ない、発熱、関節炎の増悪を繰り返し、プレドニゾロン1 mg/kg/日で維持された。免疫抑制剤として、シクロスポリン、セルセプト、タクロリムス、ビンブラスチンが試されたがいずれも効果なく、5歳時、膝関節炎、足関節炎の悪化のため歩行不可となった。2009年2月、精査目的にて横浜市立大学小児科を紹介受診、全身型若年性特発性関節炎類似疾患として、抗 IL-6 レセプター抗体であるトシリズマブ投与が開始された。トシリズマブ投与にて関節炎、関節痛は改善したが、ステロイド減量にともない、再び関節痛、関節炎が現れ、経口ステロイドは減量困難で、10 mg/日 (0.6mg/kg/日)の投与を引き続き必要とした。なお持続する炎症の精査にて尿中メバロン酸の著明高値を認め、MVK 遺伝子検査にて p.G326R ホモを認め、高 IgD 症候群と確定診断された。

2011年1月、咳嗽、CRP の上昇を認め、胸部 CT を撮像したところ、1年前にはみられなかった間質性肺炎像を認めた。CMV 感染症、真菌感染症、ニューモシスティス肺炎、マイコプラズマ感染症等感染症は否定的で、トシリズマブ投与により咳嗽の悪化を認めるため、トシリズマブ投与の副作用を考慮しトシリズマブ中止、現在経口ステロイド 10 ｍｇ/日の投与で経過観察中である。

高 IgD 症候群の病態はメバロン酸キナーゼ欠損症であり、最重症のメバロン酸尿症から軽症の高 IgD 症候群まで連続的な疾患スペクトラムである。本症例は、中枢神経症状等メバロン酸尿症に特徴的な徴候は認めずメバロン酸キナーゼ活性は2%で、1%をこえているものの、生下時より CRP 陽性が続き、全身の炎症所見、関節所見、発育状況より臨床的には重症型高 IgD 症候群と考えられる。また海外では同じ遺伝子型 (p.G326R ホモ) の症例がメバロン酸尿症として骨髄移植されており、また本邦でも同じ遺伝子型の症例が合併症で死亡している[7,8]。以上より、定義上高 IgD 症候群とはいうものの、臨床的には重症で、これまで利用可能であった副腎皮質ホルモン、免疫抑制剤、トシリズマブでは十分な抗炎症効果がえられなかった。

これまで高 IgD 症候群の治療法としては、スタチン製剤、副腎皮質ホルモン、抗 TNF-α製剤[1,9-11]、抗 IL-1 製剤[1,12-18]が治療として使用され、ある程度の効果が得られている。また最重症型のメバロン酸尿症には骨髄移植の成功している症例が存在する[8]。当患者さんにおいて、現在副腎皮質ホルモン (プレドニン 10 mg/day, 0.6 mg/kg/day) を使用しているが、上記用量の副腎皮質ホルモンでは炎症所見が改善せず(CRP 5-10 mg/dl が持続)、全身炎症及び副腎皮質ホルモンの副作用と思われる著しい発育障害を認め、また関節拘縮が徐々に進行してきている。このままの治療では、発育障害が改善されず、関節の拘縮、また2次性アミロイドーシスによる腎障害が懸念され、また副腎皮質ホルモンの増量による対応では、発育障害のさらなる助長が懸念される。また骨髄移植は治療法自体のリスクが高く、高 IgD 症候群の第一選択治療とは言えない状態である。以上の理由により、副腎皮質ホルモン以外での抗炎症治療として生物製剤を用いた治療導入を考慮しており、同疾患でこれまで報告されている2製剤、アナキンラ及びエタネルセプトを検討した。本症例では合併する間質性肺炎を抗 TNF-α製剤が増悪させる懸念が存在するため、治療薬としては不適と考えた。よってアナキンラ (商品名キネレット) を治療目的にて使用したいと考えている。

3) 目的

高 IgD 症候群の治療として、アナキンラ (キネレット) の投与を行う。

4） 対象及び方法

対象：上記した高 IgD 症候群の患者

研究実施にあたっては、ヘルシンキ宣言の精神を遵守して行うものとする。研究責任者は研究実施に際し、下記の事項について被験者に説明し、さらにアナキンラ投与の利点と欠点について別紙内容の解説書を提示する。アナキンラによる治療への参加については、文書により自由意思による同意を得るものとし、同意取得者・同意取得年月日を同意書に記入する。

i） 研究の目的および方法

ii） 予期される効果および危険性

iii） 当該疾患に対する他の治療方法の有無およびその内容

iv） 患者が研究被験者になることに同意しない場合であっても不利益を受けないこと

v） 患者が研究被験者になることに同意した場合であっても、随時これを撤回できること。その場合に、その後の治療に不利益を受けないこと。

vi） その他、患者の人権保護に関し必要な事項

方法

① アナキンラの入手方法

RHC USA Corporation 日本支社に輸入代行を依頼し、アナキンラ（商標名キネレット、製造元 SOBI）を輸入する。１バイアル 100 ｍｇを当院薬剤部にて無菌的に１回 2 mg/kg (約 34 mg)に分注する。

② アナキンラの投与法

アナキンラ 1-10 mg/kg/day を連日皮下注する。効果の判定基準として CBC, CRP, 血沈、SAA を初めの１か月間は１週に１回以上検査し、炎症反応の低下を検討するとともに、臨床症状として関節の腫脹、疼痛の変化を関節点数として記録する。その後退院してから半年間は 4 週間に１度、その後 1 か月に１度外来受診を行い、臨床所見、血液検査を行う。中枢神経系の評価として、治療前後で頭部 MRI, 発達テスト、脳波の検討を行う。

これまで使用していたプレドニゾロン等の内服薬はそのまま使用し、アナキンラの評価を行う。炎症反応、関節点数などで効果が見られればプレドニゾロンを漸減する予定である。

5） 予想される危険性、副作用およびその対策

アナキンラの治療をプレドニンと同時投与する形で開始する予定である。そのためアナキンラの投与によりさらに免疫抑制がかかり、各種感染症に罹患する危険性が増加する。そのため、対応策として治療開始１か月は入院治療として、厳重な監視下（医師の診察、血液検査等）に感染症の有無に最大限の注意を払いながら治療を行い、効果が見られれば、プレドニンを減量する予定である。その後退院してから半年間は４週間に１度、その後１か月に１度外来受診を行い、臨床所見、血液検査を行い、感染症等の副作用の有無に注意する。

他のアナキンラ投与の合併症としては、注射部位反応、感染症、好中球減少症があり、その他頻度は多くないものの頭痛、吐き気、下痢が報告されている。上記合併症の発現を臨床症状の注意深い観察、血液検査の定期的な施行（上記頻度にて）により早期発見につとめ、発現時は投与を中止し、直ちに

副作用に応じた治療を開始する。なお、健康被害が生じた場合の補償は一般診療での対処に準ずる事とする。

6） 本臨床応用実施（本邦で認可されていないアナキンラの使用）における倫理的な問題点

イ） アナキンラの危険性

アナキンラ投与の合併症として、注射部位反応、感染症、好中球減少症があり、その他頻度は多くないものの頭痛、吐き気、下痢が報告されている。同時投与する薬剤としてプレドニンがあり、アナキンラの投与によりさらに免疫抑制がかかり、各種感染症に罹患する危険性が増加する。特に結核等の慢性感染症が増悪する危険性が高く、治療前の罹患の有無の検討が重要である。

ロ） アナキンラの費用

アナキンラ投与は通常 1-2 mg/kg/day、1 日 1 回皮下注投与である。治療開始量として 1 mg/kg/day、約 17 mg（約 680 円）で治療効果を見て最大約 170mg（約 6800 円）へ増量予定である。アナキンラは 100 mg (0.67ml)ガラスシリンジ注射製剤として供給されるが、高価な薬剤であることを考慮して、一週間分をガラスバイアルに分注して、インシュリンシリンジにてとりわけ患者に投与する。その際、無菌的にアナキンラを分注するため、特殊製剤依頼として薬剤部にて分注する。その際、バイアルの費用として約 2400 円/月の負担が生じる。通常、高 IgD 症候群重症例の患者さんでは、アナキンラを継続する必要があり、費用は多大なものとなる。アナキンラ自体の費用および分注用のバイアル代は、患者家族と十分な話し合いの結果、患者/患者家族に負担頂く予定で合意している。

ハ） アナキンラの投与

高 IgD 症候群にたいする治療では、炎症が持続する状態ではアナキンラは毎日、皮下注する必要があり、誰が皮下注射をおこなうかが問題となる。まず、投与開始時は京都大学医学部附属病院にて研究責任者の監視下におこない、安全性を確認するとともに、自己注射を習得してもらう。(約２週間)。その後、緊急時の受診態勢を整えた後（福井大学附属病院小児科、県立福井病院小児科等)、自宅（福井市）において自己注射を行う。外来受診の頻度は、半年間 4 週間に 1 回、その後も 1 か月に 1 度とする。

ニ） 高 IgD 症候群に対するアナキンラ治療についての報告

本患者におけるアナキンラの治療経験は、大変貴重な医療資産であり、患者個人情報を匿名化したのち、学会・論文等で報告する可能性が存在する。

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高 IgD 症候群におけるアナキンラ投与による治療の説明

本書は、あなた（　　　　　　　　　）が受ける治療法についてあなたにその内容等を説明するものです。

あなたは、この治療法に含まれる利益と危険について十分に理解された上で、これを受けるかどうか決めてください。これは、インフォームドコンセントと呼ばれる手続きです。本書には、あなたがわかりやすく説明するために、この治療法に関する詳しい情報が記載されています。もしわかりにくい点があれば、どうぞご遠慮なく担当医にお尋ねください。この治療について十分ご理解の上、これを受ける意思があるならば、添付してある依頼書に署名捺印をしてください。

1.　アナキンラによる治療について

関節炎、新生児期からの発熱、持続する炎症反応等の症状より高 IgD 症候群が疑われ、尿中のメバロン酸を測定すると著明高値であり、また高 IgD 症候群の責任遺伝子 MVK の遺伝子解析を行ったところこれまで報告のある遺伝子異常が見つかり、高ＩｇＤ症候群であることが判明しました。また同遺伝子の酵素活性を測定したところ、低下しており同診断が間違いないことを確認しております。
近年、高 IgD 症候群の病態として炎症引き起こす IL-1β が過剰産生されることが報告されています。そしてその IL-1β を中和する生理的なタンパク質 IL-1RA（アナキンラ、商標名キネレット）を投与しその臨床症状及び検査所見の改善（発熱、発疹、炎症反応等）が見られたことが報告されています。現在の患者さんの状態は、プレドニゾロン 10 mg 連日内服等により治療しておりますが、炎症もしくは副腎皮質ホルモン投与による著明な発育障害、関節炎、関節可動域の減少、検査所見上炎症所見の高値が持続しております。今回発育障害及び関節破壊への進行及び慢性炎症によるアミロイドーシスに対する治療目的で、近年高 IgD 症候群に対して効果が報告されているアナキンラの投与を行いたいとかんがえております。

2. 方法

日本では未承認で販売されていないアナキンラ（商標名キネレット、製造元 SOBI）を入手するため、RHC USA Corporation 日本支社に輸入代行を依頼、輸入致します。患者さんの体重あたり必要な薬剤量にするため、１アンプル 100 mg から、１週間分に相当する 14 mg/kg(約 240 mg, 1.6 ml)へ、当院薬剤部にて無菌的にガラスバイアルに分注します。さらにガラスバイアルからインシュリンシリンジを用いて、 2 mg/kg（約 34 mg, 0.23 ml)を採取して皮下注射致します。

そして現在投与中の内服薬はそのまま減量することなく、アナキンラを投与し始めます。効果の判定基準として CBC, CRP, 血沈、SAA を 1-4 週に１回以上検査し、炎症反応の低下を検討するとともに、臨床症状として関節の腫脹、疼痛の変化を関節点数として記録致します。中枢神経系の評価として、治療前後で頭部 MRI, 発達テスト、脳波の検討を行います。
効果判定および感染症等の副作用を調べるため、はじめの３週間は入院治療とし、その後半年は２－４週間に１回、その後は１か月に１度の外来受診とします。また連日皮下注射を行うため、入院中に

自己注射の仕方を習得して頂きます。なお、効果判定および副作用をモニターしながら、効果が不十分であれば、アナキンラを 10mg/kg（170mg, 1.13ml）まで増量する可能性があります。

３．毒性、副作用

アナキンラの治療をこれまでの内服薬と同時投与する形で開始する予定です。患者さんの場合は、免疫抑制剤としてプレドニンをすでに内服中であり、アナキンラの投与によりさらに免疫抑制がかかる危険性があります。具体的には、各種感染症に罹患する危険性が増加致します。そのため、対応策として治療開始 3 週間は入院治療として、厳重な監視下（医師の診察、血液検査等）に感染症の有無に最大限の注意を払いながら治療を行い、効果が見られればプレドニンを減量する予定です。特に合併症として間質性肺炎がこれまで見られており、理学所見、SpO2、胸部 CT 等により厳重に増悪がないか検討します。他のアナキンラ投与の合併症としては、注射部位反応、感染症、好中球減少症があり、その他頻度は多くないものの頭痛、吐き気、下痢が報告されています。上記合併症の発現を臨床症状の注意深い観察、血液検査（血算、血液生化学、CRP 等）の定期的な施行（入院中は１週間に１度以上、退院後半年は 2-4 週間に１度、その後は１か月に１度）により早期発見につとめ、発現時は投与を中止し、直ちに副作用に応じた治療を開始致します。なおその健康被害が生じた場合の補償は一般診療での対処に準じて行うこととします。

4. 費用について

アナキンラは現在のところ、日本では高 IgD 症候群の治療薬として国内では認められていません。このため、この薬剤にかかる費用については保険で賄うことができません。費用は 1 日分 2 mg/kg 約 34 mg が 1400 円程度であり、1 日 34 mg 使用するとして月額約 42000 円、またアナキンラを分注するバイアル代として月額約 2400 円が必要となります。病状や効果によっては、1 日 10 mg/kg 約 170 mg に増量する可能性もありますが、この費用をどこで負担するか、という問題があります。先日の話し合いの結果より、この費用は患者さん及びそのご家族にご負担頂くことでご理解下さい。

5. その他

もしあなたが、本治療を望まれない場合でも、あなたは今までと同様に、継続して京都大学医学部附属病院で治療を受けることができます。また、もし本治療を受けることに承諾された後でも、いつでもその承諾の意思を変更することは可能で、変更後も今までどおりの治療は続けていきますのであなたの不利益になることはありません。

また高 IgD 症候群におけるアナキンラ治療に得られた情報は特にその副作用・有効性は大変貴重であり、患者さんの個人情報がわからないようにして、学会・論文等で報告する可能性があります。

アナキンラ治療同意書

京都大学医学部附属病院長殿
患者氏名：＿＿＿＿＿＿＿＿＿＿＿＿

私はアナキンラ治療について別紙の説明を読み、また口頭で十分な説明を受け、その利益と危険性についても十分理解しました。また、これらについて十分に質問をする機会もありました。私は、この治療に対する同意と依頼をいつでも撤回でき、その場合でも、貴院において通常受けることのできる治療が制限されないことを理解しています。

私は、貴院において行われる上記の治療に同意し、その実施を貴院に依頼します。

平成　　年　　月　　日

本人住所＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

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平成　　年　　月　　日

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研究成果の刊行に関する一覧表

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The CD40–CD40L axis and IFN-γ play critical roles in Langhans giant cell formation

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Abstract

The presence of Langhans giant cells (LGCs) is one of the signatures of systemic granulomatous disorders such as tuberculosis and sarcoidosis. However, the pathophysiological mechanism leading to LGC formation, especially the contribution of the T cells abundantly found in granulomas, has not been fully elucidated. To examine the role of T cells in LGC formation, a new *in vitro* method for the induction of LGCs was developed by co-culturing human monocytes with autologous T cells in the presence of concanavalin A (ConA). This system required close contact between monocytes and T cells, and CD4+ T cells were more potent than CD8+ T cells in inducing LGC formation. Antibody inhibition revealed that a CD40–CD40 ligand (CD40L) interaction and IFN-γ were essential for LGC formation, and the combination of exogenous soluble CD40L (sCD40L) and IFN-γ efficiently replaced the role of T cells. Dendritic cell-specific transmembrane protein (DC-STAMP), a known fusion-related molecule in monocytes, was up-regulated during LGC formation. Moreover, knock-down of DC-STAMP by siRNA inhibited LGC formation, revealing that DC-STAMP was directly involved in LGC formation. Taken together, these results demonstrate that T cells played a pivotal role in a new *in vitro* LGC formation system, in which DC-STAMP was involved, and occurred via a molecular mechanism that involved CD40–CD40L interaction and IFN-γ secretion.

Keywords: concanavalin A, granuloma, multinucleated giant cell

Introduction

Granuloma formation is a specialized inflammatory response observed in infections by certain pathogens such as *Mycobacterium* and *Cryptococcus* (1, 2). Granulomas are also found in immunodeficient conditions such as chronic granulomatous disease (CGD) (3). In addition, this response is a pathological hallmark of non-infectious idiopathic inflammatory disorders including systemic sarcoidosis and Blau syndrome/early-onset sarcoidosis (BS/EOS) (4–7). Granulomas consist of various cell types including macrophages, T cells, plasma cells and epithelioid cells, but the presence of multinucleated giant cells (MGCs) is the cardinal feature of granulomatous inflammation (1, 4). Granuloma formation is generally considered a host defense mechanism against persistent irritants or chronic infection and occurs as a consequence of the failure of the host to eliminate invading pathogens. When the irritants are large and indigestible, MGCs are formed by the fusion of monocyte–macrophage lineage cells (1, 4, 8). However, the pathophysiological mechanism of MGC development and associated granuloma formation is not well understood.

MGCs are morphologically classified into Langhans giant cells (LGCs), which show a circular peripheral arrangement of nuclei, and foreign body giant cells (FGCs), which show irregular scattering of nuclei (9). LGCs are characteristic of systemic granulomatous disorders such as tuberculosis, sarcoidosis and BS/EOS, whereas FGCs are observed as a consequence of a reaction against foreign bodies such as an implant. To explore the mechanisms of MGC formation, particularly LGC formation in systemic granulomatous disease, several *in vitro* systems of human monocyte culture have been developed. These involve the use of stimuli such as phorbol myristate acetate (10–12) and lectins such as concanavalin A (ConA) to induce LGCs (13, 14). However, a considerable degree of concurrent FGC formation is observed in systems that employ ConA.

Cytokines are also used to induce MGCs. For example, IFN-γ (15–19), IL-3 (15, 18, 19) and granulocyte macrophage colony-stimulating factor (15) are frequently used to induce LGCs, while IL-4 is known to induce FGCs (15, 20–23). Notably, IFN-γ has been regarded as a crucial factor

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for LGC formation because LGC formation is inhibited by antibodies against IFN-γ (24). However, the roles of other factors and cell types in LGC development have not been elucidated.

T cells are one of the main components of granulomas. Therefore, the present study focused on T cells and investigated their role in the development of LGCs. A novel system was developed in which LGC-dominant MGCs could be generated from freshly isolated monocytes co-cultured with autologous T cells in the presence of ConA, and this system was used to evaluate the role of the CD40–CD40 ligand (CD40L) interaction and IFN-γ in the formation of LGCs. Moreover, the hypothesis that the stimulation of monocytes with exogenous soluble CD40L (sCD40L), recombinant human IFN-γ (rhIFN-γ) and ConA would be sufficient to induce monocyte fusion into LGCs in the absence of T cells was investigated. The findings of this study revealed a pivotal role for T cells in LGC formation and suggest a new pathophysiological mechanism contributing to LGC formation in granulomatous disorders.

Methods

Reagents and antibodies

Blocking antibodies against human CD40 (clone 82102; antagonistic antibody), CD40L, IFN-γ, IL-12 and isotype control IgG and rhIL-12 were purchased from R&D systems (Minneapolis, MN, USA). ConA, LPS and PMA were purchased from Sigma Chemical Co. (St Louis, MO, USA). sCD40L was kindly provided by Dr Ashish Jain (NIAID/NIH, Bethesda, MD, USA) and also purchased from Adipogen (Incheon, Korea). rhIFN-γ was purchased from PeproTech (Rocky Hill, NJ, USA). Inhibitory reagents JSH-23, BMS-345541, U0126, JNK-inhibitor II and SB-203580 were purchased from Calbiochem (San Diego, CA, USA).

Differentiation of MGCs from peripheral CD14+ monocytes

Blood was collected from healthy volunteer donors after informed consent was obtained in accordance with the guidelines of the Institutional Review Board of Kyoto University. PBMCs were isolated from whole blood using the Lymphoprep system (Axis-Shield PoC, Oslo, Norway). PBMCs were incubated with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD14+ cells were isolated using an AutoMACS benchtop magnetic cell sorter according to the manufacturer's protocol (Miltenyi Biotec). Similarly, CD3+, CD4+ and CD8+ cells were collected in a similar fashion, using CD3, CD4 and CD8 beads (Miltenyi Biotec), respectively. The purity of the isolated cells was >95%, as demonstrated by flow cytometry using a FACS Calibur system (BD Biosciences, Franklin Lakes, NJ, USA). In some experiments, CD3+ T cells were first depleted from PBMCs, and CD14+ monocytes were isolated from the negative fraction to obtain highly purified monocytes. (Contaminating T cells comprised <0.5% of the total cell population.)

The isolated CD14+ monocytes were re-suspended at a density of 100 000 cells per well in 48-well plates in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were then cultured for 72 h with various supplemental reagents as described in Results. At the end of the culture period, cells were treated with Giemsa stain to detect nuclei. MGCs were defined as cells with more than three nuclei per cell, according to definitions established by previous studies (9–24).

The stained plates were examined under an Olympus IX70 microscope (Olympus, Tokyo, Japan) using a ×20 or ×10 objective lens with a ×10 eyepiece, and the number of nuclei and MGCs in the representative area was counted. The fusion index was calculated according to the following formula: fusion index = (number of nuclei within MGCs)/(total number of nuclei counted). More than 300 nuclei were counted for each experiment. Images were acquired with an Axio Cam camera (Carl Zeiss, Germany).

Enzyme-linked immunosorbent assay

The concentration of IFN-γ in the culture supernatants was measured by enzyme immunoassay using an OptEIA human IFN-γ ELISA set (BD Biosciences) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical staining was performed using an indirect immunofluorescent technique. Briefly, after fixation with 4% paraformaldehyde, the cells were incubated with a mouse antibody directed against-human CD3 (BD Biosciences), followed by incubation with an Alexa488-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). Cells were then stained with DAPI (Sigma) and visualized with an Olympus laser microscope IX70. Merged reconstruction images were created using Axio Vision SP4 software (Carl Zeiss).

Reverse transciption–PCR

RNA was extracted directly from culture plates using the RNeasy mini kit (Qiagen, Hilden, Germany). After DNase treatment, cDNA was obtained by reverse transcription using the Omniscript RT system (Qiagen). Reverse transcription (RT)–PCR was performed using TaKaRa LATaq (Takara, Shiga, Japan) with the following primer pairs: 5′-GATTGGGTCAGCACTTTTTG-3′ and 5′-CCTCCCAAGTGAATGGATTG-3′ for CD40L; 5′-GGGAGATTTACGTGTCTCCAAG-3′ and 5′-GGGAGGACAACACCTCTGTG-3′ for dendritic cell-specific transmembrane protein (DC-STAMP); 5′-GCCAGCTGGGAGAGAAGG-3′ and 5′-CCTGGTGGGACTTCTCCTG-3′ for triggering receptor expressed on myeloid cells 2 (TREM-2); 5′-TGGAACTCTGCCTCAGGATG-3′ and 5′-ATCACAGCCACTCCAAAAGG-3′ for dedicator of cytokinesis 180 (DOCK180); 5′-GGCAGAGAACCAGGTGAATG-3′ and 5′-GGCATACTCCGTGTGGTTG-3′ for signal regulatory protein (SIRP) α; 5′-CGCAGATCGATTTGAATATAACC-3′ and 5′-GGATTCTGTCTGTGCTGTCG-3′ for CD44; 5′-CAAGAGCATCTTCGAGCAAG-3′ and 5′-TCCTGCTCAGGGATGTAAGC-3′ for CD9; 5′-ATCTGGAGCTGGGAGACAAG-3′ and 5′-CCTCAGTACACGGAGCTGTTC-3′ for CD81; 5′-GAGACCGGTGAGCTGGATAG-3′ and 5′-GCCGTCCTGGGTGTAGAGTC-3′ for matrix metallopeptidase 9 (MMP-9) and 5′-AGGTGAAGGTCGGAGTCAAC-3′ and 5′-ACCTGGTGCTCAGTGTAGCC-3′ for glyceraldehyde 3-phosphate dehydrogenase.

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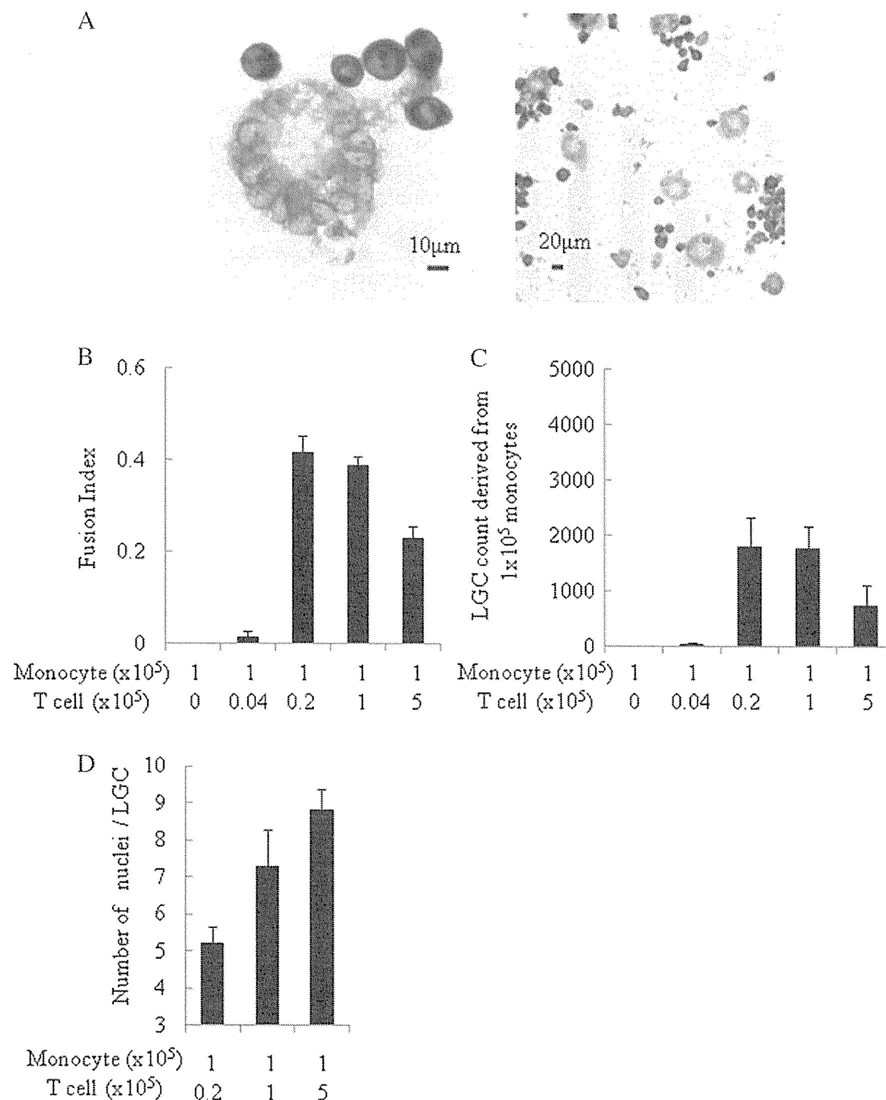

Fig. 1. Co-culture of human monocytes with autologous T cells induces LGC formation. (A) Photomicrographs of Giemsa-stained LGCs induced by co-culture of monocytes and autologous T cells stimulated with ConA. The images show typical LGCs with a circular peripheral arrangement of nuclei. (B–D) T cell-induced LGC formation depends on the ratio of T cells to monocytes. 'Monocyte' refers to the number of monocytes × 10^5 and 'T cell' refers to the number of T cells × 10^5. Values represent (B) the mean fusion index calculated without distinguishing between monocytes and T cells, (C) the mean total number of LGCs derived from 1 × 10^5 monocytes and (D) the mean number of nuclei per LGC. Error bars indicate the standard mean of the error of the indicated ratio of monocytes to T cells stimulated with ConA (5 μg ml^{-1}) (n = 3 independent co-cultures).

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siRNA transfection

Pre-designed siRNAs against DC-STAMP or control siRNAs (Applied Biosystems, Carlsbad, CA, USA) were transfected into newly isolated CD14+ cells using Lipofectamine RNAi-MAX (Invitrogen) according to the manufacturers' instructions. Additional transfections were performed after 12 and 24 h of culture.

Western blotting

Antibodies against phospho-(p)-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p38, p-p65 and p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). Isolated CD14+ cells were incubated without stimulation for 2 h, followed by stimulation with ConA, rhIFN-γ, sCD40L and LPS for 1 h. The harvested cells were lysed with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM orthovanadate) containing a protease inhibitor cocktail (Nacalai, Kyoto, Japan). Equal amounts of total protein were resolved on SDS–polyacrylamide gels and transferred onto Immobilon PVDF membranes (Millipore, Billerica, MA, USA). The membranes were treated with blocking buffer (10 mM Tris–HCl, 150 mM NaCl, 0.1% tween-20, 0.05% NaN3 and 1% FBS) and then incubated overnight with primary antibody. The membranes were then incubated for 1 h with an HRP-conjugated goat anti-rabbit secondary IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed with ECL Plus Western blotting detection kit (GE Healthcare, Uppsala, Sweden).

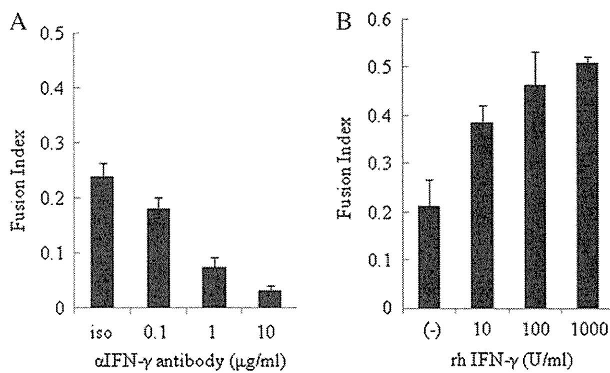

Fig. 2. IFN-γ is required for LGC formation in the co-culture of human monocytes and autologous T cells. (A) An antibody against IFN-γ abrogated LGC formation by the co-culture of monocytes and autologous T cells stimulated with ConA (2.5 μg ml^{-1}). The ratio of monocytes to T cells was 1:1. 'Iso' refers to the isotype control. (B) Addition of rhIFN-γ to the co-culture of monocytes and autologous T cells stimulated with ConA enhanced LGC formation. Values represent the mean fusion index, and error bars indicate the standard mean of the error (n = 3 independent co-cultures).

Statistical analysis

Statistical analysis was performed by applying the Student's *t*-test, and a *P* value of <0.05 was considered to be significant.

Results

Co-culturing human monocytes with autologous T cells induces LGC formation

LGCs in granulomas are a specific indication of systemic granulomatous disorders. The observation that T cells are one of the main constituents of granulomas led to the hypothesis that T cells may play a key role in LGC formation. To understand the pathophysiology underlying LGC formation, a new *in vitro* LGC formation system was developed using human monocytes and T cells. When freshly isolated peripheral blood monocytes were co-cultured with autologous T cells under ConA stimulation, LGCs formed within 3 days (Fig. 1A, left), while very few FGCs were detected (Fig. 1A, right). LGC formation, as measured by the fusion index, as well as the number of LGCs per monocyte and the number of nuclei per LGC, increased along with the number of co-cultured T cells, peaking at a monocyte:T cell ratio of 1:1 (Fig. 1B–D).

IFN-γ is required for LGC formation in the co-culture of human monocytes and autologous T cells

The cytokine IFN-γ, which is reported to be critical in LGC formation (15–19, 24), was detected in the supernatants of the ConA-stimulated co-cultures (data not shown). The addition of a neutralizing antibody against IFN-γ to the co-culture system resulted in a dose-dependent inhibition of LGC formation (Fig. 2A). Furthermore, exogenous addition of rhIFN-γ accelerated LGC formation in a dose-dependent manner (Fig. 2B). These results show that IFN-γ is important in LGC formation in this co-culture system of human monocytes and autologous T cells.

Close contact between monocytes and T cells is required for LGC formation

ConA-stimulated T cells secrete several cytokines and up-regulate the expression of surface molecules that can stimulate nearby cells. Notably, differentiated LGCs were surrounded by CD3+ T cells (Fig. 3A), suggesting that direct contact between monocytes and T cells also contributed to LGC formation. The separation of T cells and monocytes by a semipermeable membrane reduced LGC formation (Fig. 3B).

We next co-cultured monocytes with either CD4+ or CD8+ T cells and assessed whether these two T cell subtypes differed in their ability to induce LGC formation. At every T cell-to-monocyte ratio tested, CD4+ T cells induced LGCs more efficiently than an equivalent number of CD8+ T cells (Fig. 3C). Since IFN-γ was required for LGC formation, the IFN-γ concentration was measured in the supernatants of the various culture conditions. The co-cultures with CD4+ T cells contained more IFN-γ than cultures with CD8+ T cells (Fig. 3D). However, the exogenous addition of rhIFN-γ to co-cultures with CD8+ T cells did not enhance LGC formation to the levels observed with CD4+ T cells (Fig. 3E). These results showed that factors other than IFN-γ, potentially surface molecules preferentially expressed on CD4+ T cells, are required for the efficient formation of LGCs in this system.

The CD40–CD40L axis is necessary for LGC formation

A literature search for differentially expressed surface molecules between CD4+ and CD8+ T cells in the expression profiles of activated human T cells (25) identified tumor necrosis factor super family 5 (TNFSF5 or CD40L) as a candidate molecule affecting LGC formation. The preferential expression of CD40L by CD4+ cells was confirmed in our co-culture system (Fig. 4A). To evaluate whether CD40L was indeed important in LGC formation, antibodies that disrupt the CD40–CD40L interaction were added to the co-culture. Blocking antibodies against CD40 and CD40L inhibited LGC formation in a dose-dependent manner (Fig. 4B). Furthermore, exogenous addition of sCD40L to the co-culture system enhanced LGC formation in a dose-dependent manner (Fig. 4C).

The importance of IFN-γ and CD40-CD40L axis in LGC formation suggested that IL-12 might contribute to LGC formation in this co-culture system since IL-12 is produced by monocytes in response to CD40 ligation and causes T cells to produce IFN-γ (26). The blocking antibody against IL-12 failed to inhibit LGC formation (Fig. 4D), although the exogenous addition of rhIL-12 to the co-culture system enhanced LGC formation (Fig. 4E). CD40 ligation also enhanced LGC formation in the presence of anti-IL-12 antibodies (Fig. 4D). Interruption of the CD40–CD40L interaction as well as the neutralization of IFN-γ abrogated LGC formation even in the presence of exogenous IL-12 (Fig. 4E). These data together indicate that the mechanisms through which CD40/CD40L and IFN-γ enhance LGC formation under the co-culture conditions employed in this study are mostly IL-12 independent, although IL-12 may enhance the LGC formation process when it is present in a high concentration.

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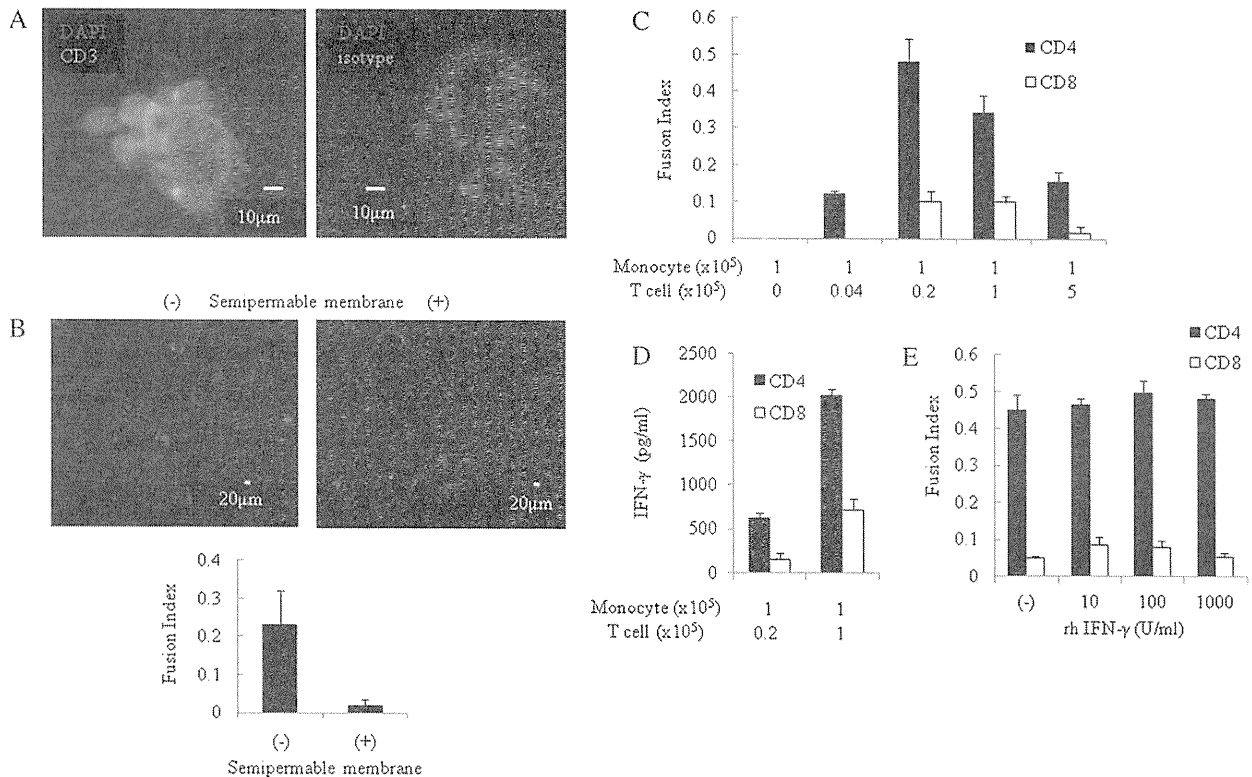

Fig. 3. Direct contact between monocytes and T cells is required for LGC formation. (A) Immunohistochemical staining of LGCs. Co-cultured cells were stained with an antibody against CD3 (green), and nuclei were stained with DAPI (blue). (B) A semipermeable membrane between the monocytes and T cells (1:1 ratio) stimulated with ConA (2.5 μg ml^{-1}) abrogated LGC formation. Nuclei were distributed in a circular pattern in the absence of the semipermeable membrane (upper left), whereas this distribution was disturbed in the presence of the membrane (upper right). The mean fusion index of the cultures are shown, and error bars indicate the standard mean of the error (n = 3 independent co-cultures) (bottom). (C) CD4+ T cells induced LGCs more efficiently than CD8+ T cells. 'Monocyte' refers to the number of monocytes $\times 10^5$, and 'T cell' refers to the number of T cells $\times 10^5$. Values represent the mean fusion index calculated without distinguishing between monocytes and T cells, and error bars indicate the standard mean of the error of the indicated ratio of monocytes to T cells stimulated with ConA (5 μg ml^{-1}) (n = 3 independent co-cultures). (D) CD4+ T cells produced more IFN-γ than CD8+ T cells. The data show the results of triplicate ELISAs for IFN-γ concentration in the supernatants of the experiment shown in Fig. 3(C). Error bars indicate the standard mean of the error of three independent cultures. (E) Addition of rhIFN-γ to the co-culture of monocytes with CD8+ T cells did not enhance LGC formation to the degree observed with CD4+ T cells. The culture condition was the same as Fig. 3(C and D), and the ratio of monocytes to T cells was 1:0.2. Values represent the mean fusion index and error bars indicate the standard mean of the error (n = 3 independent co-cultures).

T cells induce LGC formation by providing IFN-γ and CD40 stimulation

To more specifically evaluate the role of T cells in inducing LGCs, monocytes were cultured with either rhIFN-γ or sCD40L in the presence of ConA without T cells. The addition of rhIFN-γ alone did not induce LGC formation from ConA-stimulated monocytes (Fig. 5A), suggesting that T-cell factors besides or in addition to IFN-γ were required to induce the formation of LGCs. Stimulation with exogenous sCD40L did induce monocytes to differentiate into LGCs (Fig. 5B), indicating the necessity for an interaction between T cell-derived CD40L and monocyte-expressed CD40. Despite the fact that rhIFN-γ alone had no effect on LGC formation from ConA-stimulated monocytes in the absence of T cells, IFN-γ was required for LGC formation when monocytes were co-cultured with autologous T cells under ConA stimulation (Fig. 2A). Furthermore, a blocking antibody against IFN-γ abrogated LGC formation by monocytes cultured with sCD40L and ConA (Fig. 5C), indicating that the LGC formation induced by stimulating cultured monocytes with exogenous sCD40L and ConA required IFN-γ.

Because monocytes do not produce large amount of IFN-γ, it was possible that contaminating T cells were the source of IFN-γ when monocytes were stimulated with exogenous sCD40L and ConA. Indeed, when highly purified CD14+ cells were stimulated with exogenous sCD40L and ConA, significantly less LGC formation was observed than in cultures using crude monocytes (Fig. 5D). In addition, supplementing the culture medium of highly purified monocytes with rhIFN-γ as well as exogenous sCD40L and ConA enhanced LGC formation to the same extent that was observed when crude monocytes were used (Fig. 5D). These results indicate that IFN-γ produced from T cells is required for the efficient induction of LGCs.

The next hypothesis to be evaluated was that the stimulation of highly purified monocytes with rhIFN-γ and sCD40L would be sufficient to induce LGC formation. However, this was not the case; ConA was also required for LGC formation

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