 through day 50, and biweekly thereafter for the 100-day study group. Serum chemistry analysis was performed on days 0, 2, and weekly thereafter. Peripheral blood samples were taken on day 0 pre-MSC infusion, 5- and 15-minutes, 1- and 2-hours, and 1-, 2-, 3-, and 4-day time points for DNA isolation. The DNA was evaluated for the presence of GFP marked cells by an Anti-EGFP DNA PCR Elisa with digoxigenin incorporated into the product and a second step anti-digoxigenin colorimetric assay. A marrow aspirate was obtained when the platelet counts consistently reached 50,000/mm^3 and examined for the presence of GFP marked cells using the same PCR method. CMSC cultures were established to examine colony forming units (CFU), and to expand the cMSC for further Anti-EGFP PCR analysis. Upon necropsy, peripheral blood, bone marrow aspirates, and bone marrow biopsies were obtained for Anti-EGFP PCR analysis. CFU assays were performed on the bone marrow aspirates, and the Anti-EGFP PCR analysis was performed on culture-expanded cMSC. An histological analysis was performed for the presence of GFP in various tissues.

cMSC isolation, culture-expansion, transduction and cryopreservation

Bilateral bone marrow aspirates were obtained for cMSC isolation and culture establishment on week –4 for dogs CAN-07-01 and CAN-07-02 and on week –9 for dogs CAN-07-03 and CAN-07-04. Fifteen ml of marrow (7 ml from each humerus) were obtained from each dog. Dogs were anesthetized by the injection of Butorphanol followed by injection of a mixture of Diazepam and ketamine hydrochloride (Aveco Co., Inc., Fort Dodge, IA). The sites of needle insertion were scrubbed with povidone-iodine and then rinsed with alcohol. Aspirates were obtained

from each humeral condyle of each dog using a 16-gauge, 2-inch bone marrow needle. A syringe was attached to the needle, and suction was applied to remove 8 ml of marrow from each humerus. Bone marrow aspirates were transferred to 15 ml polypropylene conical tubes using sterile technique. Following the procedure, the dog was then placed on a warming pad to recover.

Five to 10 ml aliquots of bone marrow were diluted to 50 ml in Dulbecco's Phosphate Buffered Saline (DPBS) in a polypropylene culture tube. The cell suspensions underwent centrifugation at 2200 RPM for 10 minutes at room temperature (RT). Total nucleated cell counts were determined in 4% acetic acid. Cells were then diluted in DPBS for a final concentration of 20×10^6 cells/ml. Ten ml or 200×10^6 cells were loaded onto 20 ml of Percoll (sp.gr. 1.073 gm/ml) in a 50 ml conical tube and underwent centrifugation at 1300 RPM for 20 minutes. The cell interface containing mononuclear cells was washed in DPBS, resuspended in complete media, and counted to obtain a recovery percentage. The cells were then diluted in complete media, cultures were established as described below, and placed in a 37°C incubator at 5% CO_2.

Construction of bicistronic MuLV retroviral vector

The green fluorescent protein (EGFP) retrovirus was constructed by isolating EGFP-1 gene from the jellyfish *Aequorea victoria* (Clontech, CA). EGFP gene was cloned into retroviral vector pJM573-neo (resulting plasmid was named pOT-24). The plasmid pJM573-neo was derived from pN2 (*Keller et. Al., 1985, Nature 318:149*) with the following modifications: murine retroviral *gag* initiation site was substituted with an in-frame stop codon; 5'LTR and 3'LTR were constructed into the same cassette; neomycin phosphotransferase gene (*neo*) and an internal ribosomal entry site (*IRES*) were inserted into pN2. A schematic map of EGFP pOT24 plasmid is shown in Figure 8.

Preparation of Recombinant Retrovirus

pOT-24 was transfected into GP&E86 ecotropic producer cells using DOTAP (Boehringer Manheim) as suggested by manufacturer. The transfected cells were grown in DMEM-high glucose (HG) medium supplemented with 10% heat inactivated FBS, Penicillin-Streptomycin (Life Technologies) and 0.5mg/ml of protamine sulfate-G418 (Sigma) as a selective marker. Cultures were maintained up to 70% confluency at which point medium was replaced with fresh retroviral media (without G418) and cells were maintained at 32°C for 2 days. The culture medium containing the retrovirus was collected, filtered through 0.45 μm filter and stored at -70°C. Amphotropic retrovirus was prepared by transducing PA317 cells twice with ecotropic virus using a centrifugal transduction procedure followed by selection with G418 (0.5 mg/ml). Retroviral supernatant was collected. The titer of the pooled EGFP retrovirus on 3T3 cells was $1.2x10^6$ CFU/ml. GFP-retroviral supernatants were cryopreserved at -70°C.

CAN-07-01 and CAN-07-02

The washed mononuclear cells obtained at the Percoll interface were established in 10, T-185 flasks containing 30 ml of complete media and 10×10^6 cells/flask.

On days 2, 6, and 9 of culture, the media in the flasks was replaced entirely with fresh complete media. On day 12 of the primary culture photographs were taken, and the cells were taken from passage 0 (PO) to passage 1 (P1). The media was aspirated and the flasks were washed twice with 8 ml DPBS. Eight ml of trypsin was added, and flasks were placed in a 37°C incubator for 3 minutes. When the cells had lifted, the reaction was stopped by the addition of 8 ml of complete media. The cells were transferred and pooled into 50 ml conical tubes. The flasks were washed with DPBS and the pooled cells were centrifuged at RT at 2000 RPM for 5 minutes. The supernatant was removed and the cell pellets were resuspended in complete media. The cells were pooled, counted and examined for viability. Cells were plated into 15, T80 flasks containing 18 ml of complete medium and 0.4×10^6 cells per flask.

On day 15 in culture, the first transduction was performed on 15 of the 18 flasks. The media was removed. Aliquots of the retroviral supernatant were thawed and polybrene was added to a final concentration of 8 μg/ml to make the transduction cocktail. The cell medium was replaced with 10 ml of the transduction cocktail, and the flasks were centrifuged at 3000 RPM for 1 hour at 32°C. After centrifugation, 10 ml of complete media prepared using heat inactivated fetal bovine serum (FBS) was added to each flask (with the transduction cocktail) and the flasks were returned to the incubator. Three flasks were not transduced, and fresh media was replaced. On day 16 of culture, the media was replaced with fresh complete media. On day 17 of culture the transduction procedure was repeated.

On day 18 of culture, the cells were harvested as described above and taken from P1 to P2. Three x 10^6 cells were added to 100 ml of complete medium, and poured into triple-flasks (500 cm^2). Fifteen triple-flasks were prepared with transduced cells and three were prepared with untransduced cells. Any remaining cells were cryopreserved. A freeze solution was prepared containing 10% DMSO and 90% FBS. Ten x 10^6 cells were resuspended in 1 ml of freezing solution. The vials were labeled and cryopreserved in a Nalgene Cryo container for a minimum of 4 hours at –70°C, and stored at –70°C.

On day 22 of P2 culture, photographs were taken to record the cell distribution and morphology and the P2 cells were harvested and cryopreserved as described below.

CAN-07-03 and CAN-07-04

The washed mononuclear cells obtained at the Percoll interface were established in 15, T-75 flasks containing 20 ml of complete media and 12 x 10^6 cells/flask.

On day 2 of culture, the media in the flasks and in the dishes was replaced entirely with fresh complete media. On day 6 of primary culture for cMSC, the first transduction was performed as described above. Three flasks were not transduced, and fresh media was replaced on day 6. On day 7 of culture, the media was replaced with fresh media.

On day 8 of culture the transduction procedure was repeated. On day 9 in culture, photographs were taken, and the cells were passaged from P0 to P1 as described above. Three x 10^6 cells were added to 100 ml of complete medium, and poured into triple flasks. Fifteen triple flasks were prepared with transduced cells and three were prepared with untransduced cells.

The 15 ml bone marrow aspirates yielded 910, 1212, 856, and 1948 x 10^6 nucleated cells for donors CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively. Mononuclear cell counts obtained from the Percoll interface were 612, 666, 588, and 462 x 10^6, resulting in recoveries of 67.2, 55, 68.7, and 23.7%. Upon P1, the cell viability was a mean of 97.1 (range 93.3 to 100)%. Upon P2 for donors CAN-07-01 and CAN-07-02, and P1 cells for donors CAN-07-03 and CAN-07-04, the cell viability of the transduced cells was a mean of 96.7 (range 96.3 to 97.9)%. The untransduced cells were 95.4 (range 93.3 to 96.9)% viable. Upon harvest for cryopreservation of the cMSC, the viability of the transduced cells was a mean of 99.4 (range 97.4 to 100)% and the untransduced cells were 99.4 (range 97.6 to 100)% viable (Table 4).

The transduced cMSC yield per flask for donors CAN-07-01 and CAN-07-02, harvested 4 days after passage 2 and plated at 3 x 10^6 per flask was 5.9 and 6.7 x 10^6, and the untransduced cMSC yield per flask was 8.4 and 7.5 x 10^6. The transduced cMSC yield per flask for donors CAN-07-03 and CAN-07-04, harvested 4 days after passage 1 (different transduction and passage design) and plated at 3 x 10^6 per flask

was 20.0 and 14.0 x 10^6, and the untransduced cMSC yield per flask was 25.3 and 18.0 x 10^6.

CFU Assays on cMSC from P0 Cultures

CFU colony assays were prepared at the time of primary culture establishment by plating 0.5 x 10^6 cells in triplicate in 100 mm dishes containing 10 ml complete media. The dishes were incubated at 37°C and 5% CO_2. The media was replaced with fresh media each 2 to 4 days. On day 10 in culture, the CFU assay dishes were rinsed with HBSS twice, fixed with 1% gluteraldehyde for 15 minutes, rinsed with HBSS twice, and air dried. The cMSC in the dishes were then stained with 0.1% crystal violet, rinsed with deionized water three times, and air dried. Colonies were counted to calculate the number of colonies forming per 10^6 cells plated.

CFU assays plated on day of mononuclear cell isolation and culture establishment and harvested on day 10 yielded 56, 46.7, 114, and 72 colonies per 10^6 cells for dogs CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively.

On day 13 of P1 culture, photographs were taken to record the cell distribution and morphology and the P1 cells were harvested by trypsinization and cryopreserved as described below.

The media in the triple flasks was decanted, and the flasks were rinsed with 50 ml DPBS. After decanting the DPBS, 23 ml of 0.25% trypsin was added to each triple flask. The flasks were placed in a 37°C incubator for 3 minutes. After cell detachment, 23 ml complete medium was added to each flask. The cell suspensions were transferred to 50 ml conical tubes and the flasks were washed with 30 ml HBSS. The tubes were centrifuged at 2200 RPM for 5 minutes at RT. The pellets containing the transduced or untransduced cells, respectively, were pooled and counted. One aliquot of 1 x 10^7 cells was set aside for determination of the transduction percentage by an Anti-EGFP DNA PCR Elisa assay.

After harvest, the recovered P1 or P2 transduced and culture-expanded cMSCs centrifuged at 1300 RPM for 5 minutes and resuspended in 1 ml aliquots with 1 x 10^7 cMSC/ml in ice-cold cryoprotectant solution containing 85% Plasma-Lyte A (Baxter IV Therapy), 10% DMSO, and 5% autologous canine serum. Cell aliquots were dispensed into separate cryo-vials containing 1 ml each. The tubes were labeled with the canine donor number and total viable cell count. The cMSCs were cryopreserved by placing the cell vials into a Nalgene freezing container and placed in a -70°C freezer for 4 hours, then moved to storage at –70°C.

Upon cell harvest for cryopreservation of the product, aliquots of 1 x 10^7 cells were obtained for determination of the transduction efficiency. The transduction efficiency was analyzed by an Anti EGFP DNA PCR Elisa with digoxigenin incorporation into the product and a second step Anti-digoxigenin colorimetric assay.

CMSC Infusion Product

One to two hours before infusion, the vials of cMSC were thawed by swirling in a 37° water bath, sprayed with 70% ethanol, and opened in a biosafety cabinet. The cMSC product was suspended in 50 ml of infusion medium containing DMEM-LG plus 30% serum autologous to the cell donor. The viability of the cMSC product was determined by exclusion of trypan blue to determine the actual viable dose. An aliquot of each cMSC product was submitted for yeast isolate, aerobic, and non-aerobic growth. The cMSCs were evaluated for the ability to attach to tissue culture plastic and to proliferate in P2 (P3 for CAN-07-01 and CAN-07-02) culture. Aliquots of 1 x 10^6 and 0.16 x 10^6 cMSC were plated into complete canine culture medium in triplicate in T-25 plastic culture flasks. After 24 hours, the flasks plated with 1 x 10^6 cMSC and on day three, the flasks plated with 0.16 x 10^6 cMSC were harvested by trypsinization and counted.

Following TBI, the cMSC suspension was infused via a catheter inserted into the cephalic vein using a hand-held Harvard Bard Mini Infuser to deliver the 50 ml over a 15-20 minute period.

Moderately high doses of 7.49, 7.35, 10.0, and 10.0 (mean 8.7) x 10^6 viable cMSC/kg were infused on day 0 to dogs CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively. These doses represent a 4- to 10-fold increase over the typical dose that a patient would receive. Total viable cMSC infused ranged from 67.7 to 129 (mean 93.9) x 10^6 cMSC. The viability of the cells ranged from 92.1 to 97.6 (mean 94.9) as determined by trypan blue exclusion. CMSC Infusions were given between 71 and 146 (mean 110) minutes post-TBI.

Blood Sampling Post-Infusion

Blood samples (2 ml) were obtained before (pre) and during the cMSC infusion at five and fifteen minutes after the start of the infusion, as well as 1- and 2-hour, and 1-, 2-, 3-, and 4-day time points. Cell lysates were prepared using the Puregene™ (Gentra Systems, Inc.) DNA Isolation Kit for use in an Anti EGFP DNA PCR Elisa with digoxigenin incorporated into the product and a second step Anti-digoxigenin colorimetric assay to detect of the level of GFP marked cMSC in the bloodstream.

Bone marrow harvest and graft infusion

Bone marrow to be used as the transplant graft was harvested from the DLA-identical littermate prior to TBI. Aspirates were obtained from each humerus using an 11-gauge, 4-6 inch ball-top stainless steel marrow harvest needle, attached to polyvinyl tubing originating from a vacuum flask containing 100 ml Tissue Culture Medium 199 and 4 ml (4000 U) heparin. The marrow is passed through 300- and 200-um pore size, and stored at 4°C in a transfer pack container, labeled with the donor and recipient, until infusion later that day. The bone marrow total nucleated cell count (BM-TNC) of the marrow is corrected to exclude any nucleated cells which

would be present in the volume of peripheral blood obtained during the marrow harvest.

The total nucleated cell count (TNC) of the bone marrow was corrected to exclude any TNC which would be present in the volume of peripheral blood obtained during the marrow harvest. Corrected doses of marrow were 4.3, 3.5, 3.1, and 2.0 (mean 3.2) x 10^8 TNC/kg to dogs CAN-07-01, CAN-07-02, CAN-07-03, and CAN-07-04, respectively. Uncorrected bone marrow doses were 5.6, 4.2, 4.5, and 2.7 (mean 4.3) x 10^8 TNC/kg.

Twenty minutes prior to infusion, the marrow was placed at room temperature. One hour after the cMSC infusion, the marrow was infused intravenously through a butterfly needle inserted into the cephalic vein, by exerting pressure on the bag over 1 to 2 minutes.

Supportive care

On day –5, oral antibiotics (neomycin sulfate and polymyxin sulfate) were given three times daily. These oral antibiotics were administered until absolute neutrophil counts reached 500/mm^3. On day 0, the systemic antibiotic Baytril was administered intravenously twice daily and continued until absolute neutrophil counts reached 1,000/mm^3 consistently. Fluid and electrolytes lost as results of transient radiation toxicity were replaced by subcutaneous administration of 500 ml of Ringers Solution, twice daily until food and water were accepted.

Differential blood cell counts

Blood samples (2 ml) were collected from either the jugular or cephalic vein on the mornings of the marrow aspirate for isolation of cMSC, days 0 through 50 and biweekly thereafter through the end of study. The blood was transferred into a vacutainer containing EDTA. Total white blood cell (WBC) and platelet counts per

mm^3 are measured using a Sysmex E2500 and differential cell counts were determined manually after fixation and staining with Wrights stain.

Necropsy

Blood samples were obtained for CBC, Chemistry 23 analysis, and PCR evaluation. The dogs were sedated with Butorphanol followed by a mixture of Diazepam and ketamine hydrochloride. After sedation, biopsies and bilateral bone marrow aspirates were obtained from the humerii, femora, and iliac crests. Euthanasia was then completed with an overdose of the sedative sodium pentobarbital. The day-50 group of dogs (CAN-07-01 and CAN-07-02) were euthanised on day 43 in the study; the day-100 group of dogs (CAN-07-03 and CAN-07-04) were euthanised on day 100 in the study. Complete sets of the tissues were collected upon necropsy of the animals.

The collection of tissues for histological examination followed immediately. A subset of tissues was used for Anti-EGFP DNA PCR Elisa analysis. Bone marrow aspirates and biopsies were used for Anti-EGFP DNA PCR Elisa analysis, culture expansion for further PCR analysis, and CFU assays

The tissues were trimmed to about 1 inch square pieces and placed into separate labeled 50 ml conical tubes filled with 10% Neutral Buffered Formalin (pH 6.8 - 7.2). The tissues were embedded in paraffin, sectioned and stained with Hematoxylin and Eosin. Bone marrow samples were stained with Periodic Acid Schiff's stain.

Bone marrow aspirates obtained prior to necropsy were collected in 15 ml labeled tubes from the left and right humerii, femora, and iliac crests from each dog. A subset of the tissue samples were obtained during necropsy and trimmed to about 1/4 inch square pieces, wrapped in

PBS-soaked gauze and placed separately in a labeled zip-lock bag. The bone marrow aspirates were held on ice.

Preparation of bone marrow aspirates for CFU assay

Aliquots of the bone marrow aspirates from the left and right humerus, femur, and iliac crest from each canine obtained for PCR analysis were aliquoted into separate 15 ml labeled tubes. The bone marrow samples were held on ice.

CFU assay on cMSC from bone marrow obtained at necropsy

CFU colony assays performed on cMSC obtained from bone marrow obtained at necropsy were prepared by plating 0.5 x 10^6 cells in triplicate in 100-mm dishes containing 10 ml complete media. The dishes were incubated at 37°C and 5% CO_2. The media was replaced with fresh media each 2-4 days. On day 10 in culture, the CFU assay dishes were rinsed with HBSS twice, fixed with 1% gluteraldehyde for 15 minutes, rinsed with HBSS twice, and air dried. The cMSC in the dishes were then stained with 0.1% crystal violet, rinsed with deionized water three times, and air dried. Colonies were counted to calculate the number of colonies per 10^6 cells plated.

Isolation and purification of DNA

DNA was isolated from a part of each tissue. The remaining piece of the sample was cryopreserved and stored in -70°C freezer. DNA was isolated by placing samples in Phosphate Buffered Saline (PBS), adding proteinase K solution, and incubating at 55°C for 3 hrs, or until the tissue has dissolved. The samples were subsequently treated with RNase at 37°C for 60 min. The samples were cooled to room temperature and the protein was precipitated. The samples were centrifuged and the aqueous phase was gently collected in 100% isopropanol. The samples were mixed and centrifuged and the pellet was washed in 70% ethanol. The tubes were centrifuged and the supernatant was drained off and the pellets were allowed to dry for approximately 1 to 6 hrs. The DNA was allowed to hydrate overnight at room temperature and was subsequently stored at 4°C.

Peripheral blood and bone marrow samples were first lysed with RBC lysis solution (Ammonium Chloride Buffer). DNA was then isolated from the lysates as described above. DNA was quantified by the addition of 998 µl deionized H_20 and 2 µl DNA from the sample into a cuvette and vortexed. A spectrophotometer was used to determine the optical density (OD). The OD was read at 260 and 280, and the concentration of DNA was calculated for µg/ml. The DNA concentration was adjusted to 1 µg/ml using deionized water.

Anti-EGFP DNA PCR Elisa

The anti-EGFP DNA PCR Elisa assay used in these studies detects infused cMSCs utilizing oligonucleotide primers specific for GFP. For analysis of gene expression, we utilized PCR-ELISA (DIG labeling/detection) kit (Boehringer Mannheim). Briefly, PCR was performed in the presence of digoxigenin-labeled nucleotides to label the amplified product. Next, 25 µl of the PCR product was denatured and allowed to hybridize in solution to 5'-biotinylated oligonucleotide probe at 37°C in streptavidin-coated microtiter plate. The bound probe-PCR product was detected by an anti-digoxigenin peroxidase conjugate and by use of the colorimetric substrate 2,2'Azinobis (3-ethylbenzthiazoline-sulfonic Acid) (ABTS). Titration standard curves were generated using transfected control cMSC to approximate concentration of DNA per quantity of DNA used in the assay. By first correlating with an internal standard for PCR of the DLA Class II genomic DNA, and then correlating DNA concentration to cell equivalents, and assuming one retrovirus integration event per transduced cell, an estimation of cell number can be obtained.

Quantitative measurements of DNA for GFP were noted in all bone marrow scoops/biopsies.

Post-transplant blood cell recovery

The mean day to a threshold (to 3 consecutive values) of platelets to 10,000/mm^3 was 12.8 (range 11-17), to 50,000/mm^3 was 19.8 (range 16-25), and to 100,000/mm^3 23.0 (range 20-27). The mean day to a threshold value (to 3 consecutive days) of absolute neutrophil cells to 500/mm^3 was 9.3 (range 8-11), and to 1,000/mm^3 was 10.5 (range 9-13).

Interim bone marrow aspirates

When platelets recovered consistently to values greater than 50,000 per mm^3, an interim bone marrow aspirate was collected from the iliac crest. This procedure was performed on day 27 in study for CAN-07-01 and CAN-07-02, and on day 29 for CAN-07-03 and CAN-07-04.

RESULTS

Upon histopathological evaluation of all tissues from CAN-07-01 and CAN-07-02, euthanised on day 43, findings were negative for ectopic connective tissue and for subacute GVHD.

Detectable DNA signal could be found within 1 hour of infusion and again at 2 days. One sample could be quantitatively measured at 3 days post infusion for GFP DNA. This timepoint is consistent with the previous observations in the autologous canine transplant study in which signal was found at 2 and 3 days

Day 100 necropsy data in CAN-07-03 and CAN-07-04 for GFP+ cells showed GFP signal (1 GFP+ cell equivalent per 10 micrograms PCR input DNA) in the femur and humerus of CAN-07-03 and in the humerus of CAN-07-04.

In this model it was possible to detect skin graft-versus-host-disease (GVHD) by observing the redness of the eyes and ears of the animals. Using this indicator, it was determined that the animals that received mesenchymal stem cells had a lower incidence of and/or lower severity of GVHD compared to the control animals that were not treated with mesenchymal stem cells.

These results demonstrate that allogeneic MSCs can support the rapid engraftment of bone marrow hematopoietic cells. No transfusion support was needed. There was no clinical evidence of GVHD. Platelet recovery was faster than in historical controls. There was evidence of chimerism in stromal cells after allogeneic transplantation. The option to engraft allogeneic tissue by using allogeneic MSCs broadens the range of transplant material usable in clinical transplant scenarios.

Example 9

Suppression of Mixed Lymphocyte Reaction by MSC Supernatant (MLR95)

Generation of supernatants: T cells from donor 155 were purified from PBMC by negative immunomagnetic selection with anti-CD19 and anti-CD14 MicroBeads (Miltenyi Biotec). PBMC from donor 413 were X-ray irradiated with 3600 rad (12 min at 70 kV). In 24-well tissue culture plates T cells ($9x10^5$/well) were mixed with irradiated PBMCs for 3 days, then MSCs from different donors (219, 459, 461 - all at passage 5) were added into the cultures at $1.2 x10^5$ cells/well for 3 additional days. In control cultures, the same volume of medium was added instead of MSCs. In separate wells, the same number of MSCs were plated alone, and cultured for 3 additional days. After 3 days of culture (on day 6 after initiation of the primary MLR), the cells were resuspended by pipetting, and 200 µl of the cell suspensions were transferred into a 96-well plate in triplicate, and pulsed with [H^3]TdR (5 Ci/mmol, 1 µCi/well) for 18 hours to determine the level of proliferation. The remaining cells were centrifuged at 1250 rpm for 10 minutes, the supernatants were collected, aliquoted and kept frozen at -80°C.

Suppression of primary MLR by supernatants: T cells from donor 155 were purified from PBMCs by negative immunomagnetic selection with anti-CD19 and anti-CD14 MicroBeads (Miltenyi Biotec). PBMCs from donor 413 or from donor 273 were X-ray irradiated with 3600 rad (12 min at 70 kV). In 96-well tissue culture plates T cells ($1.5 x 10^5$/well) were mixed with PBMCs ($1.5 x 10^5$/well) in the presence or absence of different supernatants that were added at the initiation of cultures at the dilutions (1/8, 1/32, 1/128, 1/512, 1/2048 and 1/8192). In control cultures, the same volume of medium was added instead of supernatants. Cultures were incubated for 6 days, then pulsed with [H^3]TdR (5 Ci/mmol, 1 µCi/well) for 18 hours.

Figure 9 shows the results of the MLR between T 155 x PBMC 413. Primary MLRs in the presence of supernatants of MSCs + MLR, (#1) MLR +MSC219 (#2) MLR +MSC459 (#3) MLR +MSC461, and MSCs alone, (#8) MSC219 alone (#9) MSC459 alone (#10) MSC461 alone, were suppressed. Primary MLR in the presence of supernatant of MLR alone (#5) was not suppressed.

Suppression of an on-going MLR T cells from donor 155 were purified from PBMCs by negative immunomagnetic selection with anti-CD19 and anti-CD14 MicroBeads (Miltenyi Biotec). PBMCs from donor 413 (1.5 x 10^5/well) were X-ray irradiated with 3600 rad (12 min at 70 kV). In 96-well tissue culture plates T cells (1.5 X 10^5/well) were mixed with PBMCs (1.5 x 10^5/well) for 4 days, then supernatants were added into the cultures at different dilutions (1/8, 1/32, 1/128, 1/512, 1/2048 and 1/8192). In control cultures, the same volume of medium was added instead of supernatants. Cultures were incubated for 2 additional days (6 days after initiation of the MLR), then pulsed with [H^3]TdR (5 Ci/mmol, 1 μCi/well) for 18 h.

Figure 10 shows the results of the MLR between T 155 x PBMC 413. Supernatants from all MSCs + MLR (#1) MLR +MSC219 (#2) MLR +MSC459 (#3) MLR +MSC461 showed strong suppression of ongoing MLRs. Supernatants from MSCs alone (#8) MSC219 alone (#9) MSC459 alone (#10) MSC461 alone suppressed in a dose dependent manner with a significant effect up to 1/512 dilution. Ongoing MLR in the presence of supernatant of MLR alone (#5) was not suppressed.

Example 10

Suppression of Mixed Lymphocyte Reaction by Xenogeneic Mesenchymal Stem Cells

Suppression of Human MLRs by Baboon MSCs. Responder PBMCs from various human donors (R4, R6, R7, R11) were mixed with irradiated (3000R) allogeneic human PBMCs (S4, S6, S7, S11) in microtiter wells are 1.5 x 10^5 cells/well

for each population. Cultures were performed in standard cell culture medium containing 5% human AB serum. Baboon MSCs (bMSCs) from donor 86243 were added at 2 x 10^4 /well at the initiation of the MLR. The MSCs were not treated with IFNγ. Lymphoproliferation was determined on day 7 of culture by pulsing the cells with ^{3}H-thymidine for the final 18 hours prior to cell harvest for scintillation counting. The results illustrated in Figure 11 show that baboon MSCs suppressed robust human MLRs by greater than 50%.

Suppression of Xenogeneic MLR by Human or Baboon MSCs. Responder human T cells (hT) from donor 273 were cultured with irradiated (3000R) baboon PBMC (bPBMC) from donor 5957 or donor 5909 in standard cell culture medium containing 5% human AB serum. Human MSCs (hMSCs) from donor 244 or baboon MSCs (bMSC) from donor 6243 were added to the cultures at initiation. Lymphoproliferation was determined on day 7 of culture by pulsing the cells with ^{3}H-thymidine for the final 18 hours prior to cell harvest for scintillation counting. The results illustrated in Figure 12 (bPBMC donor 5957) and Figure 13 (bPBMC 5909) show that both human and baboon MSCs can suppress the xenogeneic human x baboon MLR.

What Is Claimed Is:

1. A method for reducing an immune response against an alloantigen, comprising contacting immune effector cells with a supernatant from a mesenchymal stem cell culture in an amount effective to reduce the immune response.

2. The method of claim 1 wherein the effector cells are T cells.

3. A method for preventing or reducing reactivation of activated T cells comprising contacting T cells that have been previously activated by an alloantigen with a supernatant from a mesenchymal stem cell culture in an amount effective to suppress re-stimulation of said activated T cells.

4. A method of reducing an immune response to a donor transplant, comprising treating the recipient with a supernatant from a mesenchymal stem cell culture in an amount effective to reduce an immune response in the recipient to the transplant.

5. The method of claim 4 wherein the mesenchymal stem cells are autologous to the recipient.

6. The method of claim 4 wherein the mesenchymal stem cells are allogeneic to the recipient.

7. The method of claim 6 wherein the mesenchymal stem cells are obtained from the donor of the transplant.

8. The method of claim 4 wherein the mesenchymal stem cells are allogeneic to both the donor of the transplant and the recipient.

9. The method of claim 4 wherein the mesenchymal stem cells are xenogeneic to both the donor of the transplant and the recipient.

10. The method of claim 4 wherein the transplant is skin.

11. The method of claim 4 wherein the supernatant is administered to the recipient prior to administration of the transplant.

12. The method of claim 4 wherein the supernatant is administered concurrently with administration of the transplant.

13. The method of claim 4 wherein the supernatant is administered as a part of the transplant.

14. The method of claim 4 wherein the supernatant is administered after the transplant.

15. The method of claim 4 wherein the supernatant is administered to the transplant recipient to treat rejection of the transplant by the recipient.

16. The method of claim 4 further comprising administering to the recipient immunosuppressive agents.

17. The method of claim 4 wherein the transplant is a solid organ.

18. The method of claim 17 wherein the solid organ is selected from heart, pancreas, kidney, lung or liver.

19. A method of treating a transplant recipient for graft versus host disease, comprising treating the recipient of a donor transplant with a supernatant from a

mesenchymal stem cell culture in an amount effective to reduce an immune response against the recipient by the transplant.

20. The method of claim 19 wherein the mesenchymal stem cells are autologous to the recipient.

21. The method of claim 19 wherein the mesenchymal stem cells are autologous to the donor transplant.

22. The method of claim 19 wherein the mesenchymal stem cells are allogeneic to both the donor and recipient.

23. The method of claim 19 further comprising administering to the recipient immunosuppressive agents.

24. A composition for reducing an adverse immune response against a donor transplant, comprising a supernatant from a mesenchymal stem cell culture in an amount effective to inhibit or reduce an adverse immune response against a donor transplant, and a pharmaceutical carrier.

25. The composition of claim 24 wherein the mesenchymal stem cells are autologous to the recipient.

26. The composition of claim 24 wherein the mesenchymal stem cells are autologous to the donor.

27. The composition of claim 24 wherein the mesenchymal stem cells are allogeneic to both the recipient and the donor.

28. The composition of claim 24 wherein the mesenchymal stem cells are xenogeneic to both the recipient and the donor.

29. A composition for reducing an adverse immune response against a graft recipient caused by a graft, comprising a supernatant from a mesenchymal stem cell culture in an amount effective to reduce the adverse immune response against the graft recipient caused by the graft, and a pharmaceutical carrier.

30. The composition of claim 29 wherein the mesenchymal stem cells are autologous to the recipient.

31. The composition of claim 29 wherein the mesenchymal stem cells are autologous to the donor.

32. The composition of claim 29 wherein the mesenchymal stem cells are allogeneic to both the recipient and the donor.

33. The composition of claim 29 wherein the mesenchymal stem cells are xenogeneic to both the recipient and the donor.

34. The process of Claim 1 wherein the supernatant is obtained from mesenchymal stem cells co-cultured with T cells undergoing a mixed lymphocyte reaction.

35. A method for reducing an immune response against an alloantigen, comprising contacting immune effector cells with xenogeneic mesenchymal stem cells in an amount effective to reduce the immune response.

36. A method for reducing an immune response of effector cells against an alloantigen, comprising contacting effector cells with a supernatant of mesenchymal

stem cells in an amount effective to reduce an immune response against an alloantigen whereby said effector cells upon contact with an alloantigen have a reduced immune response against said alloantigen.

37. The method of claim 36 wherein said effector cells are T cells previously activated and said immune response is the reactivation of said T cells.

38. The method of claim 36 wherein said immune response to be reduced is an *in vitro* immune response.

39. The method of claim 36 wherein said immune response to be reduced is an *in vivo* immune response.

40. The method of claim 36 wherein said immune response to be reduced is an immune response to a donor transplant.

41. The method of claim 40 wherein said donor transplant is a xenogeneic donor transplant.

FIG. 1

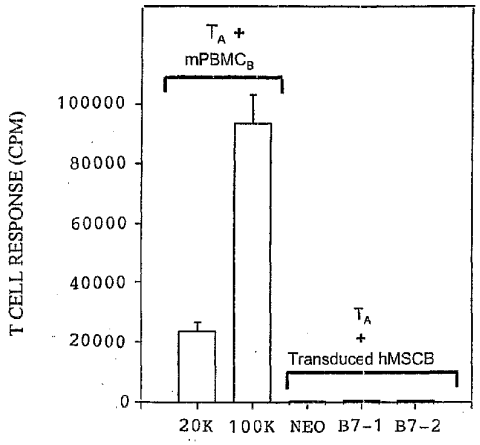

T CELL RESPONSE (CPM)
100000
80000
60000
40000
20000
0
T_A + mPBMC$_B$
T_A + Transduced hMSCB
20K
100K
NEO
B7-1
B7-2

FIG. 2

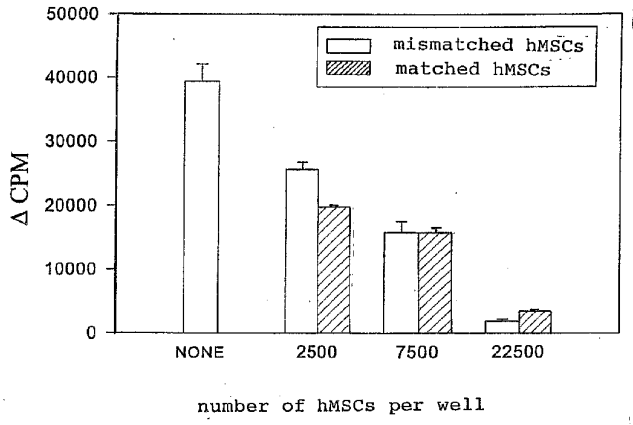

50000
40000
30000
20000
10000
0
Δ CPM
mismatched hMSCs
matched hMSCs
NONE
2500
7500
22500
number of hMSCs per well

FIG. 3

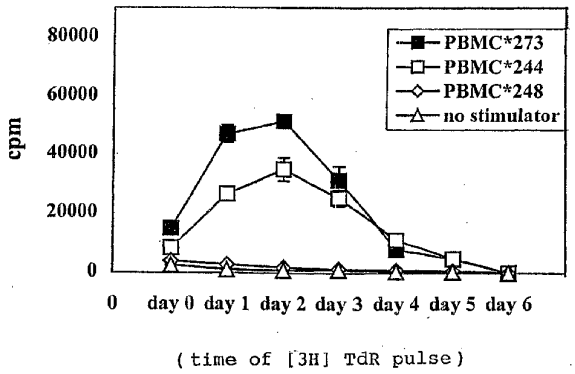

80000
60000
40000
20000
0
cpm
PBMC*273
PBMC*244
PBMC*248
no stimulator
0
day 0
day 1
day 2
day 3
day 4
day 5
day 6
(time of [3H] TdR pulse)

FIG. 4

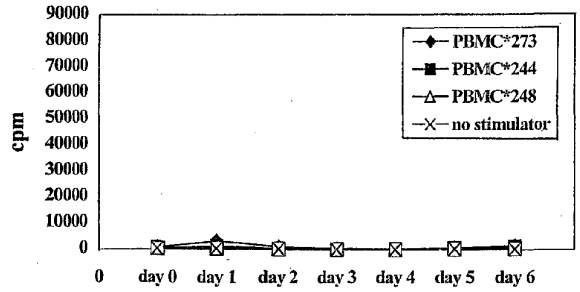

90000
80000
70000
60000
50000
40000
30000
20000
10000
0
cpm
PBMC*273
PBMC*244
PBMC*248
no stimulator
0
day 0
day 1
day 2
day 3
day 4
day 5
day 6
time of [3H] TdR pulse

FIG. 5A

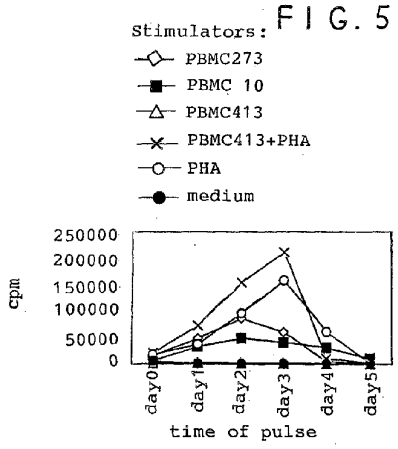

Stimulators:
PBMC273
PBMC 10
PBMC413
PBMC413+PHA
PHA
medium
250000
200000
150000
100000
50000
0
cpm
day0
day1
day2
day3
day4
day5
time of pulse

FIG. 5B

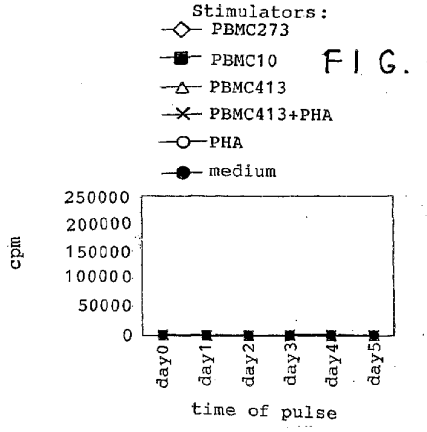

Stimulators:
PBMC273
PBMC10
PBMC413
PBMC413+PHA
PHA
medium
250000
200000
150000
100000
50000
0
cpm
day0
day1
day2
day3
day4
day5
time of pulse

FIG. 5C

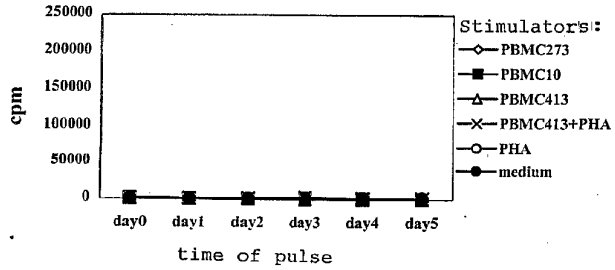

250000
200000
150000
100000
50000
0
cpm
day0
day1
day2
day3
day4
day5
time of pulse
Stimulators:
PBMC273
PBMC10
PBMC413
PBMC413+PHA
PHA
medium

FIG. 5D

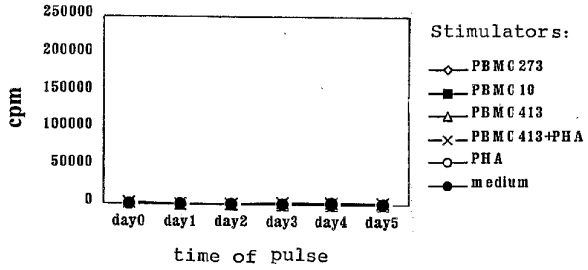

250000
200000
150000
100000
50000
0
cpm
day0
day1
day2
day3
day4
day5
time of pulse
Stimulators:
PBMC273
PBMC10
PBMC413
PBMC413+PHA
PHA
medium

FIG. 6A

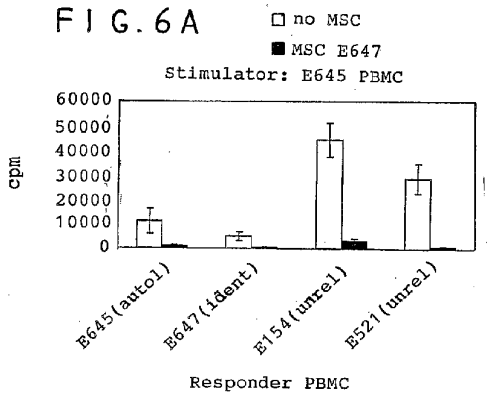

no MSC
MSC E647
Stimulator: E645 PBMC
60000
50000
40000
30000
20000
10000
0
cpm
E645(autol)
E647(ident)
E154(unrel)
E521(unrel)
Responder PBMC

FIG. 6B

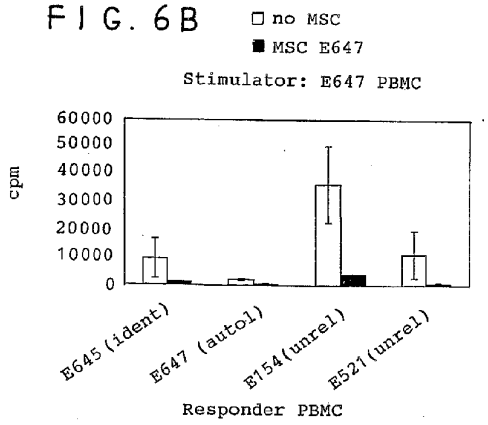

no MSC
MSC E647
Stimulator: E647 PBMC
60000
50000
40000
30000
20000
10000
0
cpm
E645 (ident)
E647 (autol)
E154(unrel)
E521(unrel)
Responder PBMC

FIG. 6C

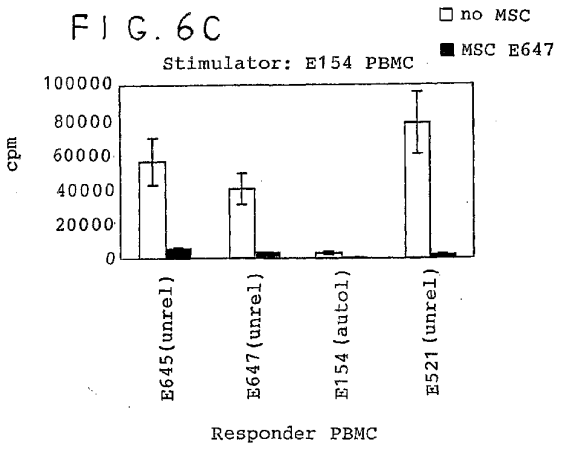

no MSC
MSC E647
Stimulator: E154 PBMC
cpm
100000
80000
60000
40000
20000
0
E645(unrel)
E647(unrel)
E154(autol)
E521(unrel)
Responder PBMC

FIG. 6D

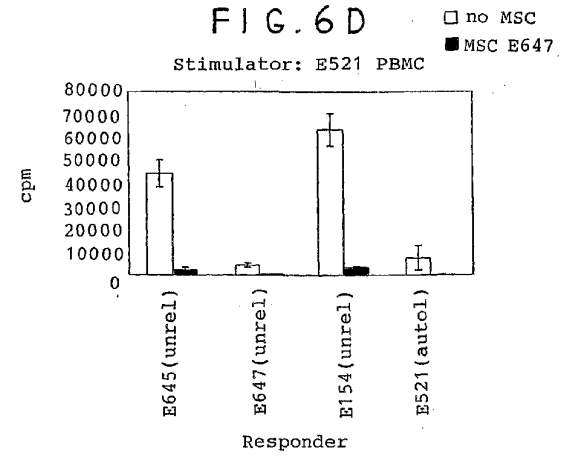

no MSC
MSC E647
Stimulator: E521 PBMC
cpm
80000
70000
60000
50000
40000
30000
20000
10000
0
E645(unrel)
E647(unrel)
E154(unrel)
E521(autol)
Responder

FIG. 7

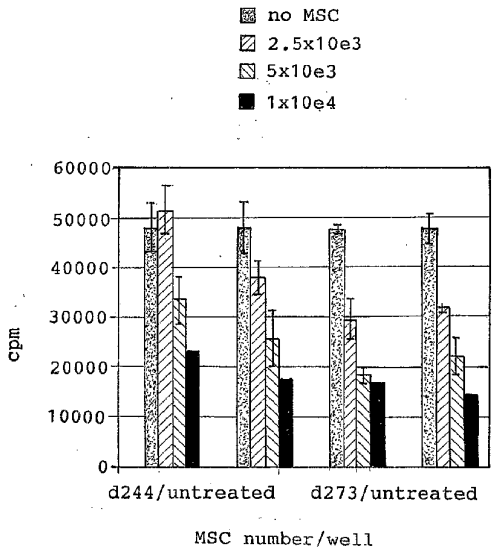

no MSC
2.5x10e3
5x10e3
1x10e4
60000
50000
40000
30000
20000
10000
0
cpm
d244/untreated
d273/untreated
MSC number/well

FIG. 8

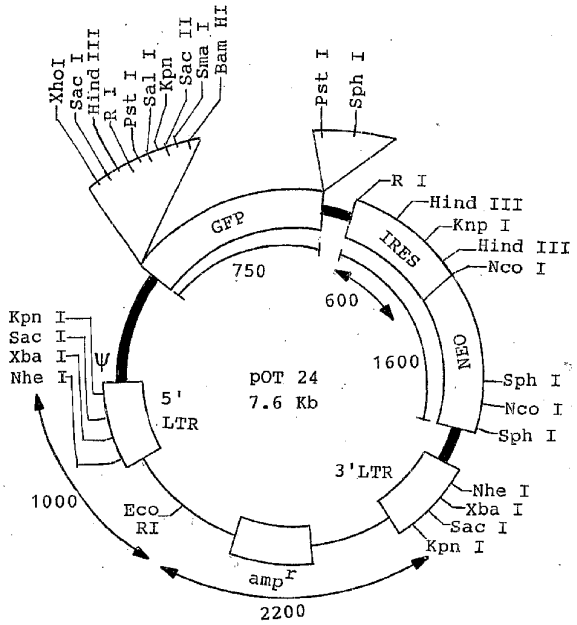

XhoI
Sac I
Hind III
R I
Pst I
Sal I
Kpn
Sac II
Sma I
Bam HI
Pst I
Sph I
R I
Hind III
Knp I
Hind III
Nco I
GFP
IRES
NEO
750
600
1600
Kpn I
Sac I
Xba I
Nhe I
Ψ
5'
LTR
pOT 24
7.6 Kb
Sph I
Nco I
Sph I
3'LTR
Nhe I
Xba I
Sac I
Kpn I
1000
Eco
RI
amp^r
2200

FIG. 9

Suppressive Effect of Supernatants Generated from hMSCs or hMSC-Suppressed MLR Cultures: Effect on Primary MLR

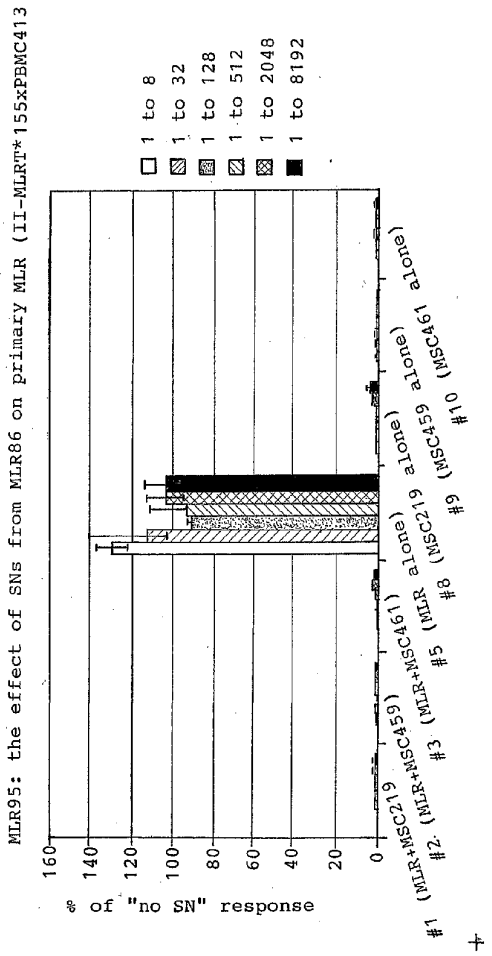


FIG. 10

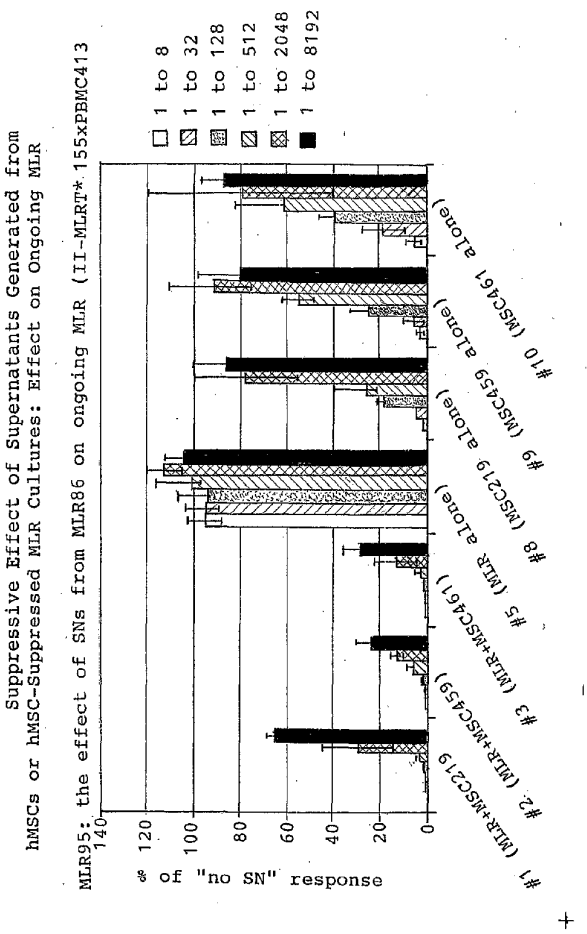

Suppressive Effect of Supernatants Generated from
hMSCs or hMSC-Suppressed MLR Cultures: Effect on Ongoing MLR
MLR95: the effect of SNs from MLR86 on ongoing MLR (II-MLRT* 155xPBMC413
1 to 8
1 to 32
1 to 128
1 to 512
1 to 2048
1 to 8192
% of "no SN" response
140
120
100
80
60
40
20
0
#1 (MLR+MSC219)
#2 (MLR+MSC459)
#3 (MLR+MSC461)
#5 (MLR alone)
#8 (MSC219 alone)
#9 (MSC459 alone)
#10 (MSC461 alone)

FIG. 11

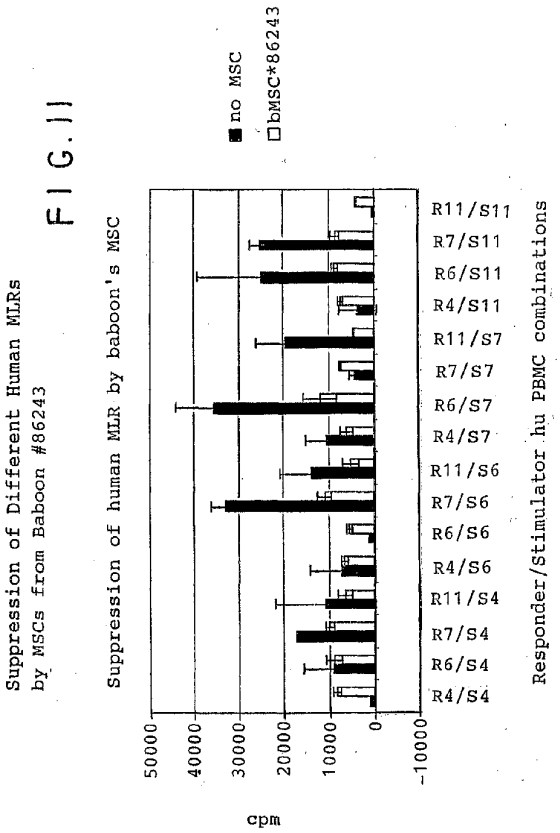

Suppression of Different Human MLRs
by MSCs from Baboon #86243
Suppression of human MLR by baboon's MSC
no MSC
bMSC*86243
R11/S11
R7/S11
R6/S11
R4/S11
R11/S7
R7/S7
R6/S7
R4/S7
R11/S6
R7/S6
R6/S6
R4/S6
R11/S4
R7/S4
R6/S4
R4/S4
50000
40000
30000
20000
10000
0
-10000
cpm
Responder/Stimulator hu PBMC combinations

FIG. 12

Suppression of Xenogeneic MLR (Human x Baboon)
by Human and Baboon MSCs

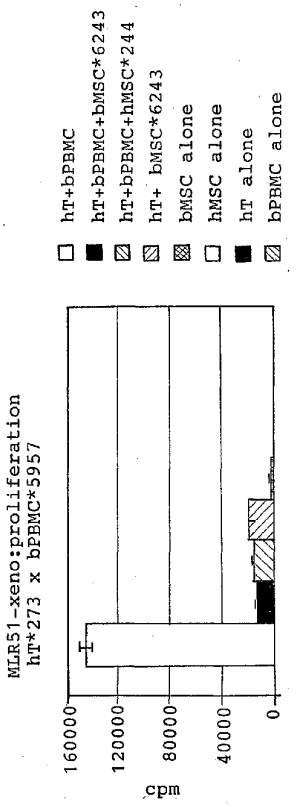
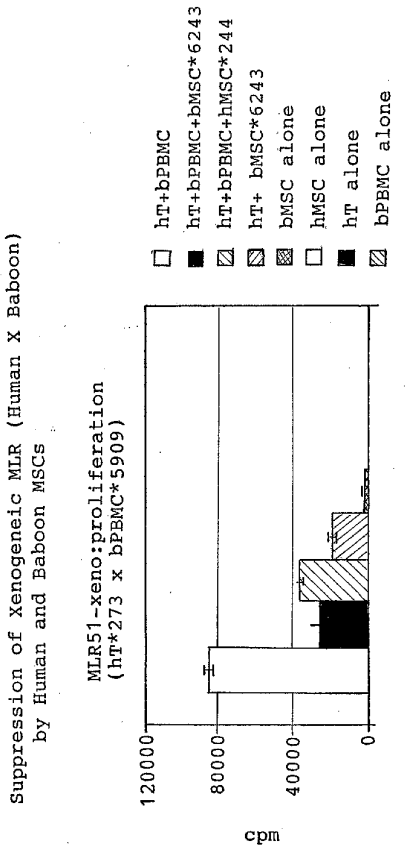


FIG. 13


Suppression of Xenogeneic MLR (Human X Baboon)
by Human and Baboon MSCs
MLR51-xeno:proliferation
(hT*273 x bPBMC*5909)
hT+bPBMC
hT+bPBMC+bMSC*6243
hT+bPBMC+hMSC*244
hT+ bMSC*6243
bMSC alone
hMSC alone
hT alone
bPBMC alone
120000
80000
40000
0
cpm

【国際調査報告】

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 00/29815

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/28 //C12N5/06

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)
IPC 6 C12N A61K

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 practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 23058 A (UNIV CASE WESTERN RESERVE) 1 August 1996 (1996-08-01) page 2, paragraph 2 -page 5, paragraph 2; claims 1-8 ---	24-33,35
X	ESHEL I ET AL: "Analysis of thymic stromal cell subpopulations grown in-vitro on extracellular matrix in defined medium ii. Cytokine activities in murine thymic epithelial and mesenchymal cell culture supernatants" JOURNAL OF IMMUNOLOGY, vol. 144, no. 5, 1990, pages 1563-1570, XP002159332 ISSN: 0022-1767 abstract page 1563, column 2, paragraph 3 -page 1564, column 1, paragraph 5 --- -/--	24-33

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 2001	22/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Muller-Thomalla, K

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INTERNATIONAL SEARCH REPORT

Int[illegible]inal Application No
PCT/US 00/29815

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,P	KLYUSHNENKOVA ET AL.: "Human mesenchymal stem cells suppress allogeneic T-cell responses in vitro: implications for allogeneic transplantion." BLOOD, vol. 92, no. 10, 15 November 1998 (1998-11-15), page 2652 XP002114436 abstract No.2652 ----	24-33,35

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International Application No. PCT/US 00/29815

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-34 and 36-41

Method of reducing an immune response comprising the use of a "supernatant" from a mesenchymal stem cell culture as well as said supernatant per se.

2. Claim : 35

Method for reducing an immune response comprising the use of xenogeneic mesenchymal stem cells.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/US 00/29815

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9623058 A	01-08-1996	US 5733542 A	31-03-1998
		AU 4704196 A	14-08-1996
		US 6010696 A	04-01-2000

Form PCT/ISA/210 (patent family annex) (July 1992)

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(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,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),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,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,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

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