C．結果

58 歳の男性の肺を CT 撮影し，本アルゴリズムを適用した．得られた細線化モデル及び世代分けを行った気道モデルをそれぞれ Fig. 10，Fig. 11 に示す．CT 画像内の目視で判断できる全ての気道を抽出することはできなかったが，Fig. 11 より本アルゴリズムによって肺の解剖学的形態が気管

Table 1　世代別の気道長さ，体積，直径

世代	平均長さ (mm)(±SD)	平均体積 (cm^3)(±SD)	平均直径 (mm)(±SD)
0	131.0(±0.0)	20.305(±0.00)	18.0(±0.0)
1	70.6(±41.4)	3.971(±1.653)	12.1(±1.2)
2	42.3(±17.9)	1.420(±1.046)	8.9(±1.4)
3	23.3(±8.4)	0.390(±0.210)	6.7(±0.9)
4	18.0(±11.7)	0.164(±0.156)	4.9(±1.2)
5	23.5(±12.6)	0.126(±0.088)	3.7(±1.0)
6	21.2(±11.2)	0.074(±0.056)	2.8(±0.9)
7	25.0(±15.8)	0.069(±0.057)	2.3(±0.8)
8	22.9(±14.0)	0.058(±0.053)	2.3(±0.8)
9	23.9(±13.7)	0.055(±0.047)	2.1(±0.7)
10	20.5(±15.5)	0.046(±0.043)	1.9(±0.8)
11	22.7(±14.7)	0.042(±0.032)	1.9(±0.6)
12	23.7(±12.1)	0.052(±0.047)	2.2(±0.7)
13	22.6(±13.1)	0.043(±0.036)	2.1(±0.5)
14	33.8(±12.8)	0.040(±0.017)	1.7(±0.1)

から第 14 世代の気道まで全体的に抽出できていることがわかる．

世代別に気道の直径を平均した値(mean±SD)を Table. 1 に示す．気道長さは第 0 世代の 131.0mm を最大値として世代の増加とともに減少し，第 10 世代の 20.5mm が最小値となっている．また第 13 世代から第 14 世代への増加が目立ち第 14 世代の気道長さは，第 2 世代の長さとほぼ同じであった．気道体積は第 0 世代が最大で 20.305cm^3 であり，世代の増加とともに減少し，第 14 世代で最小となった．気道直径は第 0 世代で最大の 18.0mm であり世代の増加とともに減少して，第 14 世代で 1.7mm と最小値となった．

この被験者の形態計測により得られた気道直径と Weibel ら [1]の研究によって解剖学的に測定された気道直径を世代ごとに平均し，それぞれ第 0 世代の直径を基準に正規化した結果を Table1 に示す．第 7 世代までの平均気道直径は Weibel ら [1]の結果とほぼ一致したが，第 8 世代以降の気道直径に関しては常に大きい値となった．

D．考察

本研究で使用した CT の空間解像度が 0.5mm であることから，直径 0.5mm 以下の気道を解像することは困難である．このことが第 8 世代以降の直径が Weibel ら [1]との結果よりも常に大きくな

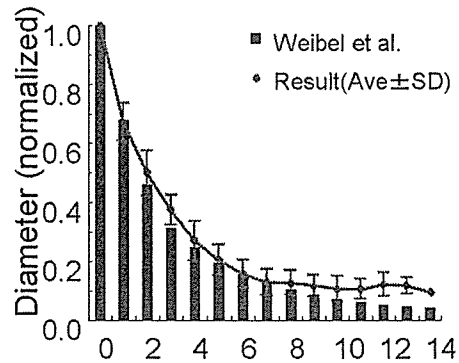

Fig. 12　計測結果と Weibel[1]との比較

ったことの原因であると考えられる．しかしながら，逆に言えば，0. 5mm 以上の直径値を有する気道に関しては本アルゴリズムによって，CT 画像の限界値までは気道追跡が可能であることが示された．

E．結論

本研究により，マルチスライス CT 画像から気道を連続的かつ自動的に評価する方法を確立できた．今後は，数値流体力学解析を用いることで抽出した肺気道モデル内の気流状態について検討し，肺気道形状と呼吸機能，更には喘鳴の発生との関係について分析を進める予定である．

参考文献

1) Weibel et al., (1963) Morphometry of the Human Lung,Academic Press Inc., New York, p.139

F．研究発表

1．論文発表

なし

2．学会発表

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G．知的財産権の出願・登録状況

1．特許取得

なし。

2．実用新案登録

なし。

3．その他

なし

アレルギー性気道炎症の成立機序及び制御機構の解明

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研究要旨

喘息患者の気道浸潤 T 細胞では、非喘息患者の T 細胞より Th1 細胞の master regulator である T-bet の発現が低下している。また、C57BL/6 背景の T-bet 欠損マウスでは、ヒト喘息と類似した好酸球性気道炎症が自然発症することも示されている。これらの研究結果は、T-bet が喘息発症の抑制機構に重要な役割を果たしていることを示唆している。そこで本研究では、喘息様病変を自然発症しない BALB/c 背景の T-bet 欠損マウスを用いて、抗原特異的なアレルギー性気道炎症における T-bet の役割を検討した。その結果、T-bet 欠損マウスでは、抗原誘発好酸球性気道炎症及び好中球性気道炎症が野生型マウスに比して増強していた。T-bet 欠損マウスにおける抗原誘発好酸球性気道炎症の増強には Th2 細胞の分化亢進が、一方、好中球性気道炎症の増強には Th17 細胞の分化亢進が関与していた。以上の研究結果は、T-bet は、Th2 細胞及び Th17 細胞の分化を抑制することにより、好酸球性及び好中球性の気道炎症の発症を抑制していることを示唆している。好中球性気道炎症は、重症喘息の病態に重要であり、本研究成果を発展させることにより、重症喘息の治療戦略に貢献できると考えられる。

A．研究目的

近年、Th1 細胞の master regulator である T-bet の遺伝子欠損マウスでは、ヒト喘息と類似した好酸球性気道炎症が自然発症することが報告された。しかし、興味深いことに T-bet 欠損マウスと同様に Th1 細胞分化に障害がある Stat4 欠損マウスでは好酸球性気道炎症が自然発症しない。これらの研究結果は、T-bet は Th1 細胞分化誘導以外の機構を介して好酸球性気道炎症の発症を抑制していることを示唆している。そこで本研究では、T-bet によるアレルギー性気道炎症の制御機構を解明し、喘息に対する新規治療法開発の基盤を構築することを目的とした。

B．方法

1）抗原特異的アレルギー性気道炎症における T-bet の役割の解明

喘息様病変を自然発症しない BALB/c 背景の T-bet 欠損マウス及び野生型マウスを卵白アルブミン(OVA)にて腹腔内感作した。感作マウスに OVA を吸入投与し、肺胞洗浄液(BALF)中への炎症細胞浸潤及びサイトカイン産生を評価した。

2）抗原特異的ヘルパーT 細胞分化における T-bet の役割の解明

OVA 特異的 TCR トランスジェニック (DO11.10+)マウス及び DO11.10+ T-bet 欠損マウスの脾細胞を Th0, Th1(IL-12), Th2 (IL-4+ anti-IFN-γ)の各条件下で抗原ペプチドで刺激し、CD4 陽性 T 細胞における IL-4、IFN-γ、IL-17 の産生を細胞内サイトカイン染色法にて評価した。

3）T-bet 依存的アレルギー性気道炎症における Stat6 の役割の解明

野生型マウス、T-bet 欠損マウス、Stat6 欠損マウス、及び T-bet/Stat6 ダブル欠損マウスを BALB/c 背景で作製した。これらのマウスを OVA にて腹腔内感作し、OVA 吸入投与後のアレルギー性気道炎症を評価した。

4）アレルギー性気道炎症における CD4 陽性 T 細胞に発現する T-bet の役割の解明

DO11.10+マウス及び DO11.10+ T-bet 欠損マウスの脾臓から MACS を用いて CD4 陽性 T 細胞を純化し、T-bet/Rag-2 ダブル欠損マウスに細胞移入した後、OVA で腹腔内感作し、OVA 吸入によるアレルギー性気道炎症を評価した。

5）アレルギー性気道炎症における IL-17 の役割の解明

DO11.10+ T-bet 欠損マウスの脾臓から純化した CD4 陽性 T 細胞を T-bet/Rag-2 ダブル欠損

マウスに細胞移入した。マウスをOVAにて腹腔内感作し、抗IL-17抗体、或はコントロール抗体の投与下に、マウスにOVAを吸入投与し、アレルギー性気道炎症を評価した。

C．結果及び考察

1）抗原特異的アレルギー性気道炎症におけるT-betの役割

OVAにて腹腔内感作したBALB/c背景のT-bet欠損マウス及び野生型マウスにOVAを吸入投与しアレルギー性気道炎症を惹起したところ、T-bet欠損マウスでは、BALF中の好酸球と好中球の浸潤が有意に増強していた(図1A)。T-bet欠損マウスでは、BALF中のIL-4, IL-5, IL-13の産生も亢進していた(図1B)。これらの結果より、T-betは、抗原特異的なアレルギー性炎症に対して抑制性に機能していることが示唆された。

2）抗原特異的ヘルパーT細胞分化におけるT-betの役割

DO11.10+マウス及びDO11.10+ T-bet欠損マウスのCD4陽性T細胞を抗原ペプチドで刺激しTh1細胞/Th2細胞分化を検討した。外因性サイトカインのないTh0条件下で抗原刺激すると、T-bet欠損CD4陽性T細胞では、T-bet野生型CD4陽性T細胞に比して、Th1細胞の分化が低下し、一方Th2細胞の分化は亢進していた(図2)。IL-12を加えたTh1条件下では、野生型CD4陽性T細胞では、Th1細胞の著明な増加が認められたが、T-bet欠損CD4陽性T細胞ではTh1細胞の増加は認められなかった(図2)。一方、IL-4と 抗IFN-γ抗体の存在下にTh2細胞の分化を誘導する条件下では、T-bet欠損CD4陽性T細胞でも野生型CD4陽性T細胞でも同程度にTh2細胞の分化が誘導された(図2)。これらの結果より、T-betは、外因性サイトカインのない状況ではTh2サイトカインの産生抑制にも重要な役割を果たしていることが示唆された。

3) T-bet依存的アレルギー性気道炎症におけるStat6の役割

次にT-bet欠損マウスで認められたアレルギー性気道炎症の増強がStat6依存的であるか否かをT-bet/Stat6ダブル欠損マウスを作製し検討した。OVAにて腹腔内感作した野生型マウス、T-bet欠損マウス、Stat6欠損マウス、及びT-bet/Stat6ダブル欠損マウスにOVAを吸入投与しアレルギー性気道炎症を評価した。その結果、T-bet欠損マウスで認められた気道好酸球浸潤の増強は、T-bet/Stat6ダブル欠損マウスでは認められなくなったが、気道好中球浸潤の増強は、T-bet/Stat6ダブル欠損マウスでも認められた(図3)。これらの結果より、T-bet欠損マウスにおける好酸球浸潤の増強と好中球浸潤の増強の誘導機構は、異なることが示唆された。

4) アレルギー性気道炎症におけるCD4陽性T細胞に発現するT-betの役割

T-bet欠損マウスで認められたアレルギー性気道炎症の増強にCD4陽性T細胞におけるT-betの欠損が関与するか否かを検討した。DO11.10+マウス及びDO11.10+ T-bet欠損マウスの脾臓CD4陽性T細胞をT-bet/Rag-2ダブル欠損マウスに細胞移入した後、OVAにて腹腔内感作し、OVA吸入投与後のアレルギー性気道炎症を評価した。その結果、T-bet欠損CD4陽性T細胞を移入したマウスでは、BALF中への好酸球と好中球の浸潤がT-bet野生型CD4陽性T細胞を移入したマウスに比して増強していた(図4A)。また、T-bet欠損CD4陽性T細胞を移入したマウスでは、BALF中のIL-13の産生が増加し、一方IFN-γの産生は低下していた(図4B)。これらの結果より、CD4陽性T細胞に発現するT-betがTh2細胞の活性化を抑制し、Th2細胞依存的な好酸球浸潤を抑制していることが示された。

5) 好中球性気道炎症におけるIL-17の役割

興味深い事にT-bet欠損CD4陽性T細胞を移入したマウスでは、BALF中のIL-17の産生が増加していた(図4B)。そこで次に、T-bet欠損CD4陽性T細胞を移入したマウスで認められた好中球性気道炎症の増強にIL-17が関与しているか否かを検討した。DO11.10+ T-bet欠損CD4陽性T細胞をT-bet/Rag-2ダブル欠損マウスに移入する上記実験系に、抗原吸入前に、抗IL-17抗体を投与し、その効果を検討した。その結果、抗IL-17抗体の投与は、好中球性気道炎症の増強を有意に抑制したが、好酸球性気道炎症は抑制しなかった(図4C)。以上の結果より、T-betはIL-17依存的な好中球性気道炎症を抑制していることが明らかとなった 。

6) Th17細胞分化におけるT-betの役割

抗原特異的なIL-17産生性CD4陽性T細胞(Th17細胞)分化におけるT-betの役割をDO11.10+マウスの実験系を用いて検討した。DO11.10+マウス及びDO11.10+ T-bet欠損マウスのCD4陽性T細胞をTh0条件下及びTh1条件下で抗原ペプチドで刺激しTh17細胞分化を

検討した。その結果、Th0 条件下においても、Th1 条件下においても、T-bet 欠損 CD4 陽性 T 細胞では、Th17 細胞の分化が亢進していた(図 5)。以上の結果より、T-bet は Th17 細胞分化を抑制し、その結果、Th17 細胞依存的な好中球性気道炎症を抑制している事が示唆された。

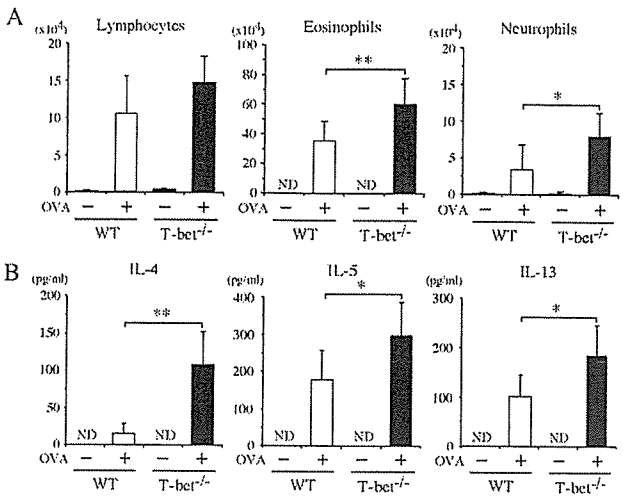

図 1. T-bet 欠損マウスにおける抗原特異的アレルギー性気道炎症の増強
OVA で腹腔内感作した野生型マウス或は T-bet 欠損マウスに OVA を吸入投与し、肺胞洗浄液(BALF)中のリンパ球、好酸球、好中球数(A)、並びに IL-4、IL-5、IL-13 濃度(B)を測定した。*p<0.05、**p<0.01。

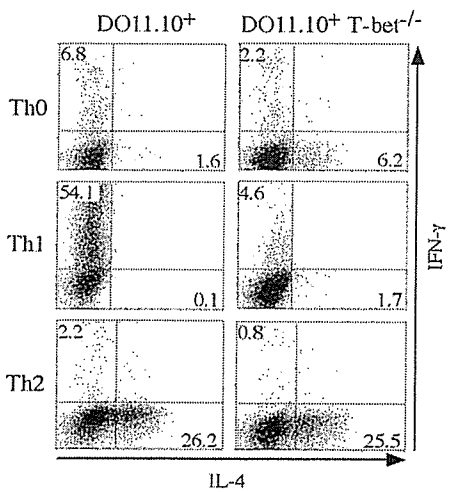

図 2. T-bet 欠損マウスにおける抗原特異的ヘルパー T 細胞分化
DO11.10+マウス、或は DO11.10+ T-bet 欠損マウスの脾細胞を、Th0 条件、Th1 条件、或は Th2 条件下に OVA ペプチドで刺激した。CD4 陽性 T 細胞における IFN-γ と IL-4 の産生を細胞内サイトカイン染色法で評価した。

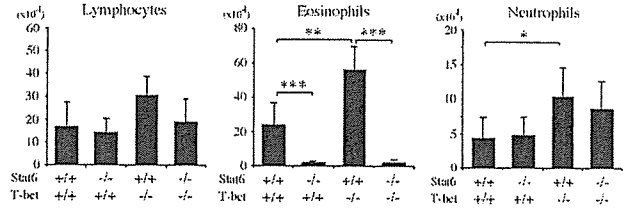

図 3. T-bet 欠損マウスのアレルギー性気道炎症増強における Stat6 の役割
OVA で腹腔内感作した野生型マウス、Stat6 欠損マウス、T-bet 欠損マウス、或は T-bet/Stat6 ダブル欠損マウスに OVA を吸入投与し、BALF 中のリンパ球、好酸球、好中球数を測定した。*p<0.05、**p<0.01、***p<0.001。

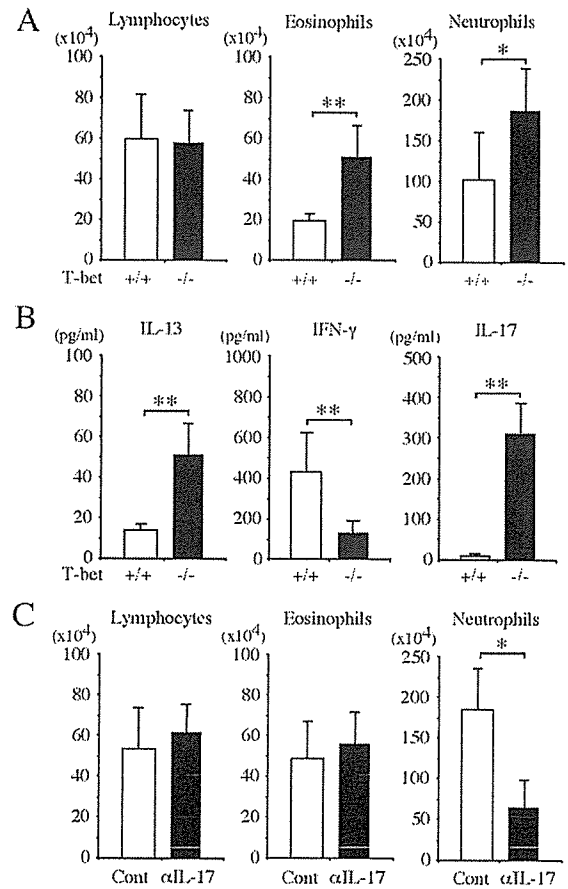

図 4. T-bet による IL-17 依存的好中球性気道炎症の抑制
A-B: DO11.10+マウス、或は DO11.10+ T-bet 欠損マウスの CD4 陽性 T 細胞を T-bet/Rag-2 ダブル欠損マウスに細胞移入した後、OVA で腹腔内感作した。OVA 吸入投与後、BALF 中のリンパ球、好酸球、好中球数(A) 、並びに IL-13、IFN-γ、IL-17 濃度(B)を測定した。*p<0.05、**p<0.005。
C: DO11.10+ T-bet 欠損マウスの CD4 陽性 T 細胞を T-bet/Rag-2 ダブル欠損マウスに細胞移入した後、OVA で腹腔内感作した。抗 IL-17 抗体、或はコントロール抗体の投与後、OVA を吸入投与し、BALF 中のリンパ球、好酸球、好中球数を測定した。*p<0.05。

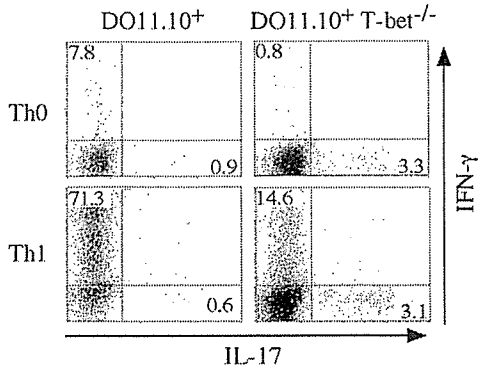

図 5. T-bet による Th17 細胞分化の抑制
DO11.10+マウス、或は DO11.10+ T-bet 欠損マウスの脾細胞を、Th0 条件及び Th1 条件下に OVA ペプチドで刺激した。CD4 陽性 T 細胞における IFN-γ と IL-17 の産生を細胞内サイトカイン染色法で評価した。

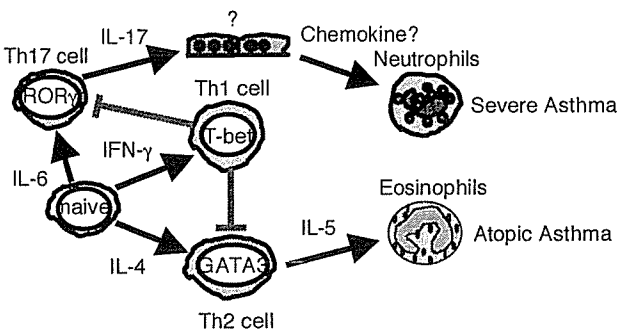

図 6. T-bet によるアレルギー性気道炎症の制御機構
T-bet は、Th2 細胞依存的な好酸球性気道炎症の抑制に加え、Th17 細胞依存的な好中球性気道炎症の抑制にも重要な役割を果たしている。

D. 結論

本研究では、アレルギー性気道炎症における T-bet の役割を検討した。そして、T-bet は、Th2 細胞依存的な好酸球性気道炎症の抑制に加え、Th17 細胞依存的な好中球性気道炎症の抑制にも重要な役割を果たしていることを明らかにした。好中球性気道炎症は、重症喘息の病態として近年注目されており、本研究成果を発展させることにより、重症喘息の治療法開発に結びつくことが期待される。

E. 健康危険情報

なし

F. 研究発表

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G. 知的財産権の出願・登録状況

なし

III. 研究成果の刊行に関する一覧表

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Prostaglandins & other Lipid Mediators 83 (2007) 42–49

PROSTAGLANDINS & other LIPID MEDIATORS

Quantification of leukotriene B4 glucuronide in human urine

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Abstract

We have developed a method for measuring leukotriene B4 glucuronide, a marker of systemic leukotriene B4 biosynthesis, in human urine. This method involves the separation of two positional isomers of leukotriene B4 glucuronide by high-performance liquid chromatography, followed by hydrolysis with β-glucuronidase and then leukotriene B4 quantification by enzyme immunoassay after purification by high-performance liquid chromatography. One of two positional isomers of leukotriene B4 glucuronide was predominantly present in urine. The concentration of the isomer increased in urine from aspirin-intolerant asthma patients after aspirin challenge. Urinary leukotriene E4 and leukotriene B4 glucuronide concentrations in 13 normal healthy adults were 94.6 pg/mg-creatinine (median) and 22.3 pg/mg-creatinine, respectively. Urinary LTE4 concentration increased during the first 3 h after allergen inhalation in atopic patients. However, allergen-induced bronchoconstriction was not associated with an increased concentration of LTB4 glucuronide in urine. The method enabled us to precisely determine urinary leukotriene B4 glucuronide concentration.

Keywords: Leukotriene E4; Leukotriene B4; Bronchial asthma

1. Introduction

Leukotrienes (LT) are a group of bioactive compounds, which play important roles in immediate hypersensitivity reaction and inflammation. The biosynthesis of LTs is initiated by the oxygenation of arachidonic acid to form 5-hydroperoxyeicosatetraenoic acid, which is subsequently converted to LTA4. This unstable epoxide LTA4 is enzymatically metabolized to LTC4, which is converted in turn to LTD4 and then to LTE4. LTB4, the alternative end product of the 5-lipoxygenase pathway, is produced from LTA4 by LTA4 hydrolase. Cysteinyl-LTs (LTC4, LTD4, and LTE4) are potent constrictors of bronchial smooth muscle and are also potent and specific chemoattractants for eosinophils. LTB4 is a potent chemoattractant for neutrophils and is considered to play a role in various inflammatory responses. There have been many studies showing that cysteinyl-LTs have considerable potential to contribute to the pathophysiological features of asthma. Significance of LTB4 in bronchial asthma is still controversial. As determined from the pharmacological properties of an LTB4 antagonist, LTB4 is considered to have no substantial impact on the pathophysiological features of bronchial asthma [1]. On the other hand, a study using LTB4 receptor knockout mice showed that LTB4 has a substantial impact on the development of Th2-type immunoresponses [2]. As mentioned above, the role of LTB4 in the development of the pathophysiological features of bronchial asthma has not been completely clarified.

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Urine has been found to be a useful biological fluid in monitoring the endogenous release of chemical mediators. If metabolic clearance systems including liver function were not altered in the disease state, urinary metabolite concentration provides an easily performed method to monitor whole-body production of the precursor. The major drawback of using urine is that a reliable metabolite of endogenous production of the mediator needs to be identified and urine analysis cannot provide any information on the cellular origin of mediators. The concentration of LTE4 in human urine is considered a good marker of LTC4 production in the human body. LTB4 itself is not a good marker of LTB4 production because of its rapid metabolism, and the metabolite that can be a good marker of LTB4 production has not been identified until very recently. Karin et al. suggested the possibility of using the concentrations of LTB4 glucuronide and 20-carboxy LTB4 in urine as markers of LTB4 whole-body production [3]. Studying the LTB4 production status in the body of asthmatic patients is useful for clarifying the clinical significance of LTB4. In a previous study, we measured LTB4 glucuronide concentration in urine and found that aspirin-intolerant asthmatic patients subjected to an aspirin tolerance test showed an increase in not only LTE4 concentration but also LTB4 glucuronide concentration in urine [4]. Because LTB4 glucuronide concentration was calculated by subtracting LTB4 concentration in urine after hydrolysis by β-glucuronidase from LTB4 concentration in urine before hydrolysis, no information on the concentration of the isomer of LTB4 glucuronide was obtained in that study. Moreover, relatively large amounts of urine samples are required for this measurement method and errors frequently occur in the evaluation of urine samples containing low concentrations of LTB4 glucuronide. The aim of this study is to develop a method of measuring the concentration of LTB4 glucuronide in human urine.

2. Materials and methods

2.1. Preparation of LTB4 glucuronide

Two isomers of LTB4 glucuronides were synthesized by reacting LTB4 with a mixture of UDP-glucuronosyltransferase cofactors containing uridine 5′-diphosphoglucuronic acid (BD Biosciences, Woburn, MA, USA) in the presence of human liver microsomes (BD Biosciences) according to manufacturer's instruction.

2.2. Aspirin challenge test

Three aspirin-intolerant asthma patients (two males and one female, 37–54 years old) received intravenous aspirin challenge. These three patients were in a clinically stable condition and all medications were stopped for at least 12 h prior to the challenge test. At the time of the study, FEV1.0 exceeded 80% of the predicted value. The challenge test was performed as reported previously [4]. Briefly, urine samples were collected at the beginning of the study. After the intravenous injection of 1 ml of saline, if FEV1.0 did not change by more than 10% from the prechallenge baseline, doubled doses of lysine aspirin (12.5, 25, 50, 100, and 200 mg equivalent of aspirin) were intravenously administered. FEV1.0 was recorded every 10 min after the administration and the time interval between administrations of increasing doses was 30 min. The challenge test was stopped when a positive reaction occurred, which was defined as a decrease in FEV1.0 by 20% or more from the baseline. Urine samples were collected for the measurement of LTB4 glucuronide and LTE4 concentrations during the following periods: the hour immediately prior to the provocation test and 0–3, 3–6, 6–9, and 9–24 h after the onset of bronchoconstriction. Urine samples were collected in polypropylene tubes containing 4-hydroxy-TEMPO and stored at −35 °C until analysis. Permission to conduct the study was obtained from the Sagamihara National Hospital Ethics Committee and all the subjects gave their informed consent.

2.3. Allergen inhalation test of atopic asthmatic patients

Bronchial provocations with cat, mite or house dust allergens were performed on seven patients on nine occasions. The patients were five female and two male, 21–73 years of age (mean, 33.9), with a mean forced expiratory volume in 1 s (FEV1.0) of about 90% of the predicted value. The patients inhaled an allergen solution in a Devilbiss nebulizer operated by air at a flow rate of 5 l/min. The nose was clipped and aerosols were inhaled through a mouthpiece during tidal breathing. After the measurement of baseline FEV1.0, patients inhaled saline for 2 min. When a change in FEV1.0 was not observed, allergen inhalation was performed. Allergen solution was inhaled for

2 min and FEV1.0 was measured 10 min after each inhalation. The starting concentration of the allergen extract for inhalation was determined from the threshold concentration on intradermal skin test. Allergen solutions at increasing concentrations were inhaled until a decrease in FEV1.0 of at least 20% was achieved. All asthma medications were withheld for at least 12 h before the provocation test. Urine samples were collected at the same interval as described above.

2.4. Measurement of urinary LTB4 glucuronide concentration

We used an Empore C18 disk cartridge (3 M, St. Paul, MN, USA) for extracting LTB4 glucuronide and LTB4, because the cartridge enabled the extract of these compounds from urine and the chromatographic effluent into a small volume of methanol with sufficient recovery. Siliconized glass tubes, polypropylene tubes and polypropylene pipettes were used throughout the study. After an aliquot of urine (2 ml) was loaded on an Empore C18 disk cartridge, the cartridge was washed with distilled water and LTB4 glucuronide was eluted with 0.5 ml of methanol. When using authentic LTB4 glucuronide, the rate of recovery of both isomers of LTB4 glucuronide from the Empore C18 disk cartridge was estimated to be more than 90%. The methanol extract was concentrated under a nitrogen stream. The residue was purified by high-performance liquid chromatography (HPLC) and the fraction corresponding to the retention times of both isomers of LTB4 glucuronide were collected. HPLC was performed using a NOVA-PAK C18 column (Waters, Milford, MA, USA) with a solvent mixture of methanol–distilled water–acetic acid (65:35:0.1, v/v/v) containing 0.1% EDTA (pH adjusted to 5.4 with ammonium hydroxide) at a flow rate of 1.0 ml/min at 37 °C. The methanol extract could be injected into the HPLC column every 20 min. After the addition of two volumes of distilled water to the effluent, LTB4 glucuronide was extracted using an Empore C18 disk cartridge. To liberate LTB4, LTB4 glucuronide was incubated with β-glucuronidase (200 U, G7646, Sigma, St. Louis, MO, USA) in 1 ml of 0.1 M phosphate buffer (pH 7.0) at 37 °C for 20 h. The solution was purified by HPLC using the same elution buffer as described above. The fraction corresponding to the retention time of authentic LTB4 was collected and LTB4 was extracted with an Empore C18 disk cartridge. LTB4 concentration was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The concentrations were expressed as picogram per milligram of creatinine.

2.5. Cross-reactivity of each isomer of LTB4 glucuronide to the antibody used in the enzyme immunoassay

Cross-reactivity of each isomer to the antibody against LTB4 was determined by incubating with LTB4 tracer and LTB4 or each isomers at increasing concentrations.

2.6. Measurement of urinary LTE4 concentration

LTE4 was quantified using an enzyme immunoassay kit (Cayman Chemical) after purification by HPLC as reported previously [5].

2.7. Normal healthy subjects

The controls (6 male and 7 female, 30–55 years of age) were non-atopic healthy hospital staff members. None of them were taking any medication.

2.8. Analysis of data

Data are expressed as median values and range in bracket unless otherwise specified. Differences between groups were evaluated using the Wilcoxon *t*-test. The Kruskal–Wallis H-test, a non-parametric statistical test, was performed for time-course experiments. When the test showed a significant difference, pairwise comparisons were performed using the Mann–Whitney *U*-test with Bonferroni's correction. A value of $p < 0.05$ was considered statistically significant.

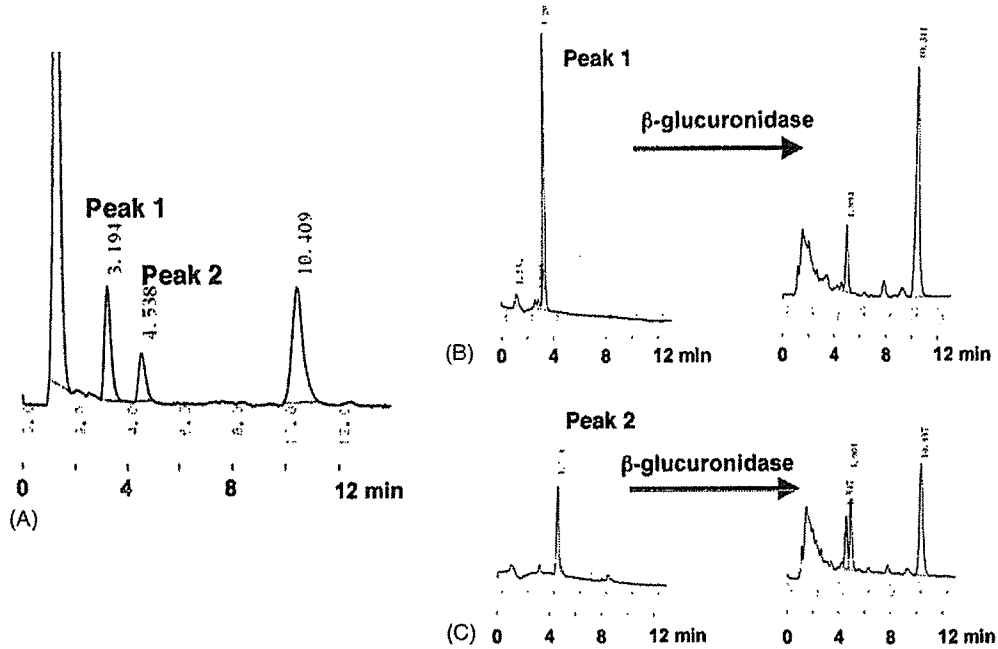

Fig. 1. The reaction mixture of LTB4, uridine 5′-diphosphoglucuronic acid and human liver microsomes was analyzed by HPLC (A). LTB4 was eluted at the retention time of about 10.4 min. LTB4 was generated by the incubation of the fraction corresponding to peak 1 (B) or that to peak 2 (C) with β-glucuronidase.

3. Results

3.1. Preparation of LTB4 glucuronide

After the incubation of LTB4 with uridine 5′-diphosphoglucuronic acid in the presence of human liver microsomes, the reaction mixture was analyzed by HPLC using the same solvent as that for LTB4 purification. Three distinct peaks appeared on the chromatogram (Fig. 1A). The peak of the fraction eluted at the retention time of approximately 10.4 min corresponds to LTB4. When the fractions for the two other peaks, which eluted at shorter retention times (3.2 and 4.5 min), were incubated with β-glucuronidase in 1 ml of 0.1 M phosphate buffer, both fractions generated LTB4, suggesting that these are isomers of LTB4 glucuronide (Fig. 1B and C).

3.2. Time course of hydrolysis of LTB4 glucuronide by β-glucuronidase

To determine the kinetics of the hydrolysis of LTB4 glucuronide, each isomer of LTB4 glucuronide was incubated with β-glucuronidase at 37 °C for various durations. LTB4 glucuronide of peaks 1 and 2 showed a weak cross-reactivity to the antibody against LTB4 in enzyme immunoassay (Fig. 2). The concentration at which each isomer reduced the binding of the LTB4 tracer by 50% was compared with the concentration of LTB4. When assessed on the basis of Fig. 2, the cross-reactivities of the isomers corresponding to peaks 1 and 2 were 0.15% and 1.8%, respectively. Since LTB4 glucuronide might coexist with LTB4 in the solution during a brief incubation, the concentration of LTB4 was measured by enzyme immunoassay after purification by HPLC. Fig. 3 shows that about 20 h was required for the completion of the hydrolysis of both isomers of LTB4 glucuronide.

3.3. Concentration of each isomer of LTB4 glucuronide

To examine the concentration of each isomer of LTB4 glucuronide, each isomer of LTB4 glucuronide was separately collected by HPLC. When the concentration of an isomer of LTB4 glucuronide was determined by enzyme immunoassay after hydrolysis with β-glucuronidase, the concentration of the fraction corresponding to peak 1, which eluted earlier

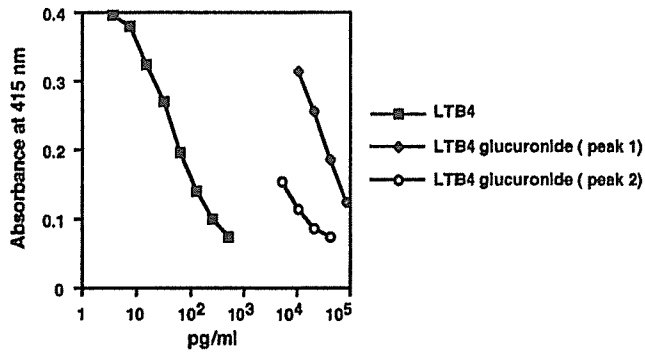

Fig. 2. Cross-reactivity of two isomers of LTB4 glucuronide to antibody against LTB4.

by HPLC, was significantly higher than that corresponding to peak 2 (20.9 pg/mg-creatinine, [4.7–76.7] versus 3.65 [0–9.6], $n = 10, p < 0.01$ analyzed by the Wilcoxon t-test). There was a significant correlation between the concentrations of both isomers ($r = 0.651, p = 0.04$).

3.4. *Quantification of urinary LTB4 glucuronide by enzyme immunoassay in combination with HPLC*

We do not know the precise concentration of LTB4 glucuronide because there has been no information about the molecular extinction coefficient of LTB4 glucuronide. Thus, we examined the recovery rate of the procedure from various aspects as follows. (1) After isolation of LTB4 glucuronide by HPLC, LTB4 glucuronide was incubated with β-glucuronidase. After extraction of LTB4 from the reaction mixture with an Empore C18 disk cartridge, LTB4 was quantified by enzyme immunoassay. Because the concentration of LTB4 was determined to be 2.8 pmol/ml, the concentration of LTB4 glucuronide in the starting solution was assumed to be 2.8 pmol/ml without considering the rate of hydrolysis with β-glucuronidase treatment. Five aliquots of LTB4 glucuronide (2.8 pmol/ml) were processed using all procedures, namely, hydrolysis with β-glucuronidase, LTB4 extraction with an Empore C18 cartridge, LTB4 purification by HPLC, and LTB4 extraction from effluent with an Empore C18 cartridge. The LTB4 concentration was determined to be 2.11 ± 0.31 pmol/ml by enzyme immunoassay, suggesting that the total recovery rate was $75.4 \pm 11.0\%$ (mean ± S.D., $n = 5$). (2) When LTB4 (600 pg) was injected into the HPLC column and the effluent containing LTB4 was extracted with an Empore C18 cartridge, the recovery rate was $90.7 \pm 4.2\%$ ($n = 5$), suggesting that LTB4 is recovered in high yield. (3) When four aliquots of an isomer (peak 1) of LTB4 glucuronide were hydrolyzed with β-glucuronidase, the concentration of LTB4 was 142 ± 9.93 ng/ml. The coefficient of variation was 7%, suggesting that LTB4 glucuronide can be reproducibly converted to LTB4 under our experimental conditions. Nevertheless, beside the recovery rate described above, LTB4 glucuronide concentration may be slightly underestimated because we do not know whether LTB4 glucuronide was fully converted to LTB4 by incubation with β-glucuronidase.

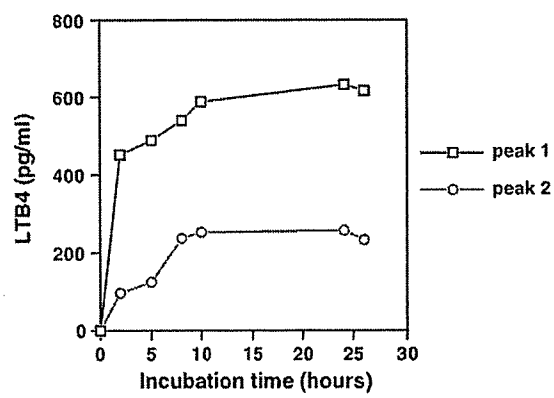

Fig. 3. Kinetics of hydrolysis of LTB4 glucuronide by incubation with β-glucuronidase; peaks 1 and 2 correspond to the fractions eluted at the retention times of 3.2 and 4.5 min shown in Fig. 1, respectively.

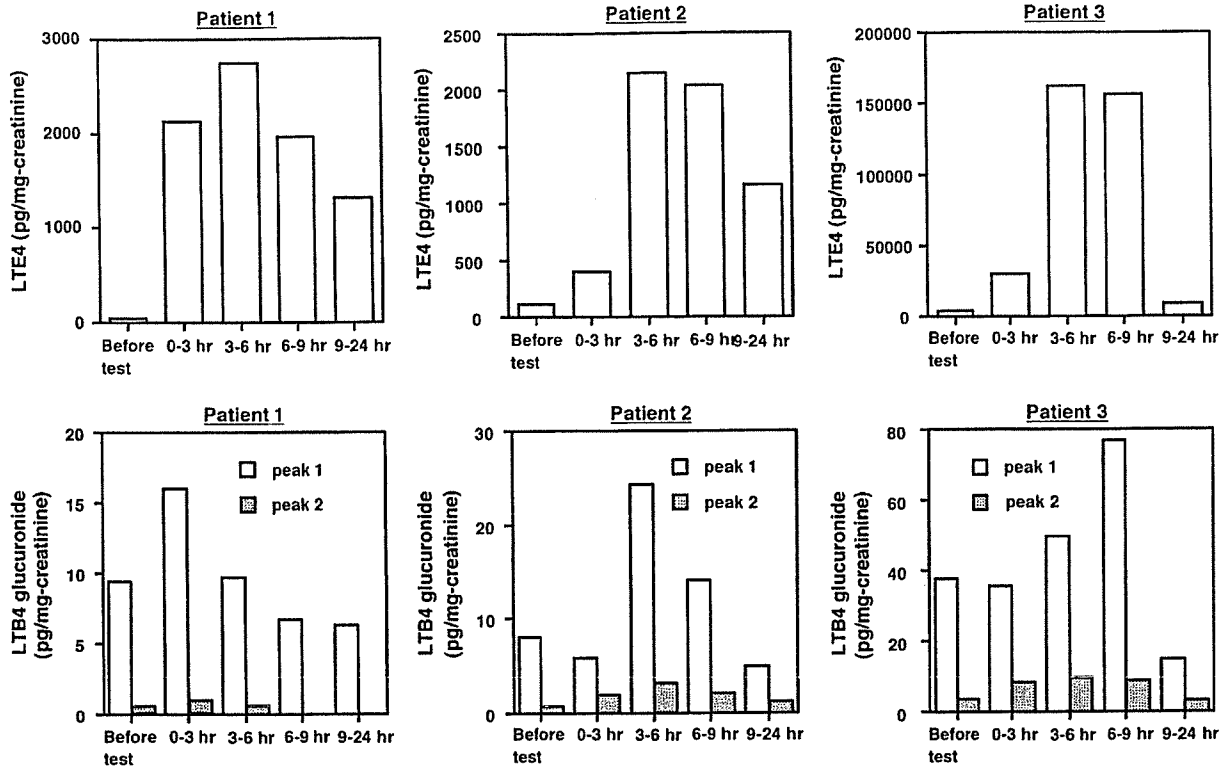

Fig. 4. Changes in concentrations of urinary LTE4 and two LTB4 glucuronide isomers in three patients with aspirin-intolerant asthma after intravenous aspirin challenge.

3.5. Concentrations of LTE4 and two LTB4 glucuronide isomers in aspirin-intolerant asthma patients after aspirin provocation

As shown in Fig. 4, a significant increase in the concentration of LTE4 was observed in urine from the three aspirin-intolerant asthma patients after the aspirin challenge. Of the two isomers of LTB4 glucuronide, a significant increase in the concentration of the isomer corresponding to peak 1 was observed. These results agreed with those in our previous report [5]. On the other hand, although the concentration of the isomer corresponding to peak 2 also increased, the increase was not apparent owing to the originally low concentration.

3.6. Concentrations of LTE4 and LTB4 glucuronide in normal healthy adults

In 13 normal healthy subjects, the concentrations of LTE4 and LTB4 glucuronide were 94.6 pg/mg-creatinine (median, range 21.4–159.9) and 22.3 pg/mg-creatinine (range 7.4–49.1), respectively. There was no correlation between LTE4 concentration and LTB4 glucuronide concentration in urine from normal healthy adults.

3.7. Concentrations of LTE4 and LTB4 glucuronide in atopic asthmatic patients after allergen inhalation

All patients developed isolated early asthmatic responses after allergen inhalation. The baseline FEV1.0 was 3.06 ± 0.861 (mean $\pm$ S.D.) and the mean percent decrease in FEV 1.0 after allergen inhalation was $67.8 \pm 17.4\%$ at 10 min. All the patients showed an increase in concentration of urinary LTE4 in the first 3 h after the challenge. There was a significant increase in urinary LTE4 concentration during the 3 h after FEV1.0 decreased by 20% or more from the baseline after allergen inhalation (96.1 pg/mg-creatinine, [61.7–346] versus 449 pg/mg-creatinine, [110–1546], $p < 0.05$). If LTB4 release is induced by allergens in atopic asthmatic patients, it is possible to detect an increase in urinary LTB4 glucuronide concentration after allergen inhalation. However, there was no significant change in urinary LTB4 glucuronide concentration after allergen inhalation (Fig. 5). These results suggest that cysteiny-LTs are

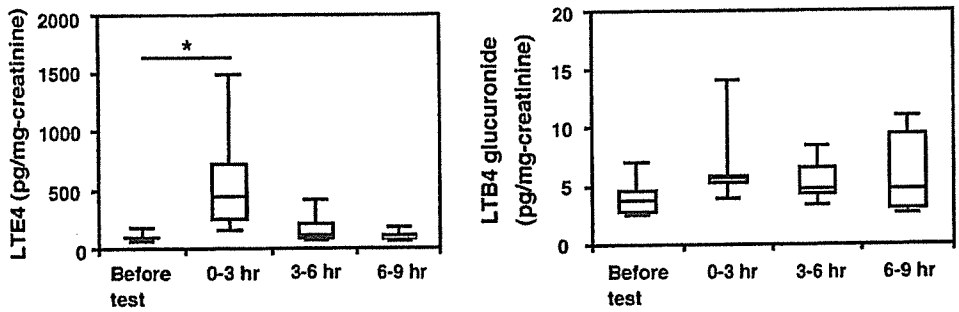

Fig. 5. Changes in urinary LTB4 glucuronide (right) and LTE4 (left) concentrations in atopic asthmatic patients after allergen inhalation. Data are presented as box plots showing medians and interquartile ranges. In the box plots, the lower boundary indicates the 25th percentile. The line within the box indicates the 50th percentile (median) and the upper boundary of the box indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles, respectively. *Significantly different from baseline concentration ($p < 0.05$).

involved in causing bronchoconstriction induced by inhalation challenge during early asthmatic responses. The absence of significantly increased concentrations of LTB4 glucuronide during bronchoconstruction suggests that LTB4 is not involved in the induction of early bronchial responses; alternatively, LTB4 is generated in amounts too small to be reflected in an increased concentration of urinary LTB4 glucuronide.

4. Discussion

When LTB4 and uridine 5′-diphosphoglucuronic acid were incubated in the presence of human liver microsomes, two new peaks appeared on the HPLC chromatogram (Fig. 1A), and this chromatogram agreed with that reported by Turgeon et al. [6]. When the fractions corresponding to the two peaks, which were distinctively eluted after the retention times of approximately 3.1 min and 4.5 min, were incubated with β-glucuronidase, both fractions generated LTB4, suggesting that these peaks correspond to LTB4 glucuronide. In human urine, the concentration of the isomer that was eluted by HPLC after a shorter retention time was significantly higher than that of the isomer that was eluted after a longer retention time. In an in vitro experiment using human liver microsomes, the height of peak 2 was approximately one-half that of peak 1 (Fig. 1A), suggesting that the isomer of LTB4 glucuronide corresponding to peak 2 was produced at a lower concentration than the isomer corresponding to peak 1. Although the distinct LTB4 glucuronide isomers show the same fragmentation pattern in LC–MS/MS analysis, the structure of LTB4 glucuronide, namely, the functional group of LTB4 to which glucuronic acid attaches, has not been clarified [6].

The possibility that the concentration of LTB4 was underestimated cannot be denied because we were not able to determine recovery rate of the entire experimental procedure, particularly the rate of hydrolysis of LTB4 glucuronide using β-glucuronidase. However, we examined the recovery rate of the procedure from various aspects and considered that there was no significant recovery loss.

The early asthmatic responses after allergen inhalation seem to be mainly induced by bronchoconstriction mediators released from cells, such as mast cells, within the airway lumen. Many studies showed that LTE4 concentration in urine increases in atopic patients following the inhalation of an allergen solution [7–11]. In our study, a significantly high concentration of LTE4 was observed in urine samples obtained during 3 h after bronchoconstriction as shown in Fig. 5. On the other hand, the concentration of LTB4 glucuronide generally did not significantly increase. This may be expected because human mast cells produce a large amount of LTC4 following IgE-dependent stimulation but produce only a small amount of LTB4 [12,13]. Moreover, LTB4 is produced by transcellular biosynthesis induced by cell-to-cell interaction [14]; however, the results shown in Fig. 5 suggest that allergen inhalation causes low, if any, LTB4 production induced by cell-to-cell interaction. Because it has been reported that only 0.2% of intravenously administered LTB4 is excreted in urine, a slight change in the concentration of LTB4 glucuronide may not be detected because of measurement errors. There is a possibility that changes in LTB4 concentration is more difficult to detect than those in LTE4 concentration except for considerable changes.

If we assume that the amount of creatinine produced in a human body is 1500 mg/day and that approximately 0.2% of LTB4 produced in a human body is excreted as LTB4 glucuronide in urine [3], the amount of LTB4 produced in the body of a healthy person can be calculated as approximately 16.7 μg/day (49.7 nmol/day), because the LTB4

glucuronide concentration in the urine sample from a healthy person was determined to be 22.3 pg/mg-creatinine in our study. On the other hand, because it was reported that approximately 5% of LTC4 is excreted as LTE4 in urine [15], the amount of LTC4 produced in the body of a healthy person can be similarly calculated as approximately 2.82 μg/day (4.5 nmol/day).

In conclusion, we established a method of measuring LTB4 glucuronide, which may be used as a marker of systemic LTB4 production. Using the method described in our previous report, we found that errors tended to occur when the concentration of LTB4 glucuronide was low because we calculated the concentration of LTB4 glucuronide from the difference between the concentration of LTB4 hydrolyzed and that not hydrolyzed. These errors rarely occur in the present method (data not shown); moreover, the advantage of this method is that it requires half the amount of a urine sample for analysis compared with that used in the previous method. By measuring the concentration of LTB4 glucuronide as well as that of LTE4, the condition of the human leukotriene pathway can be comprehensively evaluated.

Conflict of interest

The authors have no conflicting financial interests.

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IL-21–induced Bε cell apoptosis mediated by natural killer T cells suppresses IgE responses

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Epidemiological studies have suggested that the recent increase in the incidence and severity of immunoglobulin (Ig)E-mediated allergic disorders is inversely correlated with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) vaccination; however, the underlying mechanisms remain uncertain. Here, we demonstrate that natural killer T (NKT) cells in mice and humans play a crucial role in the BCG-induced suppression of IgE responses. BCG-activated murine Vα14 NKT cells, but not conventional CD4 T cells, selectively express high levels of interleukin (IL)-21, which preferentially induces apoptosis in Bε cells. Signaling from the IL-21 receptor increases the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor, resulting in Bε cell apoptosis. Similarly, BCG vaccination induces IL-21 expression by human peripheral blood mononuclear cells (PBMCs) in a partially NKT cell–dependent fashion. BCG-activated PBMCs significantly reduce IgE production by human B cells. These findings provide new insight into the therapeutic effect of BCG in allergic diseases.

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Abbreviations used: α-GalCer, α-galactosylceramide; BCG, *Mycobacterium bovis* bacillus Calmette Guerin; BM-DC, BM-derived DC; Bmf, Bcl-2–modifying factor; γc, common γ-chain; IRAK, IL-1R–associated kinase; MNC, mononuclear cell; MyD88, myeloid differentiation factor 88; PGN, peptidoglycan; TLR, Toll-like receptor.

The prevalence of IgE-mediated allergic diseases such as asthma, hay fever, and atopic dermatitis has increased dramatically over the past two decades, especially in industrialized countries (1). For example, the incidence of asthma has nearly doubled since 1980 in the United States as well as in Japan (1, 2). However, the precise mechanisms underlying the increased incidence of allergic diseases are not fully understood. One possible explanation has been termed "the hygiene hypothesis," which proposes that improved hygiene combined with the excessive use of antibiotics in industrial countries has markedly reduced the incidence of infections, particularly in children. This lack of early exposure to infectious agents is associated with accelerated IgE production and an increased incidence of allergic disorders (1–3). Epidemiological studies support this hypothesis (4–6), and bacterial and viral products have been proposed as therapeutic strategies to suppress the development of allergic responses. For example, vaccination with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) has been reported to suppress IgE production and inhibit the development of allergic diseases in mouse models (7–9) and in humans (10). Furthermore, injection of CpG oligodeoxynucleotides, bacterial DNA surrogates recognized by Toll-like receptor (TLR)9, reduces serum IgE levels in mice (11).

It has been widely accepted that IgE production is totally dependent on Th2 cells, whose functions are reciprocally inhibited by Th1 cells. Mechanistically, therefore, the hygiene hypothesis is based on an imbalance in

The online version of this article contains supplemental material.

the Th1/Th2 ratio because bacterial components stimulate Th1 responses that in turn inhibit Th2 responses and IgE production (12). On the other hand, recent findings have indicated that a spectrum of T cells with immunoregulatory properties is involved in the regulation of IgE production and the pathophysiology of allergic diseases (13). For example, $CD4^+CD25^+$ regulatory T cells inhibit Th2 responses by producing immunosuppressive cytokines that can directly inhibit B cell activation (14, 15). Furthermore, NKT cells expressing an invariant antigen receptor (Vα14-Jα281 for mice and Vα24-JαQ for humans; reference 16) suppress Th2 and IgE responses via their production of IFN-γ (17).

In addition to these cellular mechanisms, it has also been reported that IL-21 is involved in the suppression of IgE production in both mice and humans (18, 19). IL-21 is a type I cytokine produced by activated $CD4^+$ T cells and has a broad capacity to regulate lymphoid cell functions (20–22). Among these functions, IL-21 directly inhibits antibody production by IgE-bearing B (Bε) cells induced by CD40L and IL-4 (18). Conversely, IL-21R–deficient mice exhibit enhanced IgE production (23). IL-21 has been shown to specifically inhibit germ line transcription of the IgE constant region (Cε) gene but not of other isotype genes (18). However, there is no direct evidence that this inhibition of germ line transcription is responsible for the suppression of IgE production, as class switch recombination of Ig genes and subsequent antibody secretion are differentially regulated events (24). IL-21 also induces apoptosis in B cells (25, 26), which could partially explain the reduction of IgE production; however, this effect was not shown to be specific for IgE. Hence, the mechanism by which IL-21 specifically inhibits IgE production is not yet fully understood.

Here, we have investigated BCG-mediated IgE suppression and found that NKT cells specifically induced apoptosis in Bε cells through the production of IL-21, resulting in a dramatic decrease in IgE production. IL-21 increased the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor (Bmf), which is selectively expressed in Bε cells and counteracts the antiapoptotic activity of Bcl-2. We have found that similar mechanisms are operative in humans. This is the first report demonstrating that IL-21 produced by Vα14 NKT cells plays an important role in the regulation of IgE responses in both mouse and human immune systems.

RESULTS

Vα14 NKT cell–dependent IgE suppression by BCG treatment

We used an OVA-patched sensitization protocol (27) to determine if BCG activates Vα14 NKT cells. Vα14 NKT cells were detected by α-galactosylceramide (α-GalCer)–loaded CD1d tetramer staining. In control mice treated with PBS or OVA without BCG, ~15% of the liver mononuclear cells (MNCs) were Vα14 NKT cells (Fig. 1 A, left and middle). However, BCG treatment significantly increased the frequency of Vα14 NKT cells to >25% (Fig. 1 A, right). BCG treatment also increased the absolute number of Vα14 NKT cells because the total number of liver MNCs was also increased by 50–80% (not depicted). Sera were collected from these mice 1 wk after the last sensitization, and IgE levels were evaluated. In WT mice, both total and OVA-specific IgE levels were suppressed by BCG treatment (Fig. 1 B). In mice lacking the Jα18 gene (Vα14 NKT KO), there was no significant BCG-induced suppression of IgE responses, suggesting that suppression requires Vα14 NKT cells.

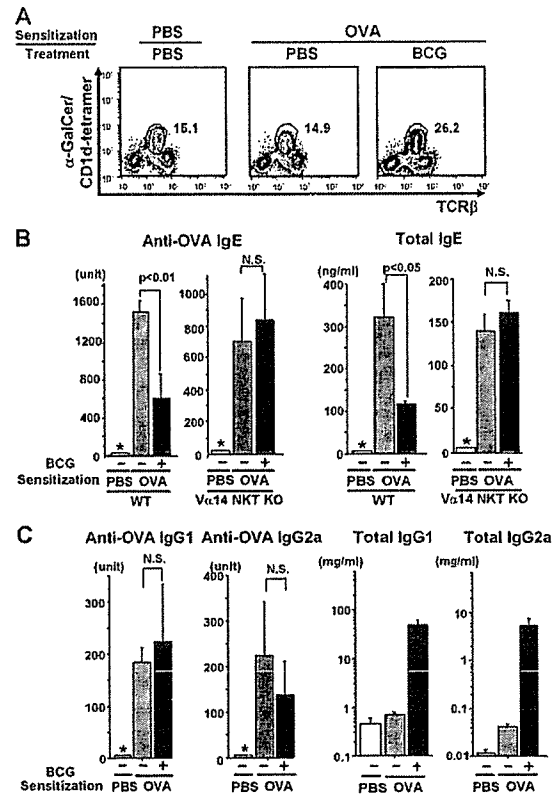

Figure 1. Requirement of Vα14 NKT cells in BCG-mediated IgE suppression. (A) FACS profiles of liver MNCs. The liver MNCs obtained 1 wk after the last immunization were stained with α-GalCer/CD1d tetramer and anti-TCRβ mAb. Three mice per each group were analyzed and representative data are shown. (B and C) Effects of BCG on antibody responses in WT and Vα14 NKT KO mice. Total and OVA-specific serum IgE (B), IgG1, and IgG2a (C) were assayed by ELISA. Five mice were used in each group. Values are expressed as mean ± SD. The asterisks (*) indicate that the amount of IgE was below the detection level for anti-OVA IgE (<31.2 U/ml), anti-OVA IgG1 (<0.002 U/ml), or anti-OVA IgG2a (<1.25 U/ml). N.S., not significant. All experiments were repeated three times with similar results.

The effect of BCG administration on Th1/Th2 responses

It is well known that the isotype commitment of B cells during Ig class switching is tightly regulated by Th1/Th2 cell cytokines (28) and that Vα14 NKT cells play a regulatory role in T cell differentiation (17, 29, 30). Therefore, we measured serum IgG2a (Th1) and IgG1 (Th2) levels to assess any changes in the Th1/Th2 balance. BCG administration did

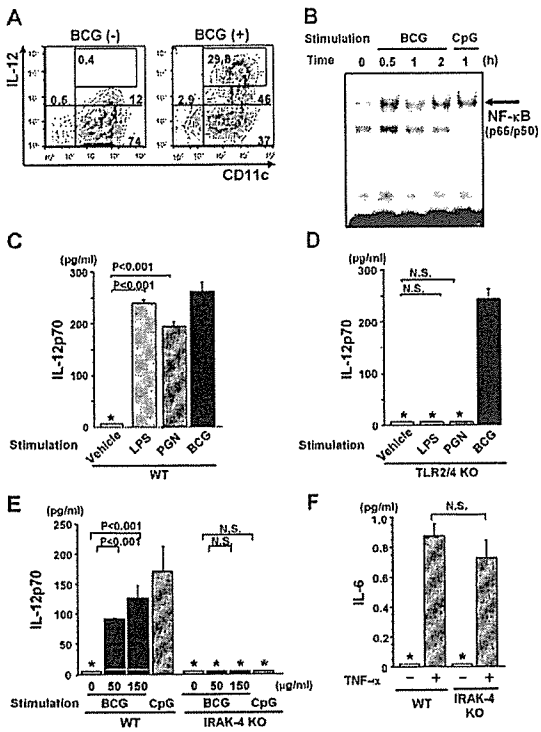

Figure 2. Activation of DCs by BCG. IL-12 production (A) and NF-κB activation (B). (A) Intracellular staining of BM-DCs with anti-IL-12p40/p70 and anti-CD11c mAbs with or without in vitro BCG (50 μg/ml) treatment for 12 h. BCG-treated BM-DCs (10,000 cells) were analyzed by FACS, and the number in each panel indicates the percentage of total cells. (B) NF-κB activation. 2 × 10^5 BM-DCs were stimulated with or without 50 μg/ml BCG or 1 μM CpG in vitro. NF-κB activity was determined by EMSA. (C and D) No requirement of TLR2 and TLR4 in BCG-mediated IL-12 production. 2 × 10^5 BM-DCs derived from WT (C) or TLR2/4 double KO (D) mice were stimulated in vitro with or without 10 μg/ml LPS, 10 μg/ml PGN, or 150 μg/ml BCG for 48 h, and IL-12p70 levels were measured by ELISA. (E and F) Requirement of IRAK-4 for IL-12 production. 2 × 10^5 BM-DCs were assayed for IL-12p70 by ELISA after stimulation with 0, 50, or 150 μg/ml BCG or 1 μM CpG (E), and for IL-6 with 10 ng/ml TNF-α stimulation for 48 h (F). In C–F, values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the levels were below the detection limits for IL-12p70 (<62.5 pg/ml) and IL-6 (<15.6 pg/ml). N.S., not significant. All experiments were repeated twice with similar results.

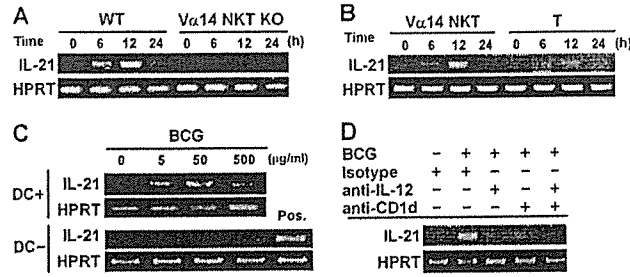

Figure 3. IL-21 expression. (A) Vα14 NKT cell–dependent IL-21 production. Liver MNCs were obtained after BCG injection (500 μg/mouse) and examined for IL-21 mRNA expression. (B) Identification of the source of IL-21. Vα14 NKT and conventional T cells were sorted from liver MNCs and examined for IL-21 mRNA expression. (C) Requirement of DCs for BCG-induced IL-21 expression by Vα14 NKT cells. Liver TCRβ$^+$ cells were cultivated in the presence of 50 μg/ml BCG with (top) or without (bottom) BM-DCs for 24 h and analyzed for IL-21 mRNA expression. Liver TCRβ$^+$ cells stimulated with 10 μg/ml anti-CD3 mAb were used as a positive (Pos.) control. (D) Requirement of IL-12– and CD1d-mediated signals for IL-21 mRNA expression upon BCG stimulation. An isotype control, anti-IL-12p40/p70, or anti-CD1d mAb (20 μg/ml) was added to the cultures of liver TCRβ$^+$ cells and BM-DCs as described in C. All experiments were repeated twice with similar results.

not significantly alter the levels of OVA-specific IgG1 or IgG2a, although total levels of both isotypes were significantly enhanced (Fig. 1 C).

Innate signaling pathway for BCG-mediated IL-12 production

During microbial infection, both CD1d- and IL-12–mediated signals are required for the rapid activation of Vα14 NKT cells (31). Thus, we assessed IL-12 production after BCG treatment. BM-derived DCs (BM-DCs) were stimulated in vitro with 50 μg/ml BCG and examined for IL-12 production by intracellular cytokine staining using an IL-12p40/p70 mAb. Upon BCG stimulation, a large fraction of CD11c^{high} cells produced IL-12 (Fig. 2 A). NF-κB activation is crucial for IL-12 production, and BCG treatment activated NF-κB to the same extent as treatment with the positive control CpG, as demonstrated by electrophoretic mobility shift assay (Fig. 2 B). These results indicate that BCG directly induces IL-12 production in DCs by activating NF-κB.

It has been reported that mycobacterial cell wall antigens such as peptidoglycan (PGN) or lipoarabinomannan induce proinflammatory gene transcription through TLR2 and TLR4 (32). However, when we compared IL-12p70 production by BCG-stimulated WT and TLR2/TLR4 double KO BM-DCs, there was no difference (Fig. 2 C). As expected, however, the TLR2/4-deficient cells failed to respond to LPS or PGN (Fig. 2 D). These results indicate that receptor(s) other than TLR2 and TLR4 are responsible for the recognition of whole BCG organisms.

To analyze intracellular signaling pathways activated by BCG, we measured IL-12p70 production by BM-DCs from WT and IL-1R–associated kinase (IRAK)-4 KO mice. BM-DCs from IRAK-4 KO mice produced less IL-12p70 than those from WT mice in response to both BCG and CpG (Fig. 2 E), whereas they produced comparable levels of IL-6 in response to TNF-α stimulation (Fig. 2 F). Similarly, BM-DCs from myeloid differentiation factor 88 (MyD88) KO mice produced nearly undetectable IL-12p70 upon BCG stimulation, whereas IL-6 production remained unchanged (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). Therefore, the recognition of BCG organisms is mediated by innate receptors other than TLR2 and TLR4 that signal through both IRAK-4 and MyD88.

BCG-induced IL-21 expression in Vα14 NKT cells

The recently identified IL-21 and its receptor (IL-21R), members of the common γ-chain (γc)-dependent cytokine family, have been shown to regulate IgE production without influencing Th2 cell differentiation (18, 20, 23). Thus, we examined the possibility that IL-21 might be induced by BCG stimulation and might suppress IgE responses in a Vα14 NKT cell–dependent manner. We first measured IL-21 mRNA expression in TCRβ$^+$ liver MNCs by a RT-PCR. IL-21 mRNA was detected in liver TCRβ$^+$ liver MNCs of WT mice within 6 h after BCG injection (Fig. 3 A). In contrast, no IL-21 mRNA was detected in the Vα14 NKT KO mice (Fig. 3 A), suggesting that Vα14 NKT cells are the source of IL-21 in response to BCG. To test this hypothesis, we separated conventional T cells and Vα14 NKT cells and found that IL-21 mRNA was more abundant in the Vα14 NKT cells after BCG injection (Fig. 3 B). Similarly, after stimulation with anti-CD3, IL-21 mRNA levels in Vα14 NKT cells were more than seven times higher than in CD4 T cells, confirming that these cells are the major source of IL-21 in this model (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1).

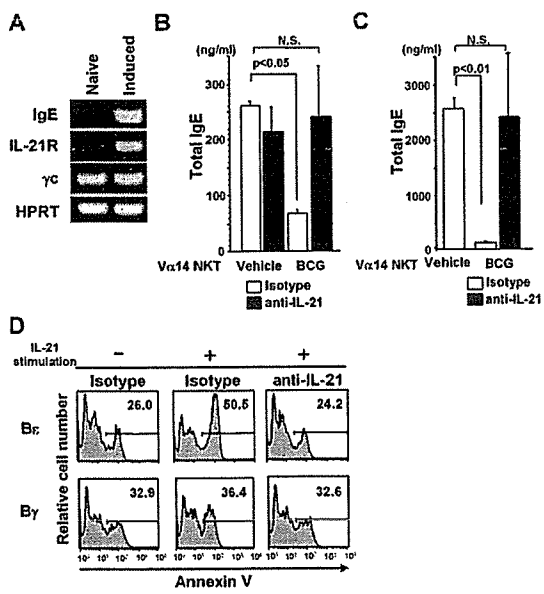

Figure 4. IL-21–mediated Bε cell apoptosis. (A) RT-PCR analysis. Expression of IgE (Cε), IL-21R, and γc was investigated in naive B (left) and Bε (right) cells. (B) Suppression of IgE production in naive B cell cultures. Naive B cells and Vα14 NKT cells (10^5 each) were cocultured in the presence of sCD40L and IL-4. (C) Suppression of IgE production in the Bε cell culture. 10^5 Vα14 NKT cells were added to the Bε cell (10^5) cultures. In B and C, 20 μg/ml anti–IL-21 mAb or isotype control mAb was added at the same time as the Vα14 NKT cells. The concentration of total IgE was measured by ELISA in triplicate. Values are expressed as mean ± SD. N.S., not significant. The experiments were repeated three times with similar results. (D) IL-21–mediated Bε cell apoptosis. 2×10^5 Bε and Bγ cells were generated and then further cultured with or without 30 ng/ml IL-21 for 30 h. Annexin V staining was then performed. The numbers represent percentage of the gated cells. Annexin V$^+$ cells among Bε and Bγ cells just before IL-21 treatment was 25.7 and 29.2%, respectively (not depicted). The experiments were repeated three times with similar results.

Requirement for IL-12 and CD1d in IL-21 expression by Vα14 NKT cells

We next analyzed the role of DCs in BCG-induced IL-21 mRNA expression. Co-culture of Vα14 NKT cells with DCs plus IL-12 strongly induced IL-21 mRNA expression, whereas no IL-21 mRNA was induced in the absence of DCs (Fig. 3 C). Furthermore, IL-21 mRNA expression was inhibited by the addition of anti–IL-12, anti-CD1d, or both into the cultures (Fig. 3 D), indicating that both IL-12 and CD1d are required for IL-21 expression by Vα14 NKT cells.

IL-21–mediated IgE suppression

To examine whether BCG-activated Vα14 NKT cells actually suppress IgE production, Bε cells were generated from naive CD19$^+$ splenic B cells using the 3-d culture system described by Snapper et al. (33). The starting population of naive B cells expressed negligible IL-21R and contained no Bε cells as defined by Cε transcripts (Fig. 4 A). However, after 3 d of the culture, the majority of CD19$^+$ B cells became Bε cells and expressed IL-21R (Fig. 4 A). We then investigated the effects of BCG treatment on B cells, before and after IgE class switching. The addition of BCG-treated liver MNCs at the onset of the naive B cell cultures significantly suppressed IgE production (~50%; Fig. 4 B). However, when BCG-activated Vα14 NKT cells were added to the Bε cell culture on day 3 and the cells were further cultivated for 5 d, IgE production was even more strongly inhibited (>90% suppression; Fig. 4 C). These results indicate that, even after B cells have undergone Cε class switching, BCG-activated Vα14 NKT cells can potently suppress IgE production. The inhibition of IgE production was IL-21 dependent, as an anti–IL-21 mAb completely abrogated the inhibitory effects (Fig. 4, B and C). When the B cells in these cultures were assessed for apoptosis by annexin V staining, there was a significant increase

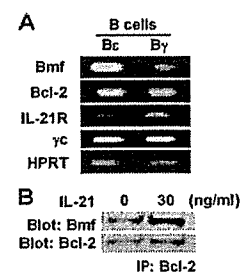

Figure 5. Bmf-mediated Bε cell apoptosis. (A) RT-PCR. RNA from Bε and Bγ cells was analyzed for its expression of the indicated genes by RT-PCR. Note that no significant differences in Bcl-2 and IL-21R expression between Bε and Bγ cells were observed. (B) Western blotting. Bε cells were stimulated with IL-21 at 37°C for 30 min, and their cell lysates (6×10^6) were subjected to immunoprecipitation with anti–Bcl-2 mAb and immunoblotting with anti-Bmf antibody (top) or anti–Bcl-2 mAb (bottom). All experiments were repeated three times with similar results.

in apoptotic Bε cells that was not observed in the Bγ cells (Fig. 4 D, middle). Apoptosis of Bε cells was abrogated by the addition of anti–IL-21, a treatment that had no significant effect on Bγ cells (Fig. 4 D, right).

Bmf-induced Bε cell apoptosis

To understand the molecular mechanisms underlying IL-21–induced IgE suppression, we performed DNA microarray analyses to compare gene expression between Bε and Bγ cells. The DNA microarray data were deposited in the Center for Information Biology Gene Expression database (CIBEX; http://cibex.nig.ac.jp/) under accession number CBX15. The proapoptotic Bmf gene (34) was dramatically up-regulated in Bε cells, a finding that was confirmed by RT-PCR (Fig. 5 A). No significant difference in the expression of IL-21R, Bcl-2, or γc was detected (Fig. 5 A), suggesting that elevated Bmf gene expression in Bε, but not in Bγ, cells may account for their differential sensitivity to IL-21–mediated apoptosis.

To investigate whether the Bmf expressed in Bε cells is functional in its proapoptotic activity, Bmf cDNA was isolated from Bε cells and used to prepare several mutants of enhanced GFP–fused Bmf. These mutations included an A69P mutation in the dynein light chain 2 binding motif and an L138A mutation in the BH3 domain. These Bmf mutants were transfected into Baf3 cells. Upon IL-3 deprivation, mock transfectants underwent apoptosis. Transfection with WT Bmf or Bmf-A69P to Baf3 cells also significantly augmented apoptosis (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). However, reduced apoptosis was seen in Baf3 cells transfected with BH3 mutants, such as Bmf-L138A or Bmf-A69P/L138A (Fig. S3), indicating that Bmf in Bε cells is functional and the BH3 domain of the protein is important for mediating its proapoptotic activity.

Based on the understanding of proapoptotic activity of Bmf expressed in Bε cells, we investigated the formation of Bmf–Bcl-2 complexes in Bε cells after activation with IL-21. Bmf in Bε cells faintly binds to Bcl-2 in unstimulated cells (Fig. 5 B, left). However, when Bε cells were stimulated with IL-21, the formation of Bmf–Bcl-2 complexes was significantly augmented (Fig. 5 B, right).

BCG-mediated IL-21 induction in human Vα24 NKT cells

To determine how widespread our findings are, we investigated whether IL-21 and Vα24 NKT cells are required for the BCG-mediated suppression of human IgE responses. When human PBMCs were stimulated with α-GalCer or BCG, a significant up-regulation of IL-21 mRNA was detected by quantitative PCR (Fig. 6 A). The BCG-induced up-regulation of IL-21 mRNA was effectively suppressed by blocking with antibodies against CD1d, IL-12p40/p70, or both (Fig. 6 B), indicating that the CD1d-restricted NKT cell–dependent suppression of IgE responses observed in mice also operates in the human immune system. IL-21 mRNA expression by anti-CD1d and anti–IL-12 treatment was significantly reduced but was not as effective as in the mouse Vα14 NKT cell system (Fig. 3 D), perhaps suggesting a significant contribution of human conventional $CD4^+$ T cells (Fig. S2 B).

To evaluate in vivo responses, we inoculated BCG into healthy volunteers and examined IL-21 mRNA levels in PBMCs 1 wk later. There was a significant up-regulation of IL-21 mRNA levels in five out of six individuals (Fig. 6 C), and, furthermore, IL-21 suppressed IgE production by human Bε cells (Fig. 6 D, left). As expected, the addition of BCG-stimulated, but not control, PBMCs significantly inhibited IgE production (Fig. 6 D, right).

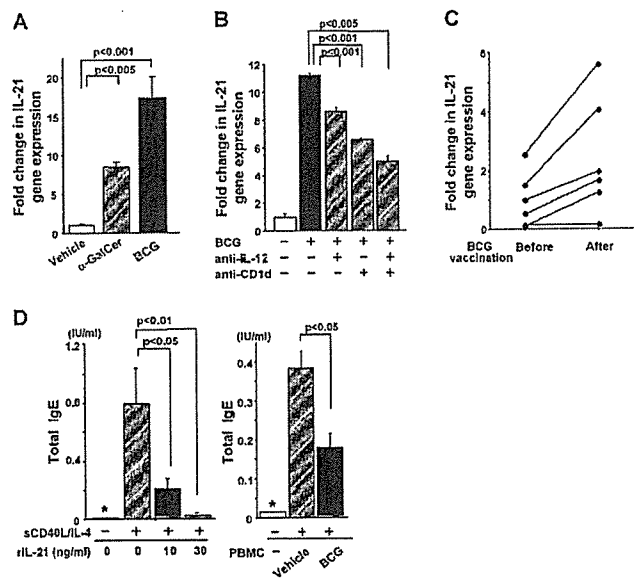

Figure 6. IL-21 mRNA expression and IL-21–induced IgE suppression in humans. (A) IL-21 mRNA expression in PBMCs. 10^6 human PBMCs were stimulated with 100 ng/ml α-GalCer or 50 μg/ml BCG and examined for IL-21 expression by quantitative real-time PCR with Taqman probes. The data are representative of five donors. (B) IL-12 and CD1d are required for IL-21 expression. 10^6 PBMCs were stimulated in vitro with 50 μg/ml BCG in the presence of 10 μg/ml anti-CD1d and/or anti–IL-12p40/p70 mAb. Representative data from five donors are shown. (C) IL-21 mRNA expression in PBMCs. Healthy volunteers were inoculated intradermally with BCG (two drops of 26.7 mg/ml of BCG emulsion per person). In A–C, the data for IL-21 expression were normalized to 18S ribosomal RNA expression, and relative expression levels are shown. Statistical analysis was performed using a matched pairs *t* test in C. (D) Suppression of IgE production. Left, suppression of IgE production by IL-21. 2×10^5 human B cells were cultured with sCD40L and IL-4 in the presence of human IL-21 for 14 d. Right, suppression of IgE production by BCG-activated human PBMCs. 10^5 Bε cells were co-cultured with 10^5 PBMCs, sCD40L, and IL-4 in the presence of 50 μg/ml BCG for 14 d. Total IgE was measured by ELISA. Values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the IgE levels are below the detection limit for total IgE (<0.014 IU/ml). Data shown are representative of three donors. Results were expressed as a fold difference in human IL-21 gene expression relative to a control sample (vehicle) after being normalized with 18S ribosomal RNA expressions in each sample.